CompCytogen 11(2): 189–201 (2017)
doi: 10.3897/CompCytogen.v11i2.11980
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Further studies on *Boreonectes* Angus, 2010, with a molecular phylogeny of the Palaearctic species of the genus

Robert B. Angus[1], Ignacio Ribera[2], Fenglong Jia[3]

1 *Department of Life Sciences (Insects), The Natural History Museum, Cromwell Road, London SW7 5BD, UK*
2 *Institut de Biologia Evolutiva (CSIC-UPF), Passeig Maritim de la Barceloneta, 37-49, 08003 Barcelona, Spain*
3 *Institute of Entomology, Life Science School, Sun Yat-sen University, Guangzhou, 570275, Guangdong, China*

Corresponding author: *Robert B. Angus* (r.angus@rhul.ac.uk)

Academic editor: *D. Lachowska* | Received 29 January 2016 | Accepted 3 March 2017 | Published 22 March 2017

http://zoobank.org/0707884B-848C-4365-8CC4-CB4E5E76DB50

Citation: Angus RB, Ribera I, Jia F (2017) Further studies on *Boreonectes* Angus, 2010, with a molecular phylogeny of the Palaearctic species of the genus. Comparative Cytogenetics 11(2): 189–201. https://doi.org/10.3897/CompCytogen.v11i2.11980

Abstract

Karyotypes are given for *Boreonectes emmerichi* (Falkenström, 1936) from its type locality at Kangding, China, and for *B. alpestris* (Dutton & Angus, 2007) from the St Gotthard and San Bernardino passes in the Swiss Alps. A phylogeny based on sequence data from a combination of mitochondrial and nuclear genes recovered western Palaearctic species of *Boreonectes* as monophyletic with strong support. *Boreonectes emmerichi* was placed as sister to the north American forms of *B. griseostriatus* (De Geer, 1774), although with low support. The diversity of Palaearctic species of the *B. griseostriatus* species group is discussed.

Keywords

Dytiscidae, *Boreonectes*, *B. emmerichi*, *B. alpestris*, molecular phylogeny, diversity, karyotypes

Introduction

The genus *Boreonectes* Angus, 2010 comprises small diving beetles and most of the species are endemic to the Nearctic Region (Angus 2010b). However, one species-complex, including the type species of the genus (*B. griseostriatus* (De Geer, 1774)) is widely distributed in the Palaearctic, where a number of chromosomally distinct species have

been recognised (Dutton and Angus 2007; Angus 2008, 2010a; Angus et al. 2015). Angus et al. (2015), in their analysis of *Boreonectes emmerichi* (Falkenström, 1936), noted that the type material, from the Kangding area of Sichuan, China, was darker than the Qinghai material they used for chromosome analysis, and in particular none of the Qinghai material had the darks marks on the pronotum as extensive as in the type material. Nevertheless, they noted that DNA (COI) data obtained by Ignacio Ribera from this material closely matched those obtained from material collected near Nam Tso (Xizang), much further south on the Plateau than the Qinghai material, and in the light of this felt that the Qinghai material could safely be referred to *B. emmerichi*. They suggested that the darker colouration of the Kangding material was perhaps associated with a more wooded environment.

In June–July 2016 one of us (R. B. Angus) had the opportunity to visit the Kangding area and collect *B. emmerichi* from its type area, for chromosome analysis. This confirms the identity of the Qinghai material as *B. emmerichi*. Then in August 2016 R. B. Angus was able to visit Switzerland and to collect *Boreonectes* from the St Gotthard and San Bernardino passes, localities where I. Ribera & A. Cieslak had in 2002 collected material considered to be *B. griseostriatus* (De Geer, 1774) (Abellán et al. 2013) Surprisingly, according to their karyotypes the Swiss populations were found to be *B. alpestris* (Dutton & Angus, 2007), although the sequenced markers were found to have identical sequences to those obtained from the 2002 material.

This study aims to clarify the identities of *B. emmerichi* (Falkenström, 1936) and *B. alpestris* Dutton & Angus, 2007, from some localities in the Alps using karyotype and molecular data. To establish their phylogenetic positions, we build a molecular phylogeny of the genus *Boreonectes* including most Palaearctic and a representation of Nearctic species.

Material and methods

The material used for chromosome preparations is listed in Table 1.

Specimens were brought back to the laboratory alive and placed in small aquaria where they were fed with living larvae of Chironomidae (Diptera). Chromosome preparation and photography were as described by Dutton and Angus (2007). In fact, most

Table 1. Material giving chromosome preparations.

Species	Locality	Material
B. emmerichi (Falkenström, 1936)	CHINA Sichuan. Kangding County Yalashenshan.30.215°N,101.757°E Small pools 4052 m a.s.l.	2 ♂♂, 4 ♀♀
B. alpestris (Dutton & Angus, 2007)	SWITZERLAND Ticino. San Bernardino pass. 46.499°N 101.755°E. Pool 2030 m a.s.l.	1♀
	SWITZERLAND Ticino. St Gotthard pass 46.559°N 8.562°E Pool 2112 m a.s.l.	1♂

of the *B. emmerichi* died before they reached the laboratory but sufficient material, all from the same locality, survived to give chromosome preparations. Survival of the Swiss *B. alpestris* was much better, but only two specimens gave preparations from which karyotypes could be assembled.

For DNA extraction and sequencing we used the same methodology as various recent works on the same family Dytiscidae (e.g. García-Vázquez et al. 2016). Briefly, specimens were directly preserved in absolute ethanol in the field, and preserved at -20°C until processed. Extractions of single specimens were non–destructive, using the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). Vouchers and DNA samples are kept in the collections of the Institut de Biologia Evolutiva (IBE). We included examples of all known species of Palaearctic *Boreonectes* with the sole exception of *B. inexpectatus* (Dutton & Angus, 2007), known from a single lake in the Alps (Dutton and Angus 2007). We also included as outgroups several species of Nearctic *Boreonectes*, including specimens of *B. griseostriatus* (De Geer, 1774) (Table 2). The tree was rooted in two species of the genus *Stictotarsus*, the closest relatives of *Boreonectes* according to the phylogeny in García-Vázquez et al. (2016).

We sequenced four mitochondrial genes in two PCR reactions: 3' end of cytochrome c oxidase subunit (COI); and a single fragment including the 3' end of the large ribosomal unit (16S), the whole tRNA–Leu gene and the 5' end of the NADH dehydrogenase 1. We also amplified fragments of one nuclear gene, histone 3 (H3) (see García-Vázquez et al. 2016 for the primers used). New sequences have been deposited in the EMBL database with Accession Numbers LT796523–LT796555 (Table 2).

We aligned the sequences using the MAFFT online v.6 and the Q–INS–i algorithm (Katoh and Toh 2008). We used Maximum Likelihood as implemented in RAxML-HPC2 (Stamatakis et al. 2008) in the CIPRES science gateway (Miller et al. 2010), using GTR+G as evolutionary model and three partitions corresponding to the three amplified fragments. Node support was assessed with 100 fast bootstrap replicas.

Results and discussion

B. alpestris (Dutton & Angus, 2007)

Published information: 2n = 54 + X0 (♂), XX (♀) (Dutton and Angus 2007). Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 1a–e. Fig. 1a shows a paratype male, Giemsa stained, Fig. 1b, c shows a male from the Colle del Nivolet, the same nucleus Giemsa stained and C-banded, and Fig. 1d, e shows the same nucleus from a female from the Paso San Bernardino, Giemsa stained and C-banded. The chromosomes in these preparations are in complete agreement with each another. Metaphase I of meiosis from a male from the St Gotthard Pass is shown in Fig. 2a (Giemsa stained) and b (the same nucleus C-banded). The C-banded preparation enables the unpaired X chromosome to be identified. Although preparations from only two of the specimens attempted resulted in complete karyotypes, several other specimens yielded almost

Table 2. Material studied for the molecular phylogeny, with voucher number, locality and collector, and EMBL accession numbers. In bold, sequences newly obtained for this study.

No	Species	Voucher	Country	Locality	Leg	COI	16S+tRNA +NAD1	H3
1	*B. alpestris* (Dutton & Angus, 2007)	IBE-RA263	Italy	Piemonte. Gran Paradiso Nat. Park. Colle del Nivolet, roadside lake at ca 2500 m. 3.9.2010	R.B. Angus	**LT796525**	**LT796551**	**LT796537**
2	*B. alpestris*	IBE-AN579	Switzerland	Col de San Bernardino, ponds	R.B. Angus	**LT796523**	-	**LT796535**
3	*B. alpestris*	IBE-AN580	Switzerland	St. Gottardo pass, summit	R.B. Angus	**LT796524**	-	**LT796536**
4	*B. alpestris*	MNCN-AI298	Switzerland	Col de San Bernardino, ponds 13.10.2002	I. Ribera & A. Cieslak	HF931186	HF931409	-
5	*B. alpestris*	NHM-IR309	Switzerland	St. Gottardo pass, summit, 27.7.00	I. Ribera & A. Cieslak	HF931275	HF931512	-
6	*B. emmerichi* (Falkenström, 1936)	IBE-AN581	China	Sichuan, Kangding County, Yalashenshan. Small pools 4052m 27.6.2016	R.B. Angus, F-L Jia, Z-Q. Li & K. Chen	**LT796531**	-	**LT796541**
7	*B. emmerichi*	IBE-AN582	China	CHINA Sichuan.Kangding County. Yalashenshan, Small pools 4074 m a.s.l. 27.6.2016	R.B. Angus, F-L Jia, Z-Q. Li & K. Chen	**LT796528**	-	**LT796538**
8	*B. emmerichi*	IBE-AN583	China	Sichuan, Kangding County Boij ta Car parking zone, Grassy and weedy pools. 3531 m a.s.l. 28.6.2016	R.B. Angus, F-L Jia, Z-Q. Li & K. Chen	**LT796529**	-	**LT796539**
9	*B. emmerichi*	IBE-AN584	China	Sichuan, Kangding County Yajiaheng, Shallow stony & peaty pools 3337 m a.s.l. 30.6.2016	R.B. Angus, F-L Jia, Z-Q. Li & K. Chen	**LT796530**	-	**LT796540**
10	*B. emmerichi*	IBE-RA1167	China	N. Qinghai Hu, Gangca, 1 km SE of Gangca Dasi, stream, 3464m 5.6.2013	R.B. Angus, F.L. Jia & Y. Zhang.	**LT796526**	**LT796555**	-
11	*B. emmerichi*	IBE-RA1168	China	Qinghai, Golo, Maduo, Roadside pools on river flats ca 20 km SE Maduo. 8.6.2013	R.B. Angus, F.L. Jia & Y. Zhang.	**LT796527**	-	-
12	*B. emmerichi*	IBE-RA891	Tibet	S Tibet, S Namtso lake 4750m, banks, 21.7.2010	J. Schmidt	**LT796532**	**LT796552**	**LT796542**
13	*B. funereus* (Crotch, 1873)	MNCN-AI1208	California (US)	California, 9.2000	Y. Alarie	HF931173	HF931393	**LT796543**
14	*B. griseostriatus* (De Geer, 1774)	MNCN-AI952	Sweden	prov. Angermanland, Hörnefors parish, Norrbyskäv island, rock pool, 23.6.2006	A.N. Nilsson	**LT796533**	**LT796553**	**LT796544**

No	Species	Voucher	Country	Locality	Leg	COI	16S+tRNA +NAD1	H3
15	*B. griseostriatus* (De Geer, 1774) cplx	MNCN-AI1160	California (US)	Napa Co., Knoxville Recreation Area, 2000	T. Berendonk	HF931168	HF931387	**LT796547**
16	*B. griseostriatus* cplx	MNCN-AI1150	California (US)	Sacramento Co., Clay Station Rd., 20.6.1999	W.D. Shepard & C.B. Barr	HF931166	HF931385	**LT796545**
17	*B. griseostriatus* cplx	IBE-RA483	California (US)	Marin Co., Seasonal Pools in Dillans Beach Dunes, Spring 2011.	D. Post	HF931317	HF931541	**LT796546**
18	*B. griseostriatus strandi* (Brinck, 1943)	MNCN-AI1082	Norway	Bugöynes, 29.7.2006	S. Ligaard & B. Andrén	HF931153	HF931372	**LT796548**
19	*B. ibericus* (Dutton & Angus, 2007)	NHM-IR22	Portugal	Sa. Da Estrela, Torre, lagoon 25.7.1998	I. Ribera	EF670064	EF670030	EF670157
20	*B. macedonicus* (Georgiev, 1959)	MNCN-AI1120	Macedonia	Macedonia, Sar (Shar) Planina, Karanikolicko ezero Black Nick's Lake, 6.9.2006	R.B. Angus	**LT796534**	**LT796554**	**LT796549**
21	*B. multilineatus* (Falkenström, 1922)	MNCN-AI115	Faroes (Isl.)	20.9.2004	J. Hansen	HF931165	HF931384	-
22	*B. riberae* (Dutton & Angus, 2007)	MNCN-AI829	Turkey	Düzce, between Kartalkaya and Çaydurt, pools in mountain pass, 1700m 23.4.2006	I. Ribera	HF931232	HF931461	**LT796550**
23	*B. striatellus* (Le Conte, 1852)	NHM-IR295	California (US)	Mono co., Long Valley 19.6.2000	I. Ribera & A. Cieslak	HF931274	HF931511	-
24	*Stictotarsus falli* Nilsson, 2001	NHM-IR334	New Mexico (US)	New Mexico, 9.2000	Y. Alarie	EF670063	EF670029	EF670155
25	*Stictotarsus roffii* (Clark, 1862)	NHM-IR335	Texas (US)	Texas, 9.2000	Y. Alarie	AJ850607	AJ850355	EF670158

Figure 1. Mitotic chromosomes from mid gut of *Boreonectes* spp., arranged as karyotypes. **a**–**e** *B. alpestris*, **a** paratype ♂, Giemsa stained (from Dutton, Angus, (2007)) **b**, **c** ♂, Colle del Nivolet **b** Giemsa stained, **c** the same nucleus C-banded (from Angus (2010)) **d**, **e** San Bernardino, ♀, **d** Giemsa stained **e** the same nucleus C-banded **f**–**k** *B. emmerichi* **f**, **g** ♂ Yalashenshan **f** Giemsa stained **g** the same nucleus C-banded **h, i** ♀ Yalashenshan **h** Giemsa stained **g** the same nucleus C-banded **j**, **k** ♂ Mado **j** Giemsa stained **k** the same nucleus C-banded (from Angus et al (2015)) **l**–**n** *B. macedonicus* ♂♂ (from Angus (2008)) **l** Crno ezero Giemsa stained **m** Karanikoličko ezero Giemsa stained **n** the same nucleus C-banded **o, p** *B. emmerichi* ♂ shown in **f, g q, r** *B. macedonicus* Crete **q** ♂Giemsa stained **r** ♀C-banded (from Angus (2008). Scale = 5 μm.

complete karyotypes and in no case were more than 55 (♂) or 56 (♀) chromosomes counted. Since *B. griseostriatus* has a karyotype with 61 or 62 chromosomes (♂, ♀) it is very unlikely that this species was present in either sample.

We extracted and sequenced two specimens of *B. alpestris* from the same St. Gotthard and San Bernardino populations used to obtain the karyotypes, which had almost identical sequences for the mitochondrial genes (with only 1 mismatch in the COI gene) as two specimens from the same localities collected in 2000 and 2002

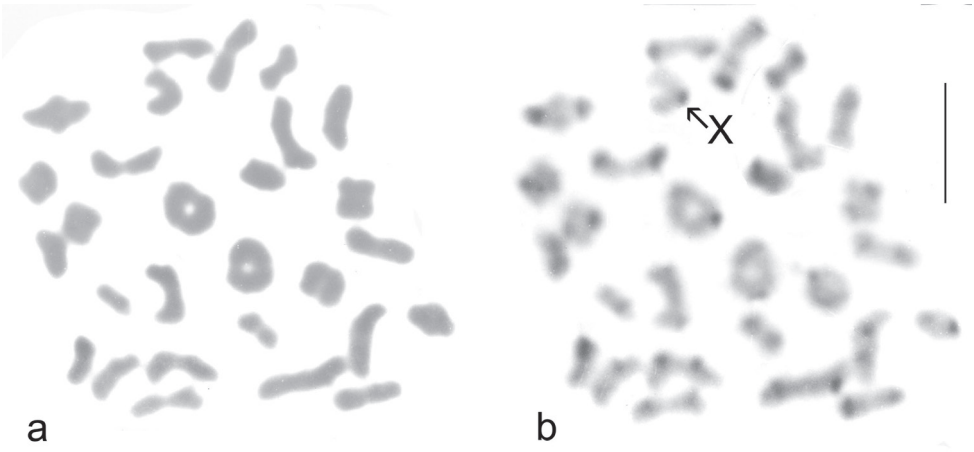


Figure 2. *B. alpestris* ♂ St Gotthard meiosis metaphase I **a** Giemsa stained **b** the same nucleus C-banded. Scale = 5 μm.

respectively (Table 2) and reported as *S. griseostriatus* in Abellán et al. (2013). These four specimens differ from one sequenced *B. alpestris* from Colle del Nivolet in five nucleotides in the COI gene (with a length of 826), and one in the 16S gene (with a length of 796 nucleotides). The nuclear gene H3 (with a length of 328 nucleotides) was identical for all sequenced specimens of *B. alpestris* (Table 1), and identical to the other Palaearctic species of the genus.

B. emmerichi (Falkenström, 1936)

Published information: 2n = 52 + X0 (♂), XX (♀) (Angus et al. 2015). Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 1f–k, n, o. The new preparations, from the Kangding area, (Fig. 1f–i) are much better than the Qinghai ones shown by Angus et al. (2015), with the chromosomes less condensed and the C-banding more clearly defined. They confirm the published features of the karyotype and also allow improved comparison of the karyotypes of *B. emmerichi* and *B. macedonicus* (Georgiev, 1959). Thus autosome pair 12 was stated to be more evenly metacentric in *B. emmerichi*, and comparison of the Sichuan male preparation (Fig. 1o, p) with a similarly elongate preparation of *B. macedonicus* from Karanikoličko ezero (Fig. 1m, n) and with Cretan *B. macedonicus* (Fig. 1q, r) confirms this. Similarly, the smaller size of autosome pair 26 when compared with pairs 24 and 25 in *macedonicus* as against the more similar sizes of these three pairs in *emmerichi* is confirmed. However, the possibly smaller X chromosome in *emmerichi*, as was suggested by preparations of mitosis but not metaphase II of meiosis, is shown not to be the case. This demonstration that these are chromosomally distinct species is in complete agreement with the degree of difference shown by the COI DNA of the two species (Fig. 5).

Variation within the species

B. alpestris (Dutton & Angus, 2007)

One of the surprises associated with collection of this species in 2016 was that the specimens from the St Gotthard pass appeared, even in the field, as noticeably larger than those from the San Bernardino. At the time it seemed that the St Gotthard specimens might be *B. griseostriatus* and those from the San Bernardino *B. alpestris*. In the event this proved not to be the case, but the size differences remain. Thus 10 ♂♂ from the San Bernardino range in length from 4.0–4.4 mm, with a mean length of 4.26 mm, while for 17 ♀♀from the same site the values are 5.2–5.1 mm, mean 4.38. From the St Gotthard the values are: 5 ♂♂, 4.3–4.6 mm, mean 4.51, and 8 ♀♀, 4.4–4.6 mm, mean 4.56 mm.

B. emmerichi (Falkenström, 1936)

As mentioned in the Introduction, the type material of *B. emmerichi* has more extensive darker markings, especially on the pronotum, than material from Qinghai, though it is matched by material from Sejilashan in southern Xizang (Angus et al. 2015). It was suggested that this more extensive darkening may be associated with wooded habitats. Latitude and longitude references for the type localities are, for Tatsienlu Tjiji 30°25' (=30.417°) N 102°40'(=102.667°) E (Balke 1992) and for Mukue Tatsienlu 30.05°N 102.03°E (Sykes et al. 2006). Google Earth gives altitudes of 1944 m a.s.l. for Tatsienlu Tjiji and 3935 m a.s.l. for Mukue Tatsienlu, with both localities in wooded areas. The localities from which *B. emmerichi* were collected in 2016 were: 1: Yalashenshan, 30.215°N 101.757°E, alt. 4052 m a.s.l.; 2: Yalashenshan, 30.205°N 101.755°E, alt. 4074 m a.s.l.; 3: Boij ta car parking area near Xinduqiao on the Kangding–Lhasa road, 30.048°N 101.569°E, alt. 3551 m a.s.l.; and 4: Yajiaheng, 29.927°N 101.995°E, alt. 3337 m a.s.l. Only the Boij ta locality (site 3) was in a wooded zone. In all localities water levels had risen recently due to the ongoing rain (see the silty water in Fig. 3e) but most of the pools had some aquatic vegetation. Only the pool shown in Fig. 3d had few beetles, suggesting that it was only recently flooded. All the chromosome preparations were from specimens collected at site 1. All this material has extensive dark markings. Fig. 4a shows the lectotype of *B. emmerichi*, while Fig. 4b, c shows two specimens from site 1. The specimen shown in "b" is a very close match for the lectotype and is the specimen from which the chromosomes shown in Fig. 1f, g were obtained. It seems clear that this more extensive darkening of the specimens is a regional phenomenon and not just a response to immediate local conditions.

Material from the northern part of the Tibetan Plateau is generally paler, and Fig. 4d shows a particularly pale specimen from Maduo, original shown as Fig. 1g by Angus et al. (2015). The palest specimen so far seen is from the western end of the Kun Lun mountains, taken by Ying Zhang. I have seen only a photograph of this specimen and this, though not good, does show the pattern (Fig. 4e).

Figure 3. *B. emmerichi* collecting sites. **a**, **b** site 1, Yalashenshan, 4052 m a.s.l, **b** with Fenglong Jia **c** site 2, Yalashenshan, 4074 m **d** site 4, Yajiaheng, 3337 m a.s.l. **e** site 3, Boij ta, 3495 m a.s.l. with Robert Angus collecting.

Figure 4. *B. emmerichi*, habitus. **a** lectotype **b**, **c** males from site 1, Yalashenshan **d** pale specimen from Maduo **e** the palest specimen seen, from the western Kun Lun mountains.

A molecular phylogeny of the Palaearctic *B. griseostriatus* complex

The analysis of the combined mitochondrial and nuclear data recovered a monophyletic *Boreonectes* with strong support (Fig. 5), although due to the reduced number of

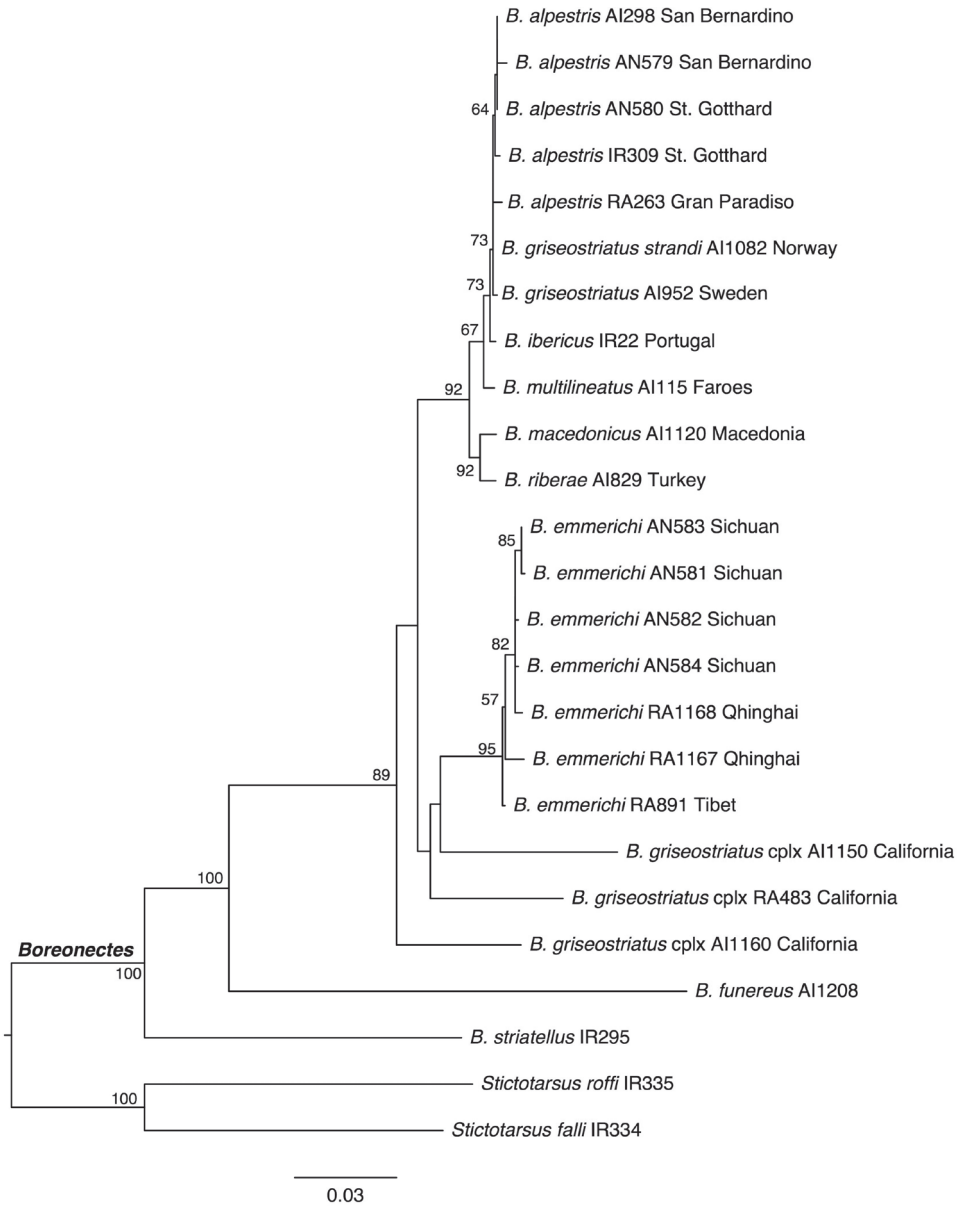


Figure 5. Phylogeny of the Palaearctic species of *Boreonectes*, obtained with maximum likelihood in RAxML using the combined mitochondrial and nuclear data. Numbers in nodes, bootstrap support. See Table 2 for details of the specimens.

outgroups its monophyly cannot be assessed adequately. The Palaearctic species, on the contrary, were not monophyletic, as *B. emmerichi* was found to be closer to some Nearctic specimens of the *B. griseostriatus* (De Geer, 1774) complex than to the western Palaearctic species, although with low support (Fig. 5). The Western Palaearctic spe-

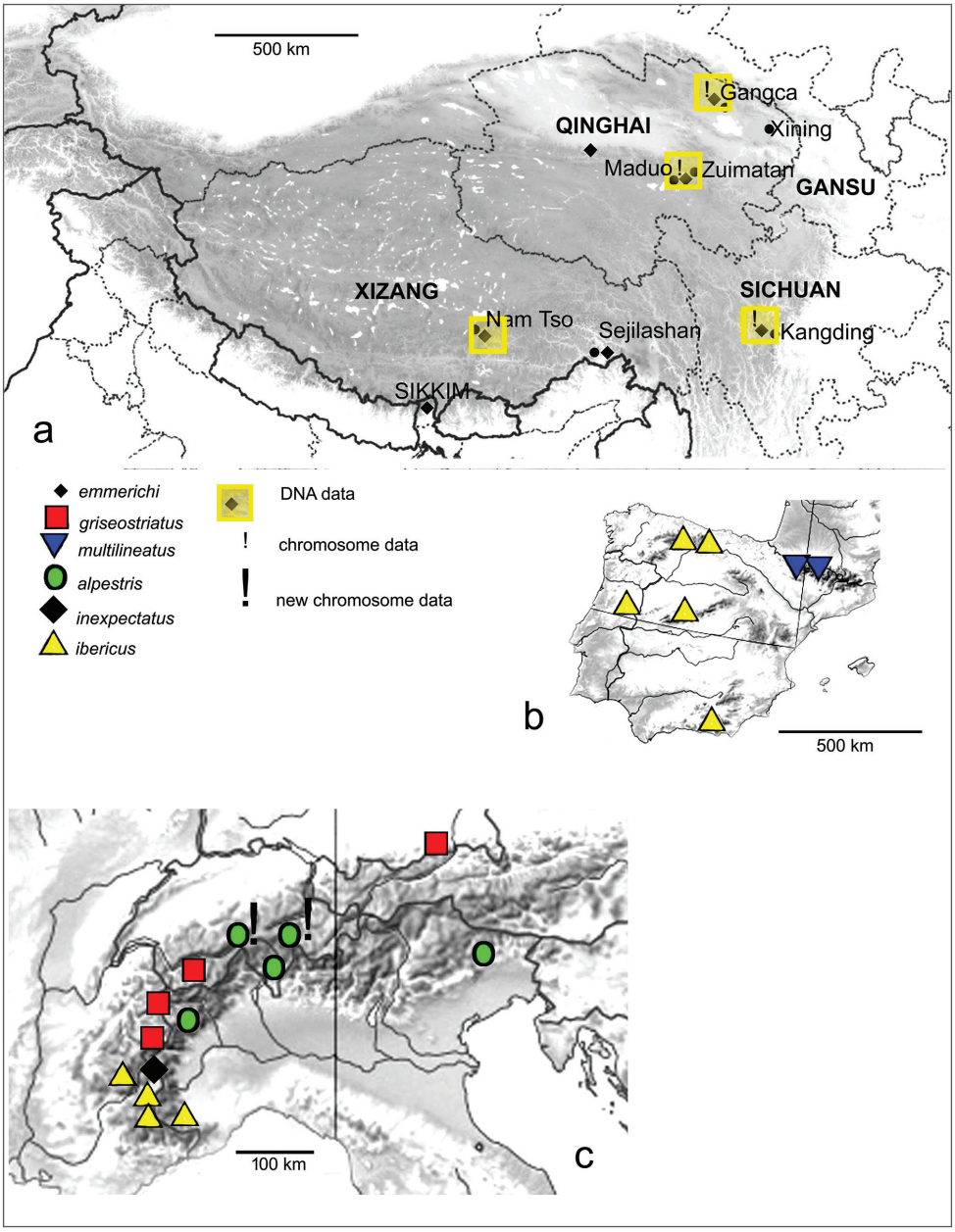


Figure 6. Maps of the distributions of various *B. griseostriatus* group species. **a** the Tibetan Plateau showing the localities of studied material of *B. emmerichi* **b** the Iberian Peninsula with the distributions of *B. ibericus* and *B. multilineatus* **c** The Alps with the distributions of *B. ibericus*, *inexpectatus*, *griseostriatus* and *alpestris.*

cies were recovered as monophyletic with strong support, and divided into the eastern Mediterranean *B. macedonicus* (Gerogiev, 1959) and *B. riberae* (Dutton & Angus, 2007) on one clade and all the western species on the other. Within the latter clade,

species seem to be very close to each other, with poor resolution among them and no variation in the nuclear marker (H3, see above), suggesting a very recent expansion and differentiation between them. Contrary to the lack of genetic variation among the Western Palaearctic species of the group, the three studied Nearctic specimens show a high divergence, with ca. 13% of variable positions in the COI gene. There are no available data on the karyotypes of these Nearctic populations, but most probably they represent a complex of undescribed species.

The Palaearctic species show a clearly uneven distribution of their diversity (Fig. 6). Thus, all the material from the Tibetan Plateau is *B. emmerichi*, with only limited variation in COI haplotypes. This can be compared with the situation in the Iberian Peninsula, occupying an area similar in size to the eastern part of the Tibetan Plateau, from which the *B. emmerichi* data have been obtained. Here again only one species, *B. ibericus* (Dutton and Angus 2007) is present, and this species has a wider distribution, also occurring in the French Alpes-Maritimes and Italian Alpi Marittime (Dutton and Angus 2007; Angus 2008), on Corsica (Angus 2010a) and in the Middle Atlas of Morocco (Angus 2010b). The occurrence of *B. multilineatus* (Falkenström, 1922) in the Pyrenees (Angus 2010b) reflects higher diversity, and this species is also known from inland Sweden and the British Isles (Dutton and Angus 2007) and, from COI data, from the Faroe Islands. The COI of this species appears sufficiently distinct (Fig. 5) to give confidence to the identification, although the study of more material is needed. However, the centre of high diversity is the Alps, where four chromosomally distinct species occur. One of these, *B. inexpectatus* is known only from a single lake, the Lac de Lauzet Inférieur (Dutton and Angus 2007) but the other three, *B. ibericus*, *B. griseostriatus* and *B. alpestris*, are more widely distributed, though apparently always allopatric. It is possible that the diversity of this group of species in Western Europe is, in part at least, a result of faunal movements associated with Pleistocene climatic fluctuations. *B. griseostriatus* group species are known as fossils in deposits dating from the Last Glaciation in the English Midlands (e.g. Coope et al. 1961), as well as from Starunia in the Ukrainian Carpathians (Angus 2010a), both areas outside the current known ranges of these species.

Acknowledgements

We thank the Natural History Museum London, Royal Holloway University of London and the Biology School of Sun Yat-sen University, Guangzhou for research facilities, Dandan Zhang for her help for booking hotel and flight tickets for the Kangding collection, Zhiqiang Li and Mr. Kai Chen for their help when we were working on field, A. Izquierdo, R. Alonso and A. Cardoso (IBE) for laboratory work, and Ying Zhang for information and the photograph of *B. emmerichi* from the Kun Lun mountains. We thank José Serrano and one anonymous reviewer for helpful suggestions. Molecular work was funded by projects CGL2010-15755 and CGL2013-48950-C2-1-P to IR.

References

Abellán P, Sánchez-Fernández D, Picazo F, Millán A, Lobo JM, Ribera I (2013) Preserving the evolutionary history of freshwater biota in Iberian National Parks. Biological Conservation 162: 116–126. https://doi.org/10.1016/j.biocon.2013.04.001

Angus RB (2008) Further Karyosystematic investigation of the *Stictotarsus griseostriatus* (De Geer) group of sibling species (Coleoptera: Dytiscidae). Comparative Cytogenetics 2(2): 151–156. http://www.zin.ru/journals/compcyt/pdf/2/Angus.pdf

Angus RB (2010a) A third karyosystematic investigation of the *Stictotarsus griseostriatus* (De Geer) group of sibling species (Coleoptera: Dytiscidae). Comparative Cytogenetics 4(1): 13–20. https://doi.org/10.3897/compcytogen.v4i1.27

Angus RB (2010b) *Boreonectes* gen. n. a new genus for the *Stictotarsus griseostriatus* (De Geer) (Coleoptera: Dytiscidae), with additional karyosystematic data on the group. Comparative Cytogenetics 4: 123–131.https:// doi.org/10.3897/compcytogen.v4i2.46

Angus RB, Angus EM, Jia FL, Chen Z-n, Zhang Y (2015) Further karyosystematic studies on the *Boreonectes griseostriatus* (De Geer) group of sibling species (Coleoptera, Dytiscidae)–characterisation of *B. emmerichi* (Falkenström, 1936) and additional European data. Comparative Cytogenetics 9(1): 133–144. https://doi.org/10.3897/CompCytogen.v9i1.4463

Balke M (1992) Systematische und faunistische Untersuchungen an paläarktischen, orientalischen und afrotropischen Arten von *Rhantus* Dejean (Coleoptera: Dytiscidae). Mitteilungen der Schweizerischen entomologischen Gesellschaft 65(3-4): 283–296. http://doi.org/10.5169/seals-402489

Coope GR, Shotton FW, Strachan I (1961) A late Pleistocene fauna and flora from Upton Warren, Worcestershire. Philosophical Transactions of the Royal Society B 244: 379–421. https://doi.org/10.1098/rstb.19610012

Dutton LA, Angus RB (2007) A karyosystematic investigation of a group of sibling species related to *Stictotarsus griseostriatus* (De Geer) (Coleoptera: Dytiscidae) Comparative Cytogenetics 1(1): 3–16. http.//www.zin.ru/journals/compcyt/pdf/1/DuttonAngus.pdf

García-Vázquez D, Bilton DT, Alonso R, Benetti CJ, Garrido J, Valladares LF, Ribera I (2016) Reconstructing ancient Mediterranean crossroads in *Deronectes* diving beetles. Journal of Biogeography 43: 1533–1545. https://doi.org/10.1111/jbi.12740

Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Briefings in Bioinformatics 9: 286–298. https://doi.org/10.1093/bib/bbn013

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), 14 November 2010, New Orleans, LA, 1–8. https://doi.org/10.1109/GCE.2010.5676129

Sykes DR, Madge RB, Trumbo ST (2006) Revision of *Nicrophorus* in part: new species and inferred phylogeny of the *nepalensis* group based on evidence from morphology and mitochondrial DNA. (Coleoptera: Silphidae: Nicrophorinae). Invertebrate Systematics 20(3): 30. https://doi.org/10.1071/is05020

CompCytogen 11(2): 203–212 (2017)
doi: 10.3897/CompCytogen.v11i2.10282
http://compcytogen.pensoft.net

SHORT COMMUNICATION

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Chromosome mapping in *Abracris flavolineata* (De Geer, 1773) (Orthoptera) from the Iguaçu National Park – Foz do Iguaçu, Paraná, Brazil

Mariana Bozina Pine[1], Raquel Bozini Gallo[1], Carlos Roberto Maximiano da Silva[1], Larissa Forim Pezenti[1], Fernando Campos De Domenico[2], Vilma Loreto[3], Renata da Rosa[1]

1 *Departamento de Biologia Geral, CCB, Universidade Estadual de Londrina (UEL), Londrina, Paraná, Brazil* **2** *Museu de Zoologia, Instituto de Biociências, Universidade de São Paulo (USP), São Paulo, São Paulo, Brazil* **3** *Departamento de Genética, CCB, Universidade Federal de Pernambuco, Recife, Brazil*

Corresponding author: *Renata da Rosa* (renata-darosa@uel.br)

Academic editor: *A. Bugrov* | Received 24 August 2016 | Accepted 1 February 2017 | Published 31 March 2017

http://zoobank.org/C0BB769F-53A6-4B31-8226-CC4DBF3890B5

Citation: Bozina Pine MB, Gallo RB, da Silva CRM, Pezenti LF, De Domenico FC, Loreto V, Rosa R (2017) Chromosome mapping in *Abracris flavolineata* (De Geer, 1773) (Orthoptera) from the Iguaçu National Park – Foz do Iguaçu, Paraná, Brazil. Comparative Cytogenetics 11(2): 203–212. https://doi.org/10.3897/CompCytogen.v11i2.10282

Abstract

In this paper, we present the cytomolecular analysis of a population of *Abracris flavolineata* collected in the largest fragment of the Brazilian Atlantic forest, the Iguaçu National Park. The diploid number in males was 23 (22+X0), with two large pairs (1–2), 7 medium (3–9), 2 small (10–11) and the X chromosome of medium size. Heterochromatic blocks were evident in the pericentromeric regions of all chromosomes. Heterogeneity in the distribution of heterochromatin was observed, with a predominance of $DAPI^+$ blocks. However, some chromosomes showed CMA_3^+ blocks and other $DAPI^+/CMA_3^+$ blocks. The 18S rDNA sites were distributed on the short arms of 5 pairs. In two of these pairs, such sites were in the same chromosome bearing 5S rDNA, and one of the bivalents, they were co-located. Histone H3 genes were found on one bivalent. The results added to the existing cytogenetic studies provided evidence of great karyotypic plasticity in the species. This pliancy may be the result of vicariant events related to the geographical distribution of different populations of *A. flavolineata*.

Keywords

Acrididae, Brazilian Atlantic forest, chromosome banding, fluorescence *in situ* hybridization, grasshopper

Introduction

The family Acrididae is one of the most speciose, heterogeneous and conceivably the most recent in the Acridoidea group (Song et al. 2015). Among its subfamilies, Ommatolampidinae comprises 9 tribes and over 50 genera, with geographical distribution in North, Central and South America (Amédégnato 1974, Carbonell 1977). This subfamily is associated with secondary growth of vegetation in dry forest areas (Amédégnato and Decamps 1980, Rowell 1987), or even in clearings within Tropical rainforests (Braker 1991, Sperber 1996). The genus *Abracris* Walker, 1870 belongs to this subfamily and is distributed in the Neotropical region, from Mexico to Argentina (Roberts and Carbonell 1981).

Although there are several cytogenetic studies of Neotropical Acrididae species, especially of the subfamily Ommatolampidinae, there are few studies that elucidate the molecular structure of chromosomes. Specific C-banding techniques for the identification and classification of chromosomes have been applied more often in *Abracris flavolineata* species (De Geer, 1773) (Cella and Ferreira 1991, Rocha et al. 2011).

According to Cella and Ferreira (1991), *Abracris flavolineata* shows 2n=24/23 (females/males), with the XX/X0 sex chromosome system. Seven subtelocentric, two metacentric and two submetacentric pairs, and the subtelocentric chromosome X make up the karyotype of *A. flavolineata*. In addition, this species displayed B chromosomes (Cella and Ferreira 1991, Bueno et al. 2013). Molecular markers were applied to understand their molecular composition and mechanisms of evolution (Bueno et al. 2013, Milani and Cabral-de-Mello 2014, Palacios-Gimenez et al. 2014).

Knowledge of Ommatolampidinae is limited, considering the diversity of the subfamily. Few cytogenetic tools have been applied, and the only samples that have been analyzed were from southeastern Brazil (Rio Claro/São Paulo state). To improve the knowledge about this species, in particular about this group, it was necessary, not only to use more cytogenetic tools but also study samples from other regions of Brazil. Thus, this work aimed to study specimens of *A. flavolineata* collected in the Iguaçu National Park (Southern Brazil), a paramount area of the Brazilian Atlantic, using different cytogenetic markers in order to understand the evolutionary mechanisms present in this group of insects.

Material and methods

The current study used twenty male specimens of *Abracris flavolineata* collected from the Iguaçu National Park, Foz do Iguaçu, Paraná State, Brazil – 25°37'40.67"S; 54°27'45.29"W (DDM). The individuals were identified and deposited in the Museu de Zoologia da Universidade de São Paulo (MZUSP) and collected with the permission of Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio, protocol number 31946-2. The insects were anesthetized and dissected before fixing their testes in methanol: acetic acid (3:1). Chromosome preparations for mitotic and meiotic

analyses were made through cell suspension by maceration in one drop of 45% acetic acid. Heterochromatin distribution was analyzed by Giemsa C-banding (Sumner 1972). The GC- and AT-rich bands were detected with chromomycin A_3 (CMA_3) and 4'-6-diamino-2-phenylindole (DAPI), respectively (Schweizer et al. 1983).

In addition to the karyotype studies, genomic DNA was extracted from the muscle tissue of a male specimen using the phenol/chloroform procedure described by Sambrook and Russel (2001). Unlabelled 18S rDNA, 5S rDNA and Histone H3 gene probes were generated by polymerase chain reaction (PCR) using the primers: 18S rDNAF 5'-CCTG AGAAACGGCTACCACATC-3' and 18S rDNAR 5'-GAGTCTCGTTCGTTATCGGA-3'(Whiting 2002); 5SrDNAF 5'-AACGACCATACCACGCTGAA-3' and 5SrDNAR 5'-AA GCGGTCCCCCATCTAAGT-3' (Loreto et al. 2008a); H3F 5'-ATATCCTTRGGCATRAT RGTGAC-3' and H3R 5'-ATGGCTCGTACCAAGCAGACVGC-3' (Colgan et al. 1998). The probes isolated by PCR were labeled with digoxigenin-11-dUTP and biotin-11-dATP by PCR. The *in situ* hybridization procedure was performed according to Pinkel et al. (1986).

Results and discussion

All samples of *Abracris flavolineata* collected in the Iguaçu National Park presented 2n=23 (♂) and an XX/X0 sex-determination system (Fig. 1). The chromosomes were classified into two large pairs (1–2), 7 medium pairs (3–9) and 2 small pairs (10–11). The X chromosome is medium-sized. Pairs 1 to 7 and the X chromosomes are subtelo-acrocentric; pairs 8 and 9 are submetacentric, and pairs 10 and 11 are metacentric (Fig. 1a). The X chromosome presented itself univalent at diakinesis (Fig. 1b).

The karyotype structure observed in this study is conserved regarding the diploid number and morphology of the A chromosomes, corroborating the data already established for the species by Cella and Ferreira (1991) and by Bueno et al. (2013) studying specimens from Rio Claro (southeastern Brazil). However, these authors reported the occurrence of supernumerary chromosomes, which were not found in samples from National Iguaçu Park. The sex-determination pattern considered the ancestor of grasshoppers was present in specimens of *Abracris flavolineata*, with males with the X0 and females with the XX sex chromosome system. In Acrididae, some species, such as *Rhammatocerus brasiliensis* (Bruner, 1904), *R. brunneri* (Giglio-Tos, 1895), *R. palustris* Carbonell, 1988, *R. pictus* (Bruner, 1900), *Orthoscapheus rufipes* (Thunberg, 1824), among others, show a karyotype similar to that of *Abracris flavolineata*, with 2n = 23,X0 and 24,XX, being considered the ancestral pattern of the family (White 1973; Loreto et al. 2008b; Rocha et al. 2011).

Heterochromatic blocks were shown in the pericentromeric regions of all chromosomes (Fig. 1d–i). Chromosome 4 showed a slight heterochromatic block in the proximal region of the long arm (Fig. 1f). Nevertheless, this block was also viewed in most meiotic stages. It was possible note discrete heterochromatic bands in some chromosomes (Fig. 1d–f). These bands appear in some cells and not others, and this

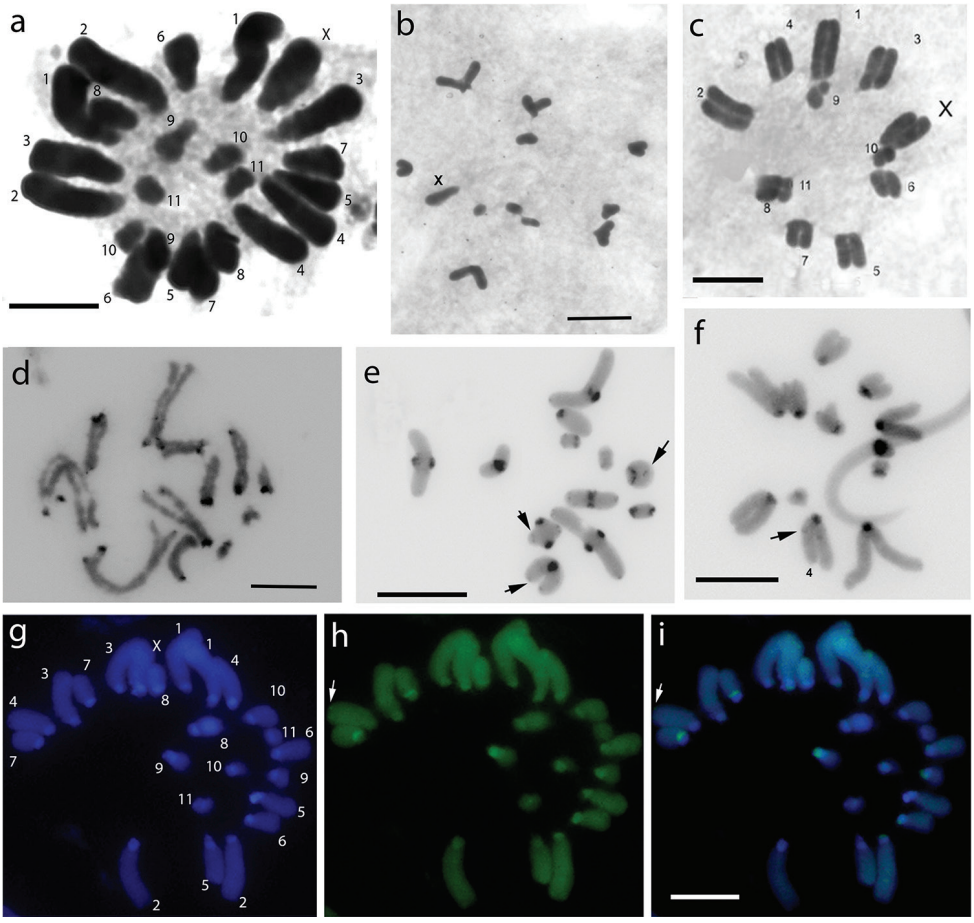


Figure 1. Mitotic and meiotic stages of *Abracris flavolineata* by Giemsa conventional staining (**a–c**), C-banding (**d–f**) and fluorochromes staining (**g–i**): **a** mitotic metaphase **b** diakinesis **c** metaphase II **d** pachytene **e** diakinesis **f** metaphase II **g** mitotic metaphase by DAPI staining **h** mitotic metaphase by CMA_3 staining **i** overlapping DAPI/CMA_3. The numbers correspond to autosomes. X corresponds to the sex chromosome. The arrows indicate the discrete heterochromatic bands. Bar= 5μm.

may be the result of differences in the condensation of chromosomes in different cells or a technical artifact. The position of heterochromatin at pericentromeric regions is a common feature in Acrididae, being observed in several species (Loreto and Souza 2000, Rocha et al. 2004). The heterochromatin plays a very important role in the evolution of karyotypes. The pericentromeric preferential distribution may be related to equilocal transfer between non-homologous chromosomes of similar size by positioning in the nucleus by bouquet configuration (Schweizer and Loidl 1987).

Staining with fluorochromes CMA_3/DAPI showed heterogeneity in the distribution of heterochromatin on autosomes: (i) $DAPI^+$ bands located at pericentromeric regions in most chromosomes; (ii) pericentromeric $DAPI^+/CMA_3^+$ bands on 2 and 6

chromosomes; (iii) a discrete CMA_3^+ band in the terminal region on chromosome 4; and (iv) $DAPI^+$ adjacent to CMA_3^+ blocks on three pairs (5, 7 and 9). The X chromosome showed adjacency of $DAPI^+$-CMA_3^+-$DAPI^+$ bands (Figure 1g-i). The distribution of these blocks can be best demonstrated in the ideogram (Figure 3).

There are three patterns of distribution of GC-rich blocks in grasshoppers: (i) CMA_3^+ bands related to NORs location (Souza et al. 1998, Loreto and Souza 2000, Rocha et al. 2012); (ii) CMA_3^+ in all chromosomes (Souza et al. 1998, Pereira and Souza 2000); and, (iii) GC-rich blocks in some chromosomes (Loreto and Souza 2000, Souza et al. 2003, Loreto et al. 2005, Souza and Melo 2007, Rocha et al. 2012). AT-rich heterochromatin is rarely encountered in Acrididae, as is the case *Arcyptera fusca* (Pallas, 1773), *A. tornosi* Bolívar, 1884 (Bella and Gosálvez 1991) and *Dociostaurus genei* (Ocskay, 1832) (Rodríguez Iñigo et al. 1993). In *Abracris flavolineata*, studies show that most heterochromatin is GC-rich and there are no AT-rich blocks (Bueno et al. 2013). Thus, the pattern observed in specimens of *A. flavolineata* in this study shows a considerable karyotypic differentiation, where heterochromatinization processes, such as heterochromatin spreading, can be responsible for the differentiation of the Iguaçu National Park population, as in the grasshopper of the genus *Tropidacris* Scudder, 1869 (Rocha et al. 2015). Moreover, this differentiation of heterochromatin may be related to action of transposable elements (Grewal and Jia 2007).

Although the position of rDNA sites was similar to that previously reported for other populations of this species, on terminal region, the number of sites was different from that observed in previous studies. The 18S rDNA sites were distributed on terminal regions of the short arms of all analyzed specimens of 5 pairs (1, 2, 4, 7 and 10), with no variations. Two of these sites were on the same chromosome as 5S rDNA sites. These sites were co-located on one of the bivalents (Fig. 2a and b). Bueno et al. (2013) reported a variation from 5 to 9 chromosomes bearing 18S rDNA in the species of the southeastern population. Conversely, the 5S rDNA sites were distributed in the terminal portion of the bivalent 1, in the interstitial region of bivalent 2 and pericentromeric region of 7. This pattern was more conservative where two sites (1 and 2) were similar to those observed by Bueno et al. (2013). A difference in the present study was the co-location of 18S rDNA and 5S sites on pair 7, where these sequences are located in heterochromatic regions. Cabral-de-Mello et al. (2011a) analyzed the distribution of 45S and 5S rDNA in 29 species of family Acrididae, and noted the predominance of the location of sites on separate chromosomes (80.3% of the clusters). Considering this study, the co-location of DNA sites may be associated with recent evolutionary processes in the species from the Iguaçu National Park.

Histone H3 genes were located on a corresponding bivalent of pair 8 (Fig. 2c and d). This location is similar in number to that found in most species of grasshoppers, distributed on a single pair of chromosomes (Cabrero et al. 2009, Cabral-de-Mello 2011a, 2011b, Neto et al. 2013). However, our data differ from those reported for the population of *A. flavolineata* of southeastern Brazil, in which these genes were observed on all chromosomes, including the X chromosome, but not on B chromosomes (Bueno et al. 2013). As in our results, the distribution of the histone gene was

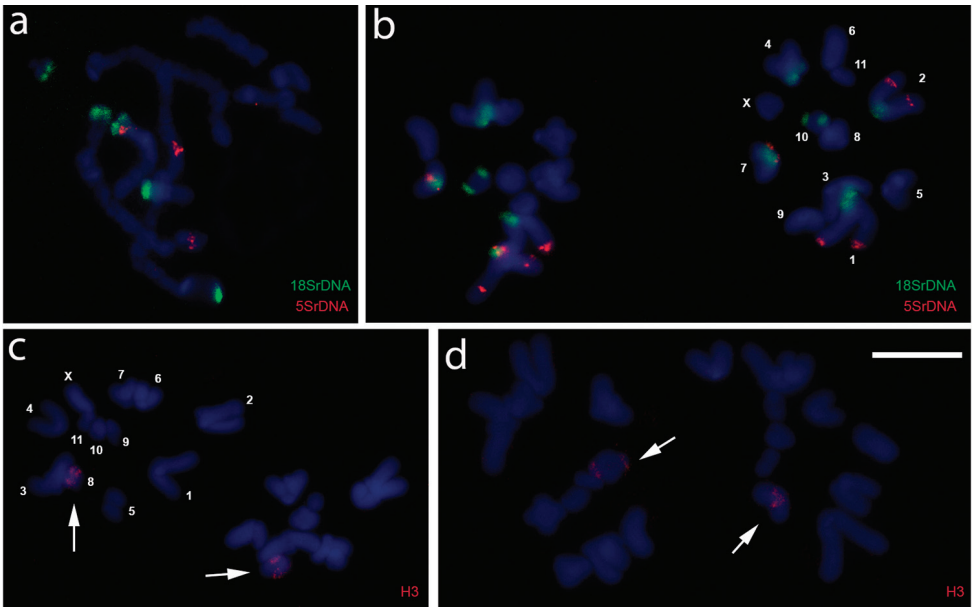


Figure 2. Fluorescent *in situ* hybridization of *A. flavolineata* in meiocytes: **a, b** 18S rDNA (green) and 5S rDNA (red) probes **c, d** histone H3 gene probe: **a** pachytene **b–d** two cells in metaphase II. The numbers correspond to autosomes. X corresponds to the sex chromosome. The arrows indicate the histone H3 gene sites. Bar= 5μm.

adjacent to the CMA_3^+ and $DAPI^+$ heterochromatic blocks (Fig. 3), these genes are probably intercalated to heterochromatin blocks. Thus, the repetitive DNA present in the heterochromatin may play an important role in the dispersion of this sequence leading to differences in distribution between different histone genes of *A. flavolineata* populations studied herein.

Although the chromosome number and karyotype formula of the specimens studied here were the same as those described in the southeastern population (Cella and Ferreira 1991, Bueno et al. 2013), a considerable variation in the distribution of AT and GC-rich bands and specific gene sequences (18S, 5S and H3) was found. This variation refers to significant chromosomal changes, as spreading of heterochromatin can be involved in the evolution of this species. The populations studied by Cella and Ferreira (1991) and Bueno et al. (2013) inhabit Atlantic rainforest fragments, which once formed a continuous biome. The forest fragments mentioned above are approximately 790 km away from the population studied here (Iguaçu National Park). Such distance, coupled with the fact that these species probably underwent vicariance events that led to the disruption of these populations, caused reproductive isolation that led to chromosomal differences. The data obtained, together with the existing cytogenetic studies, allow us to suggest that *Abracris flavolineata* has an extensive karyotype plasticity.

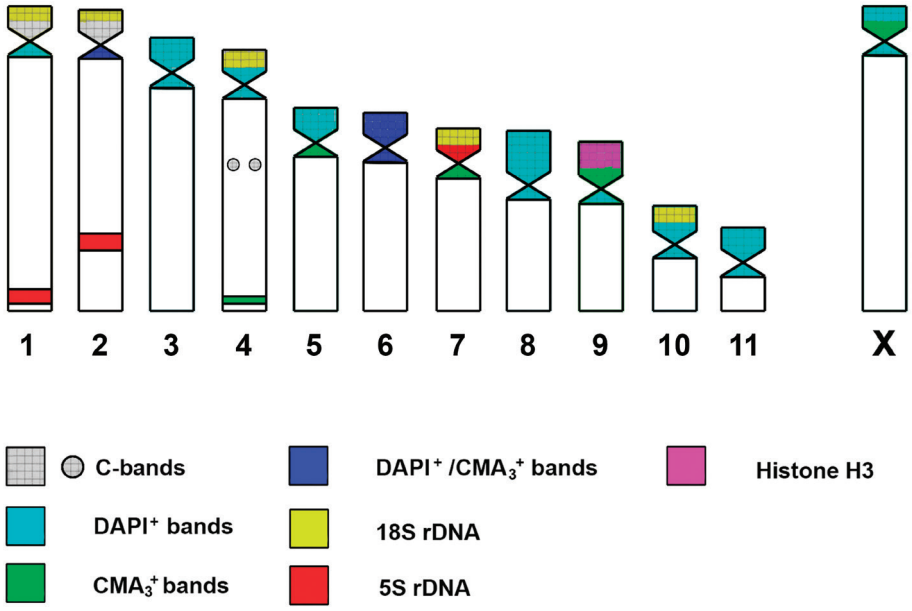


Figure 3. Idiogram showing the mapping of different sequences studied in *Abracris flavolineata* from the Iguaçu National Park.

Acknowledgements

The authors are grateful to the National Iguaçu Park staff members for their technical assistance; to Edson Mendes Francisco for his help with the sample collection. This work was supported by Universidade Estadual de Londrina. The researchers received permission from Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio to collect grasshoper specimens.

References

Amédégnato C (1974) Les genes d'acridiens néotropicaux, leur classification par families, sous-families et tribus. Acrida 3: 193–204.

Amédégnato C, Decamps M (1980) Évolution dês populations d'orthoptéres d'amazonie du nord-ouest dans les cultures traditionneles et hes formations secondaries d'origine anthropique. Acrida 9: 1–33.

Bella JL, Gosálvez J (1991) C-banding with specific fluorescent DNA-ligands: a new approach to constitutive heterochromatin heterogeneity. Biotechnic and Histochemistry 1: 44–52. https://doi.org/10.3109/10520299109110549

Braker E (1991) Natural history of a neotropical gap-inhabiting grasshopper. Biotropica 23: 41–50. https://doi.org/10.2307/2388686

Bueno D, Palacios-Gimenez OM, Cabral-de-Mello DC (2013) Chromosomal mapping of repetitive DNAs in the grasshopper *Abracris flavolineata* reveal possible ancestry of the B chromosome and H3 histone spreading PLoS ONE 8: e66532. https://doi.org/10.1371/journal.pone.0066532

Cabral-de-Mello DC, Cabrero J, López-León MD, Camacho JPM (2011a) Evolutionary dynamics of 5S rDNA location in acridid grasshoppers and its relationship with H3 histone gene and 45S rDNA location. Genetica 139: 921–931. https://doi.org/10.1007/s10709-011-9596-7

Cabral-de-Mello DC, Martins C, Souza MJ, Moura RC (2011b) Cytogenetic mapping of 5S and 18S rRNAs and H3 histone genes in 4 ancient Proscopiidae grasshopper species: contribution to understanding the evolutionary dynamics of multigene families. Cytogenetic and Genome Research 132: 89–93. https://doi.org/10.1159/000317476

Cabrero J, López-León MD, Teruel M, Camacho JPM (2009) Chromosome mapping of H3 and H4 histone gene clusters in 35 species of acridid grasshoppers. Chromosome Research 17: 397–404. https://doi.org/10.1007/s10577-009-9030-5

Carbonell CS (1977) Origin, evolution and distribuition of the neotropical acridomorph fauna (Orthoptera): A preliminary hypothesis. Revista de la Sociedad Entomológica Argentina 36: 153–175.

Cella DM, Ferreira A (1991) The cytogenetics of *Abracris flavolineata* (Orthoptera, Caelifera, Ommatolampinae, Abracrini). Revista Brasileira Genética 14: 315–329. http://www.gmb.org.br/Revistas/V14/v14a23.pdf

Colgan DJ, McLauchlan A, Wilson GDF, Livingston SP, Edgecombe GD, Macaranas J, Cassis G, Gray MR (1998) Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. Australian Journal of Zoology 46: 419–437. https://doi.org/10.1071/ZO98048

Grewal SIS, Jia S (2007) Heterochromatin revisited. Nature Reviews Genetics 8: 35–46. https://doi.org/10.1038/nrg2008

Loreto V, Souza MJ (2000) Karyotype, constitutive heterochromatin and nucleolar organizer regions (NORs) in *Belosacris coccineipes* (Acrididae-Leptysminae). Genetics and Molecular Biology 23: 575–579. https://doi.org/10.1590/S1415-47572000000300013

Loreto V, Stadtler E, de Melo NF, Souza MJ (2005) A comparative cytogenetic analysis between the grasshopper species *Chromacris nuptialis* and *C. speciosa* (Romaleidae): constitutive heterochromatin variability and rDNA sites. Genetica 125: 253–260. https://doi.org/10.1007/s10709-005-0368-0

Loreto V, Cabrero J, López-León MD, Camacho JPM, Souza MJ (2008a) Possible autosomal origin of macro B chromosomes in two grasshopper species. Chromosome Research 16: 233–241. https://doi.org/10.1007/s10577-007-1188-0

Loreto V, Cabrero J, López-León MD, Camacho JPM, Souza MJ (2008b) Comparative analysis of rDNA location in five Neotropical gomphocerine grasshopper species. Genetica 132: 95–101. https://doi.org/10.1007/s10709-007-9152-7

Milani D, Cabral-de-Mello DC (2014). Microsatellite organization in the grasshopper *Abracris flavolineata* (Orthoptera: Acrididae) revealed by FISH mapping: remarkable spreading in the A and B Chromosomes. PloS ONE 9: e97956. https://doi.org/10.1371/journal.pone.0097956

Neto MSR, Souza MJ, Loreto V (2013) Chromosomal evolution of rDNA and H3 histone genes in representative Romaleidae grasshoppers from northeast Brazil. Molecular Cytogenetics 6: 41. https://doi.org/10.1186/1755-8166-6-41

Palacios-Gimenez OM, Bueno D, Cabral-de-Mello DC (2014) Chromosomal mapping of two Mariner-like elements in the grasshopper *Abracris flavolineata* (Orthoptera: Acrididae) reveals enrichment in euchromatin. European Journal of Entomology 111(3): 329–334. https://doi.org/10.14411/eje.2014.052

Pereira LG, Souza MJ (2000) Nature and distribution of constitutive heterochromatin and NOR location in the grasshopper *Phaeoparia megacephala* (Romaleidae: Orthoptera). Cytobios 103: 111–119.

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences USA 83: 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Roberts HR, Carbonell CS (1981) A revision of the neotropical genus *Abracris* and related genera (Orthoptera, Acrididae, Ommatolampinae). Proceedings of the Academy of Natural Sciences of Philadelphia 133: 1–14. http://www.jstor.org/stable/4064766

Rocha MF, Souza MJ, Moura RC (2004) Karyotype analysis, constitutive heterochromatin and NOR distribution in five grasshopper species of the subfamily Leptysminae (Acrididae). Caryologia 57: 107-116. https://doi.org/10.1080/00087114.2004.10589378

Rocha MF, Melo NF, Souza MJ (2011) Comparative cytogenetic analysis of two grasshopper species of the tribe Abracrini (Ommatolampinae, Acrididae). Genetics and Molecular Biology 34: 214–219. https://doi.org/10.1590/S1415-47572011000200008

Rocha MF, Souza MJ, Moura RC (2012) Karyotypic analysis, constitutive heterochromatin and NOR distribution in five grasshopper species of the subfamily Leptysminae (Acrididae). Caryologia 57: 107–116. https://doi.org/10.1080/00087114.2004.10589378

Rocha MF, Pine MB, Oliveira EFAS, Loreto V, Gallo RB, Da Silva CRM, De Domenico FC, Da Rosa R (2015) Spreading of heterochromatin and karyotype differentiation in two *Tropidacris* Scudder, 1869 species (Orthoptera, Romaleidae). Comparative Cytogenetics 9(3): 435–450. https://doi.org/10.3897/CompCytogen.v9i3.5160

Rodriguez-Iñigo E, Bella JL, La Vega CG (1993) Heterochromatin differentiation between two species of the genus *Dociostaurus* (Orthoptera: Acrididae). Heredity 70: 458–465. https://doi.org/10.1038/hdy.1993.67

Rowell CHF (1987) The biogeography of Costa Rican acridid grasshoppers, in relation to their phylogenetic origins and ecology. In: Baccetti BM (Ed.) Evolutionary biology of orthopteroid insects. Ellis Horwood, Chicester, 470–482.

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, New York, 1659 pp.

Schweizer D, Loidl J (1987) A model for heterochromatin dispersion and the evolution of C-bands patterns. In: Stahl A, Luciani JM, Vagner-Capodano AM (Eds) Chromosomes Today. Allen & Unwin, London, 61–74. https://doi.org/10.1007/978-94-010-9166-4_7

Schweizer D, Mendelak M, White MJD, Contreras N (1983) Cytogenetics of the parthenogenetic grasshopper *Warramaba virgo* and its bisexual relatives. X. Pattern of florescent banding. Chromosoma 88: 227–236. https://doi.org/10.1007/BF00285625

Song H, Amédégnato C, Cigliano MM, Desutter-Grandcolas L, Heads SH, Huang Y, Otte D, Whiting MF (2015) 300 million years of diversification: elucidating the patterns of orthopteran evolution based on comprehensive taxon and gene sampling. Cladistics. 1–31. https://doi.org/10.1111/cla.12116

Souza MJ, Rufas JS, Orellana J (1998) Constitutive heterochromatin, NOR location and FISH in the grasshopper *Xyleus angulatus* (Romaleidae). Caryologia 51: 73–80. https://doi.org/10.1080/00087114.1998.10589122

Souza MJ, Haver PRO, Melo NF (2003) Karyotype, C- and florescence banding patterns,NOR location and FISH in the grasshopper *Xestotrachelus robustus* (Romaleidae). Caryologia 56: 261–267. https://doi.org/10.1080/00087114.2003.10589333

Souza MJ, Melo NF (2007) Chromosome study in *Schistocerca* (Orthoptera-Acrididae-Cyrtacanthacridinae): Karyotypes and distribution patterns of constitutive heterochromatin and nucleolus organizer regions (NORs). Genetics and Molecular Biology 30: 54–59. https://doi.org/10.1590/S1415-47572007000100011

Sperber CF (1996) Field diet of the grasshopper *Abracris dilecta* Walker (Orthoptera, Acrididae). Revista Brasileira de Zoologia 13: 127–135. https://doi.org/10.1590/S0101-81751996000100012

Sumner AT (1972) A simple techinique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

White MJD (1973) Animal cytology and evolution. 3rd ed. Cambridge University Press, London, 455p.

Whiting MF (2002) Mecoptera is paraphyletic: multiple genes and phylogeny of *Mecoptera* and *Siphonaptera*. Zoologica Scripta 31: 93–104. https://doi.org/10.1046/j.0300-3256.2001.00095.x

CompCytogen 11(2): 213–223 (2017)
doi: 10.3897/CompCytogen.v11i2.11572
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotype characterization and comparison of three hexaploid species of *Bromus* Linnaeus, 1753 (Poaceae)

Leonardo Luís Artico[1], Ana Cristina Mazzocato[1], Juliano Lino Ferreira[1], Carlos Roberto Carvalho[2], Wellington Ronildo Clarindo[3]

1 *Setor de Plantas Forrageiras, Embrapa Pecuária Sul. CEP: 96.401-970, Bagé – RS, Brazil* **2** *Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa. CEP: 36.570-000, Viçosa – MG, Brazil* **3** *Laboratório de Citogenética e Cultura de Tecidos Vegetais, Departamento de Biologia, Universidade Federal do Espírito Santo. CEP: 29.500-000, Alegre – ES, Brazil*

Corresponding author: *Wellington Ronildo Clarindo* (welbiologo@gmail.com)

Academic editor: *M. Dhar* | Received 21 December 2016 | Accepted 27 February 2017 | Published 7 April 2017

http://zoobank.org/2A957CAE-A700-4752-BAA8-D365D7398765

Citation: Artico LL, Mazzocato AC, Ferreira JL, Carvalho CR, Clarindo WR (2017) Karyotype characterization and comparison of three hexaploid species of *Bromus* Linnaeus, 1753 (Poaceae). Comparative Cytogenetics 11(2): 213–223. https://doi.org/10.3897/CompCytogen.v11i2.11572

Abstract

Chromosome morphometry and nuclear DNA content are useful data for cytotaxonomy and to understand the evolutionary history of different taxa. For the genus *Bromus* Linnaeus, 1753, distinct ploidy levels have been reported, occurring from diploid to duodecaploid species. The geographic distribution of *Bromus* species has been correlated with chromosome number and ploidy level. In this study, the aims were to determine the nuclear genome size and characterize the karyotype of the South American *Bromus* species: *Bromus auleticus* Trinius ex Nees, 1829, *Bromus brachyanthera* Döll, 1878 and *Bromus catharticus* Vahl, 1791. The mean nuclear 2C value ranged from 2C = 12.64 pg for *B. catharticus* to 2C = 17.92 pg for *B. auleticus*, meaning a maximum variation of 2C = 5.28 pg, equivalent to 41.70%. Despite this significant difference in 2C value, the three species exhibit the same chromosome number, 2n = 6x = 42, which confirms their hexaploid origin. Corroborating the genome size, the chromosome morphometry (total, short- and long-arm length) and, consequently, the class differed among the karyotypes of the species. Based on the first karyograms for these *Bromus* species, some morphologically similar and several distinct chromosome pairs were found. Therefore, the karyotype characterization confirmed the hexaploid origin of the studied *Bromus* species, which differ in relation to the karyogram and the nuclear 2C value. Considering this, cytogenetics and flow cytometry can be used to discriminate *Bromus* species, contributing to taxonomy and systematic studies and providing information on the evolutionary history of this taxa.

Keywords
Karyogram, nuclear genome size, polyploidy, forage grasses

Introduction

The genus *Bromus* Linnaeus, 1753, family Poaceae comprises more than 160 species of annual and perennial grasses (Acedo and Liamas 2001). This taxon is widely distributed around the world (Williams et al. 2011), demonstrating the adaptability of its species (Martinello and Schifino-Wittmann 2003). The genus *Bromus* includes important forage grasses, such as *Bromus auleticus* Trinius ex Nees, 1829, *Bromus brachyanthera* Döll, 1878 and *Bromus catharticus* Vahl, 1791 (Puecher et al. 2001, Martinello and Schifino-Wittmann 2003, Iannone et al. 2010).

The basic chromosome number of the genus *Bromus* is x = 7, and its species possess karyotypes varying from 2n = 2x = 14 (diploid) to 2n = 12x = 84 (duodecaploid) (Fedorov 1969, Armstrong 1984, 1987, Klos et al. 2009, Williams et al. 2011). Most of the species are diploid (2n = 2x = 14) or tetraploid (2n = 4x = 28) (Martinello and Schifino-Wittmann 2003), but large variation in chromosome number among *Bromus* species has been found, such as: *Bromus cappadocicus* Boissier et Balansa, 1857, and *B. tomentosus* Trinius, 1813, with 2n = 2x = 14; *B. erectus* Huds., 1762, *B. biebersteinii* Roemer et Schultes, 1817, and *B. stenostachyus* Boissier, 1884, with 2n = 4x = 28; *B. tomentellus* Boissier, 1846, *B. variegatus* M. Bieberstein, 1819 (Sheidai et al. 2008), *B. auleticus* (Martinello and Schifino-Wittmann 2003), *B. bonariensis* Parodi et J. H. Camara, 1963, *B. brevis* Steudel, 1854, *B. parodii* Covas et Itria, 1968, *B. brachyanthera* and *B. catharticus* Vahl, 1791 (Schifino and Winge 1983, Naranjo 1985) with 2n = 6x = 42. Variation in chromosome number has also been found within the same species, such as in *Bromus kopetdagensis* Drobow, 1925, (2n = 6x = 42 in Tehran and 2n = 10x = 70 in Emamzadeh-Hashem; Sheidai et al. 2008) and in *Bromus setifolius* J. Presl, 1830, (2n = 10x = 70 for 'Pictus' and 'Brevifolius', and 2n = 4x = 28 for 'Setifolius'; García et al. 2009). So, cytogenetic and plant morphology data supported the classification of the *B. setifolius* lines as separate species (García et al. 2009).

Karyotype characterization showed that the chromosomes of the *Bromus* species are similar in relation to total length and class (Joachimiak et al. 2001), with the occurrence of metacentric and submetacentric chromosomes being reported (Sheidai et al. 2008). This way, the distinction between karyotypes of *Bromus* species with the same chromosome number is generally carried out based on the size of the satellite portions (Armstrong 1983, Joachimiak et al. 2001) and heterochromatin distribution (Klos et al. 2009).

Stebbins (1981) reported that the genus *Bromus* originated in Eurasia. During the Pliocene, three sections were originated: *Ceratochloa* P. Beauvois, 1812, *Pnigma* Dumort, 1823 and *Neobromus* Shear, 1900, being that *Ceratochloa* and *Neobromus* spread to

Americas. Given this hypothesis, Stebbins argued that the geographic region Eurasia was also the differentiation center of diploid, tetraploid and, most likely, hexaploid species.

Differentiation of allohexaploid *Bromus* species in South America proceeded in the Pleistocene. Meanwhile, in North America, allopolyploidy events also occurred, leading to new ones with higher ploidy level (8x, 12x) (Stebbins 1947, 1981). This way, the *Bromus* species in South America is somewhat restricted to hexaploid species, differently from those found in North America, which are octoploids and duodecaploids (Massa et al. 2004).

According to current knowledge, the South American species (as *B. auleticus*, *B. brachyanthera* and *B. catharticus*) have chromosome number of 2n = 6x = 42 (Schifino and Winge 1983, Naranjo 1985, Martinello and Schifino-Wittmann 2003). Nevertheless, it is still necessary to confirm and understand the chromosomal changes among these species. Because chromosomal changes constitute an important mechanism of diversification and speciation (Stace 2000, Peer et al. 2009, Weiss-Schneeweiss et al. 2013), the investigation of this aspect in *Bromus* species of South America may generate knowledge on the speciation processes in this genus.

Numerical and structural chromosomal rearrangements have been reported to trigger changes in karyotype in various plant taxa. Due to these changes, the nuclear genome size varies between phylogenetically related species (Raskina et al. 2008, Bonifácio et al. 2012). Thus, nuclear DNA content measurements have increasingly been employed in taxonomic, systematic and evolutive approaches using flow cytometry (FCM). In addition to its practicality and reproducibility, FCM is useful to reveal differences among taxa, especially those that exhibit conserved chromosome number (Mabuchi et al. 2005). The nuclear genome size was measured for *Bromus* hexaploid (2n = 6x = 42) species. In spite of the same chromosome number, the seven species showed distinct nuclear 2C values, varying from 2C = 12.72 pg in *Bromus willdenowii* Kunth, 1829, to 2C = 15.10 pg in *Bromus lithobius* Trin., 1836 (Klos et al. 2009).

Hence, karyotype and nuclear 2C value are relevant data for the taxonomy and systematics of *Bromus*, as well as for understanding the evolutionary history of the genus and the relationships within the taxa. Thus, the aims of the present study were to measure the nuclear 2C value, determine the chromosome number and characterize the karyotype of the South American *Bromus* species *B. auleticus, B. brachyanthera* and *B. catharticus*.

Material and methods

Plant samples

Seeds of *B. auleticus, B. brachyanthera* and *B. catharticus* were provided by the South Forage Germplasm Bank (BAG) of Embrapa South Livestock, Brazil (BRA 00059183-4, 00080317-1 and 00059197-4, respectively). The seed samples were collected from several individuals of each species, occurring in the Brazilian Pampa biome, state of Rio Grande do Sul, Brazil. Copies of the species were deposited in the Herbarium CNPO

Embrapa (voucher numbers CNPO 4408 for *B. auleticus*, CNPO 4412 for *B. brachyanthera*, and CNPO 4408 for *B. catharticus*).

Nuclear 2C DNA measurement

Nuclear suspensions were prepared from leaf fragments (2 cm^2) obtained from each specimen of *B. auleticus, B. brachyanthera* or *B. catharticus* (samples), together with the internal standard *Pisum sativum* L. (2C = 9.16 pg; Praça-Fontes et al. 2011). For nuclear extraction, chopping was performed in 0.5 mL of OTTO-I buffer (Otto 1990) supplemented with 2 mM dithiothreitol and 50 µg mL^{-1} RNAse. Next, 0.5 mL of OTTO-I buffer was added, and the suspensions were filtered through nylon mesh of 30 µM, placed in microtube and centrifuged at 100 *x*g for 5 min. The pellet was resuspended in 100 µl OTTO-I buffer and incubated for 10 min (Praça-Fontes et al. 2011). The nuclei suspensions were stained with 1.5 ml of OTTO I:OTTO II (1:2 – Otto 1990; Praça-Fontes et al. 2011) supplemented with 2 mM dithiothreitol, 50 µg ml^{-1} propidium iodide and 50 µg ml^{-1} RNase (Praça-Fontes et al. 2011). The suspensions were kept in the dark for 30 min, filtered through a 20-µM nylon mesh, and analyzed on a flow cytometer Partec PAS II / III (Partec GmbH, Germany) equipped with a laser source (488 nm). For determination of the nuclear DNA content, histograms were analyzed with the Max Partec Flow software tools. Six independent repetitions, accounting for more than 10,000 nuclei, were carried out in each analysis. The genome size of *Bromus* species was calculated according to the formula:

$$2C_D = \left(\frac{C1}{C2}\right).2C_S$$

Wherein: $2C_D$: value of 2C DNA content (pg) of each *Bromus* species; C1: average G_0/G_1 peak channel of the *Bromus* species; C2: average G_0/G_1 peak channel of *P. sativum*; $2C_S$: value of 2C DNA content of *P. sativum* (2C = 9.16 pg).

Karyotype characterization and morphometric analysis

The seeds were aseptically scarified, disinfested, inoculated into medium composed of half-strength MS salts, 10 ml l^{-1} MS vitamins (Murashige and Skoog 1962), 30 g l^{-1} sucrose and 2.8 g l^{-1} Phytagel, and grown in photoperiod of 16 h at 25 ± 2°C. Roots of the seedlings were excised and treated with 4 µM amiprophos-methyl (APM, Sigma) for 4 h (B.O.D., 30°C). Root apical meristems were washed in dH_2O, fixed in methanol:acetic acid (Merck®) solution (3:1), and stored at -20°C. After 24 h, the meristems were washed in dH_2O and macerated in enzyme solution pool (96.6% pectinase, 0.4% hemicellulose, 1.0% macerozyme and 4.0% cellulose, Sigma®) 1:20 (pool:dH_2O) for 2 h at 34°C. Again, the meristems were washed with dH_2O, fixed,

and stored at -20°C. Subsequently, slides were prepared by dissociation of the macerated meristems and air-drying (Carvalho et al. 2007). The slides were stained with 5% Giemsa (Merck®) for 6 min, washed twice in dH_2O, and dried under heating plate. All slides were examined under a microscope Nikon Eclipse Ci-S (Nikon). Mitotic images were captured with the objective 100x and CCD camera (Nikon Evolution™) coupled to the microscope. Morphometric analysis of the chromosomes of the three *Bromus* species was performed for the total length, length of the long and short arms, arm ratio, and chromosome class. The latter was determined as proposed by Levan et al. (1964) and reviewed by Guerra (1986).

Results

FCM nuclear suspensions resulted in G_0/G_1 fluorescence peaks with a coefficient of variation of less than 5% for *Bromus* species and *P. sativum*. Thereby, FCM procedures provided suspensions with adequate amount of isolated, intact and stoichiometrically stained nuclei. The 2C nuclear DNA content was measured for the *Bromus* species through analysis of the histograms. 2C value of *B. catharticus* was 2C = 12.64 ± 0.00 pg, *B. brachyanthera* was 2C = 16.73 ± 0.16 pg, and *B. auleticus* was 17.92 ± 0.44 pg. Mean value for *B. auleticus* was 41.70% higher than for *B. catharticus*, and 7.10% greater than for *B. brachyanthera*. In turn, *B. brachyanthera* presented 2C value 32.36% higher than that of *B. catharticus*. These values reflect interspecific variation among the nuclear genome sizes of the analyzed species.

Root meristems treated with amiprophos-methyl and macerated in enzyme pool solution resulted in adequate metaphase chromosomes. Metaphases were chosen based on the following criteria: well-spread chromosomes with well-defined constriction, no chromatin deformations and no cytoplasmic background noise. These features allowed accurate chromosome counting, karyotype measurements, chromosome class determination and karyogram assembly. All three *Bromus* species showed a conserved number of 2n = 42 chromosomes (Figure 1).

Based on the morphometric data, the chromosome class was determined and the differences between the karyotypes of the three species were verified. *B. auleticus* presented eleven metacentric (1, 3, 4, 5, 6, 8, 9, 11, 15, 18 and 19) and ten submetacentric chromosomes (2, 7, 10, 12, 13, 14, 16, 17, 20 and 21). *B. brachyanthera* exhibited 13 metacentric (1, 2, 4, 5, 6, 9, 10, 13, 15, 16, 17, 19 and 21) and eight submetacentric chromosomes (3, 7, 8, 11, 12, 14, 18 and 20). Finally, *B. catharticus* displayed eleven metacentric (1, 4, 5, 7, 8, 9, 10, 11, 14, 17 and 20) and ten submetacentric chromosomes (2, 3, 6, 12, 13, 15, 16, 18, 19 and 21) (Table 1). Groups of morphologically similar chromosome pairs were also identified for all *Bromus* species: 3–4 in *B. auleticus*, 11–12 and 15–16 in *B. brachyanthera*, and 12–13 in *B. catharticus* (Figure 1, Table 1).

The three *Bromus* species presented only metacentric and submetacentric chromosomes. Despite belonging to the same class, the chromosomes differed intra- and inter-

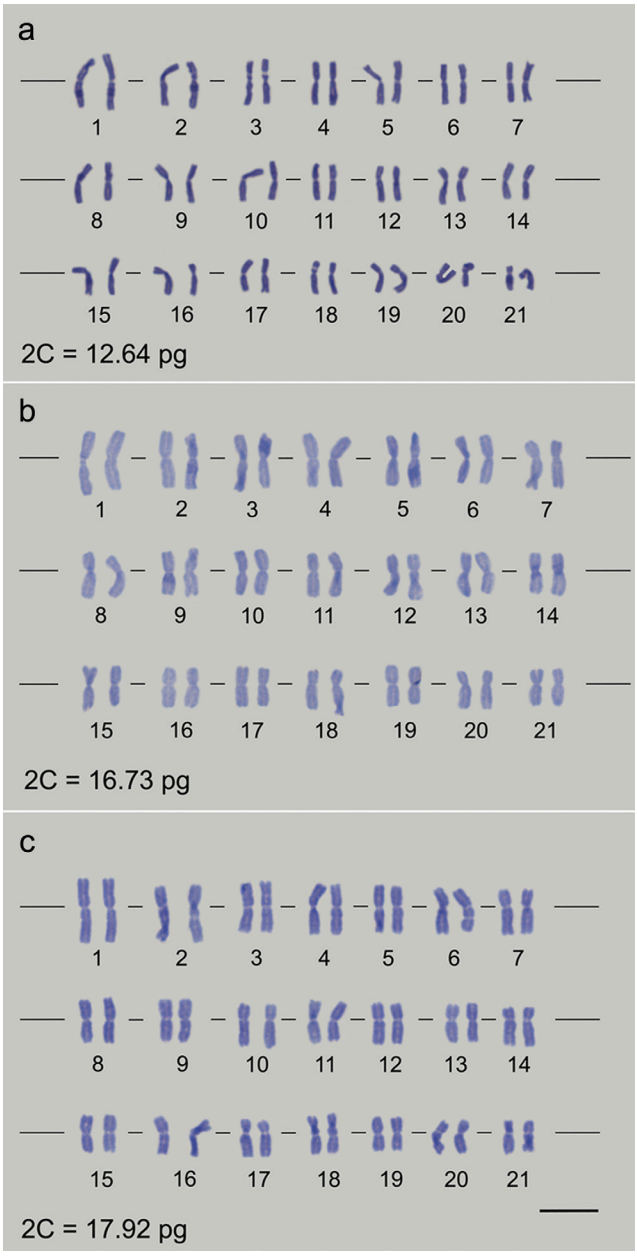


Figure 1. First karyograms of *Bromus* species, displaying 2n = 6x = 42 chromosomes: **a** *B. catharticus* **b** *B. brachyanthera* and **c** *B. auleticus*. **a** *B. catharticus* displayed eleven metacentric (1, 4, 5, 7, 8, 9, 10, 11, 14, 17 and 20) and ten submetacentric chromosomes (2, 3, 6, 12, 13, 15, 16, 18, 19 and 21) **b** *B. brachyanthera* exhibited 13 metacentric (1, 2, 4, 5, 6, 9, 10, 13, 15, 16, 17, 19 and 21) and eight submetacentric chromosomes (3, 7, 8, 11, 12, 14, 18 and 20) **c** *B. auleticus* presented eleven metacentric (1, 3, 4, 5, 6, 8, 9, 11, 15, 18 and 19) and ten submetacentric chromosomes (2, 7, 10, 12, 13, 14, 16, 17, 20 and 21). Note the morphologically similar chromosomes: **a** 12–13 in *B. catharticus* **b** 11–12 and 15–16 in *B. brachyanthera*, and **c** 3–4 in *B. auleticus*. Bar = 5 μm.

Table 1. Morphometry of the metaphasic chromosomes of *B. auleticus, B. brachyanthera* and *B. catharticus*.

Chrom.	*B. auleticus*						*B. brachyanthera*						*B. catharticus*					
	Total (µm)	Arms		r	Class	Size (%)	Total (µm)	Arms		r	Class	Size (%)	Total (µm)	Arms		r	Class	Size (%)
		Short	Long					Short	Long					Short	Long			
1	5.867	2.667	3.200	1.20	M	7.55	5.233	2.467	2.767	1.12	M	6.41	4.567	2.233	2.333	1.04	M	6.96
2	4.833	1.733	3.100	1.79	SM	6.22	4.767	2.133	2.633	1.23	M	5.84	3.833	1.500	2.333	1.56	SM	5.85
3	4.333	2.067	2.267	1.10	M	5.58	4.733	1.867	2.867	1.54	SM	5.80	3.800	1.500	2.300	1.53	SM	5.80
4	4.333	1.967	2.367	1.20	M	5.58	4.533	1.900	2.633	1.39	M	5.55	3.700	1.633	2.067	1.27	M	5.64
5	4.100	1.867	2.233	1.20	M	5.28	4.500	2.067	2.433	1.18	M	5.51	3.467	1.567	1.900	1.21	M	5.29
6	3.967	1.700	2.267	1.33	M	5.11	4.100	1.967	2.133	1.08	M	5.02	3.467	1.367	2.100	1.54	SM	5.29
7	3.900	1.433	2.467	1.72	SM	5.02	4.000	1.433	2.567	1.79	SM	4.90	3.400	1.667	1.733	1.04	M	5.19
8	3.900	1.667	2.233	1.34	M	5.02	3.933	1.500	2.433	1.62	SM	4.82	3.367	1.600	1.767	1.10	M	5.13
9	3.733	1.667	1.967	1.18	M	4.68	3.900	1.933	1.967	1.02	M	4.78	3.233	1.300	1.933	1.49	M	4.93
10	3.633	1.400	2.333	1.67	SM	4.80	3.867	1.833	2.033	1.11	M	4.74	3.167	1.567	1.600	1.02	M	4.83
11	3.600	1.633	1.967	1.20	M	4.63	3.800	1.467	2.333	1.59	SM	4.66	3.100	1.300	1.800	1.38	M	4.73
12	3.567	1.333	2.233	1.68	SM	4.59	3.800	1.467	2.333	1.59	SM	4.66	3.067	1.210	1.857	1.53	SM	4.68
13	3.333	1.300	2.033	1.56	SM	4.29	3.600	1.567	2.033	1.30	M	4.41	2.967	1.170	1.797	1.54	SM	4.52
14	3.333	1.100	2.233	2.03	SM	4.29	3.467	1.367	2.100	1.54	SM	4.25	2.967	1.267	1.700	1.34	M	4.52
15	3.333	1.567	1.767	1.13	M	4.29	3.433	1.533	1.900	1.24	M	4.21	2.800	1.033	1.767	1.71	SM	4.27
16	3.300	1.300	2.000	1.54	SM	4.25	3.433	1.500	1.933	1.29	M	4.21	2.733	0.867	1.867	2.15	SM	4.17
17	3.167	1.100	2.067	1.88	SM	4.08	3.400	1.533	1.867	1.22	M	4.17	2.733	1.300	1.433	1.10	M	4.17
18	3.000	1.467	1.533	1.05	M	3.86	3.400	1.200	2.200	1.83	SM	4.17	2.700	1.067	1.633	1.53	SM	4.12
19	2.933	1.300	1.633	1.26	M	3.78	3.300	1.500	1.800	1.20	M	4.04	2.367	0.800	1.567	1.96	SM	3.61
20	2.833	1.133	1.700	1.50	SM	3.65	3.267	1.033	2.233	2.16	SM	4.00	2.267	0.933	1.333	1.43	M	3.46
21	2.700	1.067	1.633	1.53	SM	3.47	3.167	1.300	1.867	1.44	M	3.88	1.867	0.600	1.267	2.11	SM	2.85
Sum	77.700	32.470	45.230	-	-	100.00	81.630	34.570	47.070	-	-	100.00	65.570	27.480	38.090	-	-	100.00

Chrom – chromosome; r – arm ratio (long/short); Size – % size in relation to sum of the mean values of total length; M – metacentric; SM – submetacentric; Sum – sum of the mean values.

specifically based on their morphology, which was characterized by occurrence of well-defined telomere and centromere portions and relatively low chromatin compaction level (Figure 1). As summarized in Table 1, the majority of the chromosomes could be distinguished by at least one morphometric parameter: total length, short- and long-arm length, ratio between arms, and/or relative chromosome size (%) in relation to the sum of the total length (Table 1). In addition, some chromosomes showed the secondary constriction in the interstitial region of the short arm, such as the chromosome 3 of *B. catharticus* (Figure 1a) or 18 of *B. auleticus* (Figure 1c).

Discussion

According to the chromosome number found here and the complement set x = 7 (Stebbins 1981), *B. auleticus*, *B. brachyanthera* and *B. catharticus* are hexaploid species (2n = 6x = 42). This result is in accordance with previous data reported for the three species (Schifino and Winge 1983, Naranjo 1985, Martinello and Schifino-Wittmann 2003). The genus *Bromus* originated in Eurasia, whereas the hexaploid species emerged in South America during the Pleistocene, from the subgenus *Ceratochloa* (Stebbins 1981). Thus, the chromosome number of the three species has remained conserved in relation to the ancestors, supporting Stebbins's hypothesis on the diversification of the *Bromus* species.

The mean nuclear 2C value divergences were corroborated by chromosome morphometry. *B. catharticus* clearly differed from the other species, being that its relatively small genome size correlated with the sum of the total chromosome length. Differently, for *B. auleticus* and *B. brachyanthera* this relation was not observed, as a result of the low compaction level of the chromatin in the latter species (Figure 1, Table 1). The large differences among nuclear 2C values for the same chromosome number suggests that the three species diverged through chromosomal rearrangements. In a study of hexaploid *Bromus* species, Klos et al. (2009) also reported interspecific variations (2C = 12.72 to 15.10 pg) in relation to the nuclear genome size. According to these authors, chromosomal changes occurred during the evolution of the hexaploid *Bromus* species, most likely through the gain or loss of highly repeated DNA sequences. Regarding this, karyotype and nuclear genome size should be considered together when comparing *Bromus* species.

Despite the similar morphology of some chromosome pairs (12–13 in *B. catharticus*, 11–12 and 15–16 in *B. brachyanthera*, and 3–4 in *B. auleticus*; Figure 1, Table 1), differences were found for most pairs of the three hexaploid *Bromus* species (Figure 1, Table 1). For the genus *Bromus*, Stebbins (1981) classified all species as allohexaploids. The allopolyploidy in *Bromus* was also found in *Bromus hordeaceus* L., 1753, which was classified as an allotetraploid (Ainouche et al. 1999). In that sense, Klos et al. (2009) highlighted that the *Bromus* section *Ceratochloa* includes a number of closely related allopolyploid species originated by three ancestors AABBCC.

Conclusion

The nuclear 2C value and karyotype characterization allowed differentiating the three *Bromus* species, thus contributing to the cytotaxonomy and evolutional understanding in this taxon. As also demonstrated by other authors, these data provide insights about the evolutionary process and diversification of the polyploid *Bromus* species.

Acknowledgments

We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília – DF, Brazil), the Fundação de Amparo à Pesquisa do Espírito Santo (FAPES, Vitória – ES, Brazil), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília – DF, Brazil) for financial support. Scholarship holder associated to the project MP1 – 01.15.02.002.05.08 of the Rede Nacional de Recursos Genéticos, Vertente Vegetal, Projeto Componente "Bancos ativos de Germoplasma de Forrageiras".

References

Acedo C, Liamas F (2001) Variation of morphological characters of lemma and palea in the genus *Bromus* (Poaceae). Annales Botanici Fennici 38: 1–14. https://doi.org/10.2307/i23726794

Ainouche ML, Bayer RJ, Gourret JP, Defontaine A, Misset MT (1999) The allotetraploid invasive weed *Bromus hordeaceus* L. (Poaceae): genetic diversity, origin and molecular evolution. Folia Geobot 34: 405–419. https://doi.org/10.1007/BF02914919

Armstrong KC (1983) The relationship between some Eurasian and American species of Bromus section Pnigma as determined by the karyotypes of some F1 hybrids. Canadian Journal of Plant Science 61: 700–707. https://doi.org/10.1139/b83-080

Armstrong KC (1984) Chromosome pairing affinities between Old and New World species of *Bromus* section Pnigma. Canadian Journal of Plant Science 62: 581–585.

Armstrong KC (1987) Chromosome numbers of perennial *Bromus* species collected in the USSR. Canadian Journal of Plant Science 67: 267–269. https://doi.org/10.4141/cjps87-038

Bonifácio EM, Fonsêca A, Almeida C, Santos KGB, Pedrosa-Harand A (2012) Comparative cytogenetic mapping between the lima bean (*Phaseolus lunatus* L.) and the common bean (*P. vulgaris* L.). Theoretical and Applied Genetics 124: 1513–1520. https://doi.org/10.1007/s00122-012-1806-x

Carvalho CR, Clarindo WR, Almeida PM (2007) Plant cytogenetics: still looking for the perfect mitotic chromosomes. Nucleus 50: 453–462.

Fedorov AA (1969) Chromosome Numbers of Flowering Plants. Russian Academy of Sciences, Leningrad, 926 pp.

García AM, Schrauf GE, González G, Poggio L, Naranjo CA, Dupal MP, Spangenberg GC, Forster JW (2009) Use of AFLP and RAPD molecular genetic markers and cytogenetic analysis to explore relationships among taxa of the Patagonian *Bromus setifolius* complex. Genetics and Molecular Biology 32: 312–319. https://doi.org/10.1590/S1415-47572009005000029

Guerra MS (1986) Reviewing the chromosome nomenclature of Levan et al. Revista Brasileira de Genética 9: 741–743.

Iannone LJ, WhiteJr JF, Giussani LM, Cabral D, Novas MV (2010) Diversity and distribution of *Neotyphodium*-infected grasses in Argentina. Mycological Progress 10: 9. https://doi.org/10.1007/s11557-010-0669-2

Joachimiak A, Kula A, Sliwinska E, Sobieszczanska A (2001) C-banding and nuclear DNA amount in six *Bromus* species. Acta Biologica Cracoviensia Series Botanica 43: 105–115.

Klos J, Sliwinska E, Kula A, Golczyk H, Grabowska-Joachimiak A, Ilnicki T, Szostek K, Stewart A, Joachimiak AJ (2009) Karyotype and nuclear DNA content of hexa-, octo-, and duodecaploid lines of *Bromus* subgen. *Ceratochloa*. Genetics and Molecular Biology, 32: 528–537. https://doi.org/10.1590/S141547572009005000046

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Mabuchi T, Kokubun H, Mii M, Ando T (2005) Nuclear DNA content in the genus *Hepatica* (Ranunculaceae). Journal of Plant Research 118: 37–41. https://doi.org/10.1007/s10265-005-0191-9

Martinello GE, Schifino-Wittmann MT (2003) Chromosomes of *Bromus auleticus* Trin. ex Nees (Poaceae). Genetics and Molecular Biology 26: 369–371. https://doi.org/10.1590/S1415-47572003000300024

Massa AN, Jensen KB, Larson SR, Hole DJ (2004) Morphological variation in *Bromus* sect. Ceratochloa germplasm of Patagonia. Canadian Journal of Plant Science 82: 136–144.

Murashige T, Skoog FA (1962) A revised médium for a rapid growth and bioassays with tobacco tissues cultures. Plant Physiol 15: 473–479. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Naranjo CA (1985) Estudios citogeneticos, bioquimicos y sistemáticos en algunas especies americanas del genero *Bromus* (Gramineae). PhD Thesis, Universidade de Buenos Aires, Buenos Aires.

Otto FJ (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewiez Z, Crissman HA, Robinson JP (Eds) Methods in cell biology. San Diego Academic Press, San Diego, Canadá, 105–110. https://doi.org/10.1016/s0091-679x(08)60516-6

Peer YV, Maere S, Meyer A (2009) The evolutionary significance of ancient genome duplications. Nature Reviews Genetics 10: 725–732. https://doi.org/10.1038/nrg2600

Puecher L, Claudio G, Robredo L, Ra´ul D, Rios L, Rimieri P (2001) Genetic variability measures among *Bromus catharticus* Vahl. Populations and cultivars with RAPD and AFLP markers. Euphytica 121: 229–236. https://doi.org/10.1023/A:1012068415647

Praça-Fontes MM, Carvalho CR, Clarindo WR, Cruz CD (2011) Revisiting the DNA C-values of the genome size-standards used in plant flow cytometry to choose the ‘‘best

primary standards". Plant Cell Reports 30: 1183–1191. https://doi.org/10.1007/s00299-011-1026-x

Raskina O, Barber JC, Nevo E, Belyayev A (2008) Repetitive DNA and chromosomal rearrangements: speciation-related events in plant genomes. Cytogenetic and Genome Research 120: 351–377. https://doi.org/10.1159/000121084

Schifino MT, Winge H (1983) Karyotypes and nuclear DNA content of species of the *Briza* complex and some other genera of Poeae (Gramineae). Revista Brasileira de Genética 6: 245–259.

Sheidai M, Saeidi S, Atri M (2008) Taxonomic applications of seed proteins in the genus *Bromus* L. (Poaceae). Iran Journal of Botanical 14: 126–131.

Stace CA (2000) Cytology and cytogenetics as a fundamental taxonomic resource for the 20th and 21" centuries. Taxon 49: 451–477. https://doi.org/10.2307/1224344

Stebbins GL (1947) The origin of the complex of *Bromus carinatus* and its phylogeographic implications. Contributions from the Gray Herbarium of Harvard University 165: 42–55.

Stebbins GL (1981) Chromosomes and evolution in the genus *Bromus* (Gramineae). Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie 102: 359–379.

Weiss-Schneeweiss H, Emadzade K, Jang TS, Schneeweiss GM (2013) Evolutionary consequences, constraints and potential of polyploidy in plants. Cytogenetic and Genome Research 140: 137–150. https://doi.org/10.1159/000351727

Williams WM, Stewart AV, Williamson ML (2011) *Bromus*. Wild crop relatives: genomic and breeding resources, millets and grasses. In: Kole C (Ed.) Springer-Verlag Berlin Heidelberg, 15–30. https://doi.org/10.1007/978-3-642-14255-0_2

CompCytogen 11(2): 225–237 (2017)
doi: 10.3897/CompCytogen.v11i2.11579
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

The *Mugil curema* species complex (Pisces, Mugilidae): a new karyotype for the Pacific white mullet mitochondrial lineage

Mauro Nirchio[1,2], Claudio Oliveira[3], Zoila R. Siccha-Ramirez[3], Viviani F. de Sene[3], Luciana Sola[4], Valentina Milana[4], Anna Rita Rossi[4]

1 *Escuela de Ciencias Aplicadas del Mar, Núcleo de Nueva Esparta, Universidad de Oriente, Apartado 174, Porlamar, Isla de Margarita, Venezuela* **2** *Universidad Técnica de Machala, Av. Panamericana km 5½, Via Pasaje, Machala, El Oro, Ecuador* **3** *Departamento de Morfologia, Instituto de Biociências Universidade Estadual Paulista, 18618-970 Botucatu, São Paulo, Brazil* **4** *Dipartimento di Biologia e Biotecnologie "C. Darwin", Sapienza - Università di Roma, Via Alfonso Borelli 50, 00161, Rome, Italy*

Corresponding author: *Valentina Milana* (valentina.milana@uniroma1.it)

Academic editor: *E. Gornung* | Received 3 January 2017 | Accepted 10 February 2017 | Published 7 April 2017

http://zoobank.org/6E4E2B0D-DB63-48E5-B9C4-2A5324595310

Citation: Nirchio M, Oliveira C, Siccha-Ramirez ZR, de Sene VF, Sola L, Milana V, Rossi AR (2017) The *Mugil curema* species complex (Pisces, Mugilidae): a new karyotype for the Pacific white mullet mitochondrial lineage. Comparative Cytogenetics 11(2): 225–237. https://doi.org/10.3897/CompCytogen.v11i2.11579

Abstract

Recent molecular phylogenetic analyses have shown that the *Mugil curema* Valenciennes, 1836 species complex includes *M. incilis* Hancock, 1830, *M. thoburni* (Jordan & Starks, 1896) and at least four "*M. curema*" mitochondrial lineages, considered as cryptic species. The cytogenetic data on some representatives of the species complex have shown a high cytogenetic diversity. This research reports the results of cytogenetic and molecular analyses of white mullet collected in Ecuador. The analyzed specimens were molecularly assigned to the *Mugil* sp. O, the putative cryptic species present in the Pacific Ocean and showed a 2n = 46 karyotype, which is composed of 2 metacentric and 44 subtelocentric/acrocentric chromosomes. This karyotype is different from the one described for *M. incilis* (2n = 48) and from those of the two western Atlantic lineages *Mugil curema* (2n = 28), and *Mugil margaritae* (2n = 24). Data suggest the need for a morphological analysis to assign a species name to this Pacific lineage.

Keywords

Fish, Mugilidae, cytochrome oxidase subunit I, cytotaxonomy, molecular systematics

Introduction

The family Mugilidae currently comprises 20 genera and 74 species (Eschmeyer and Fong 2016), which are widely distributed in various tropical, subtropical and temperate coastal regions of the world (Thomson 1997, Froese and Pauly 2016). These fishes show highly conserved morphological and anatomical characteristics, which are often associated with wide distribution ranges and, thus, the family has undergone many taxonomic revisions, both at the genus and species levels (Thomson 1997).

In the last decade, molecular phylogenetic and phylogeographic analyses have revealed that the morphological features commonly used to identify species seem to be insufficient, both to describe the great diversity of species within Mugilidae and to infer the phylogenetic relationships among the species (Durand et al. 2012, Durand and Borsa 2015). More specifically, Durand et al. (2012) showed that a proportion of the species with large distribution ranges, such as *Mugil cephalus* Linnaeus, 1758 and *M. curema* Valenciennes, 1836 consists of cryptic species. Specifically referring to *M. curema,* different mtDNA lineages had been previously identified along the American Atlantic coasts by Heras et al. (2006, 2009) and Fraga et al. (2007). Unfortunately these studies did not adopt a uniform nomenclature for the lineages (see Rossi et al. 2016 for a detailed review) and did not cover the entire species range, that includes both the Eastern and Western Atlantic coasts and the Eastern Pacific coast (Froese and Pauly 2016). Durand et al. (2012) and Durand and Borsa (2015) showed that these lineages are part of a *Mugil curema* species complex which includes *M. incilis* Hancock, 1830 and *M. thoburni* (Jordan & Starks, 1896), and at least four "*M. curema*" mitochondrial lineages, considered as cryptic species. The first lineage is distributed along the Atlantic coast of the Americas and retains the name of *M. curema*, as the type locality of the original *M. curema* is Bahia, Brazil; the second lineage is present along the Atlantic African coasts and is indicated as *Mugil* sp. M. The third lineage, indicated as *Mugil* sp. N, is present in Venezuela and has recently been formally described as a new species, named *M. margaritae* Menezes, Nirchio, Oliveira & Siccha-Ramirez, 2015 (Menezes et al. 2015). The fourth lineage is distributed along the Pacific coast of the Americas, from the USA to Ecuador, and is indicated as *Mugil* sp. O.

Cytotaxonomy has been proven to be a powerful tool in revealing different lineages/species within Mugilidae. For example, the presence of different cytogenetic features (Nirchio et al. 2003) provided the basis for the identification of an undescribed species, *M. rubrioculus* Harrison, Nirchio, Oliveira, Ron & Gaviria, 2007 (Harrison et al. 2007), as well as the first hints about the existence of cryptic species among mullets, which, until then, had been reported under the name of *M. curema* (Nirchio et al. 2005). Although only two of the four mitochondrial lineages of "*M. curema*" have been cytogenetically investigated to date, they have been found to differ from each other in diploid number and chromosome formula, as well as differing from all the other mugilids investigated to date (see Rossi et al. 2016 for a review). *M. curema sensu strictu* shows a karyotype composed of 2n = 28 chromosomes (LeGrande and Fitzsimons 1976, Nir-

chio et al. 2005) and *M. margaritae* shows a karyotype composed of 2n = 24 chromosomes (Nirchio and Chequea 1998, Nirchio et al. 2005).

This paper reports the cytogenetic analysis of samples of the white mullet collected in Ecuador (Pacific Ocean); according to Durand and Borsa (2015), it is reasonable to assume that they belong to the *Mugil* sp. O. The aim of the study is to describe the karyotype and the cytogenetic features of this *Mugil* sp. and to detect specific cytotaxonomic markers that could be useful for its identification. To verify that all the analyzed specimens belong to the *Mugil* sp. O, sequences of the mitochondrial cytochrome oxidase I (COI) gene were also produced and compared to those previously obtained by Durand et al. (2012) and Durand and Borsa (2015).

Materials and methods

Seventeen juvenile specimens (undetermined sex), morphologically classified as white mullet (*Mugil curema*) according to Harrison (1995), were caught by cast net at Puerto Hualtaco, at the border between Ecuador and Perú (3°26'S; 80°13'W), and transported alive to the laboratory. The fishes were sacrificed with an overdose of benzocaine (250 mg/l), following the guidelines of the AVMA (2013). Small pieces of muscle and cephalic kidneys were removed from all specimens, and nine individuals preserved in 70% ethanol were deposited as voucher specimens (Table 1).

Total genomic DNA was extracted from muscle according to Aljanabi and Martínez (1997).

A 546 base-pair (bp) fragment of the mitochondrial cytochrome oxidase subunit I gene (COI) was amplified by PCR using primers FishF1 and FishR2 (Ward et al. 2005) and the procedures reported in Milana et al. (2011). The obtained sequences were aligned using the program MEGA5 (Tamura et al. 2011) and submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/Genbank) under accession numbers KU504271–KU504272 (see Table 1 for details). BLAST (Basic Local Alignment Search Tool) software was used for similarity searching of the COI sequences in GenBank.

Tree reconstructions were conducted using neighbor-joining (NJ), maximum-likelihood (ML) and Bayesian inference (BI) analyses. The NJ and ML analyses (1000 bootstrap replicates) were performed using MEGA5 and PhyML v3.0 (Guindonet al. 2010), respectively. The Bayesian analyses were carried out as implemented in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001); two independent runs of four Markov chains each for 10^6 generations were performed. Modeltest v3.7 (Posada and Crandall 1998) and MrModeltest v2.3 (Nylander 2008) were used to select the evolutionary models for the ML and the BI analyses, respectively, according to the Akaike information criterion. All 37 COI sequences of *Mugil* sp. obtained by Durand et al. (2012) and Durand and Borsa (2015), and five COI sequences of *M. incilis,* previously obtained from Venezuelan specimens by our research group (Hett et al. 2011), were included in the phylogenetic analyses (Table 1).

Table 1. GenBank accession number (A.N.), sampling areas and references of the *Mugil* sp. COI sequences used in phylogenetic analyses.

Individual (Voucher number)	A.N.	Sampling area	Reference
0102 (UTMACH0102)	KU504271	Ecuador	Present paper
0103 (UTMACH0103)	KU504271	Ecuador	Present paper
104	KU504271	Ecuador	Present paper
105	KU504271	Ecuador	Present paper
119	KU504272	Ecuador	Present paper
120	KU504271	Ecuador	Present paper
121	KU504271	Ecuador	Present paper
122	KU504271	Ecuador	Present paper
123	KU504271	Ecuador	Present paper
124	KU504271	Ecuador	Present paper
76104 (LBP 76104)	KU504271	Ecuador	Present paper
76105 (LBP 76105)	KU504271	Ecuador	Present paper
76107 (LBP 76107)	KU504271	Ecuador	Present paper
76129 (LBP 76129)	KU504271	Ecuador	Present paper
76130 (LBP 76129)	KU504271	Ecuador	Present paper
76131(LBP 76131)	KU504271	Ecuador	Present paper
76132 (LBP 76132)	KU504271	Ecuador	Present paper
415	JQ060604	El Salvador	Durand et al. 2012
426	JQ060600	El Salvador	Durand et al. 2012
429	JQ060601	El Salvador	Durand et al. 2012
430	JQ060602	El Salvador	Durand et al. 2012
432	JQ060603	El Salvador	Durand et al. 2012
293	JQ060573	Western Panama	Durand et al. 2012
294	JQ060574	Western Panama	Durand et al. 2012
413	JQ060592	Perù	Durand et al. 2012
420	JQ060595	Ecuador	Durand et al. 2012
423	JQ060597	Western Mexico	Durand et al. 2012
425	JQ060599	Western Mexico	Durand et al. 2012
406	JQ060588	Western Mexico	Durand et al. 2012
422	JQ060596	Western Mexico	Durand et al. 2012
396	JQ060580	Togo	Durand et al. 2012
397	JQ060581	Togo	Durand et al. 2012
390	JQ060575	Senegal	Durand et al. 2012
391	JQ060576	Senegal	Durand et al. 2012
392	JQ060577	Senegal	Durand et al. 2012
393	JQ060578	Benin	Durand et al. 2012
394	JQ060579	Benin	Durand et al. 2012
399	JQ060582	Venezuela	Durand et al. 2012
400	JQ060583	Venezuela	Durand et al. 2012
401	JQ060584	Venezuela	Durand et al. 2012
403	JQ060585	Venezuela	Durand et al. 2012
414	JQ060593	Venezuela	Durand et al. 2012
408	JQ060590	Brazil	Durand et al. 2012
411	JQ060591	Guadeloupe	Durand et al. 2012

Individual (Voucher number)	A.N.	Sampling area	Reference
419	JQ060594	Belize	Durand et al. 2012
404	JQ060586	Eastern USA	Durand et al. 2012
407	JQ060589	Eastern Mexico	Durand et al. 2012
417	JQ060605	Uruguay	Durand et al. 2012
418	JQ060606	Uruguay	Durand et al. 2012
405	JQ060587	Honduras	Durand et al. 2012
6435	JX559534	Galapagos Is.	Durand et al. 2012
6445	JX559535	Galapagos Is.	Durand et al. 2012
299	JQ060609	French Guyana	Durand et al. 2012
302	JQ060608	French Guyana	Durand et al. 2012
780	HQ285928	Venezuela	Hett et al. 2011
782	HQ285929	Venezuela	Hett et al. 2011
785	HQ285930	Venezuela	Hett et al. 2011
786	HQ285931	Venezuela	Hett et al. 2011
788	HQ285927	Venezuela	Hett et al. 2011

LBP: Laboratório de Biologia e Genética de Peixes, UNESP, Botucatu (São Paulo State, Brazil); UTMACH: Laboratorio de Acuicultura, Universidad Técnica de Machala, Ecuador.

Cell suspensions were obtained from the cephalic kidney, following the procedure reported by Nirchio and Oliveira (2006). Nucleolus organizer regions (NORs) were identified by silver (Ag) nitrate staining (Howell and Black 1980), and C-banding patterns were obtained following the protocol described by Sumner (1972).

Fluorescence in situ hybridization (FISH) was accomplished according to Pinkel et al. (1986). (TTAGGG)n, major (18S rDNA) and minor (5S rDNA) ribosomal probes were amplified by a polymerase chain reaction (PCR) from the genomic DNA of *Eigenmannia* sp. 2, using primers available from the literature (Ijdo et al. 1991, White et al. 1990, Pendás et al. 1994, respectively). The 18S rDNA sequences were labelled during PCR with Digoxigenin-11-dUTP; the 5S rDNA and (TTAGGG)n probes were labelled with biotin-16-dUTP. The detection of hybridization signals was performed using conjugated avidin-fluorescein (FITC) for the 18S rDNA probe and anti-digoxigenin-rhodamine for the 5S rDNA and (TTAGGG)n probes. The chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide.

The mitotic figures were photographed using an Olympus BX61 photomicroscope equipped with the appropriate selective filters for FISH and with a DP70 digital camera. The images were digitally edited with Adobe Photoshop CS6 Extended.

Results

Similarity searching of the obtained COI sequences in the GenBank database, using the BLAST function, provided 99.6–100% similarity with those obtained by Durand et al. (2012) and Durand and Borsa (2015) for the Pacific white mullet, i.e., the *Mugil* sp. O. These data were confirmed by the phylogenetic tree topology, obtained by NJ,

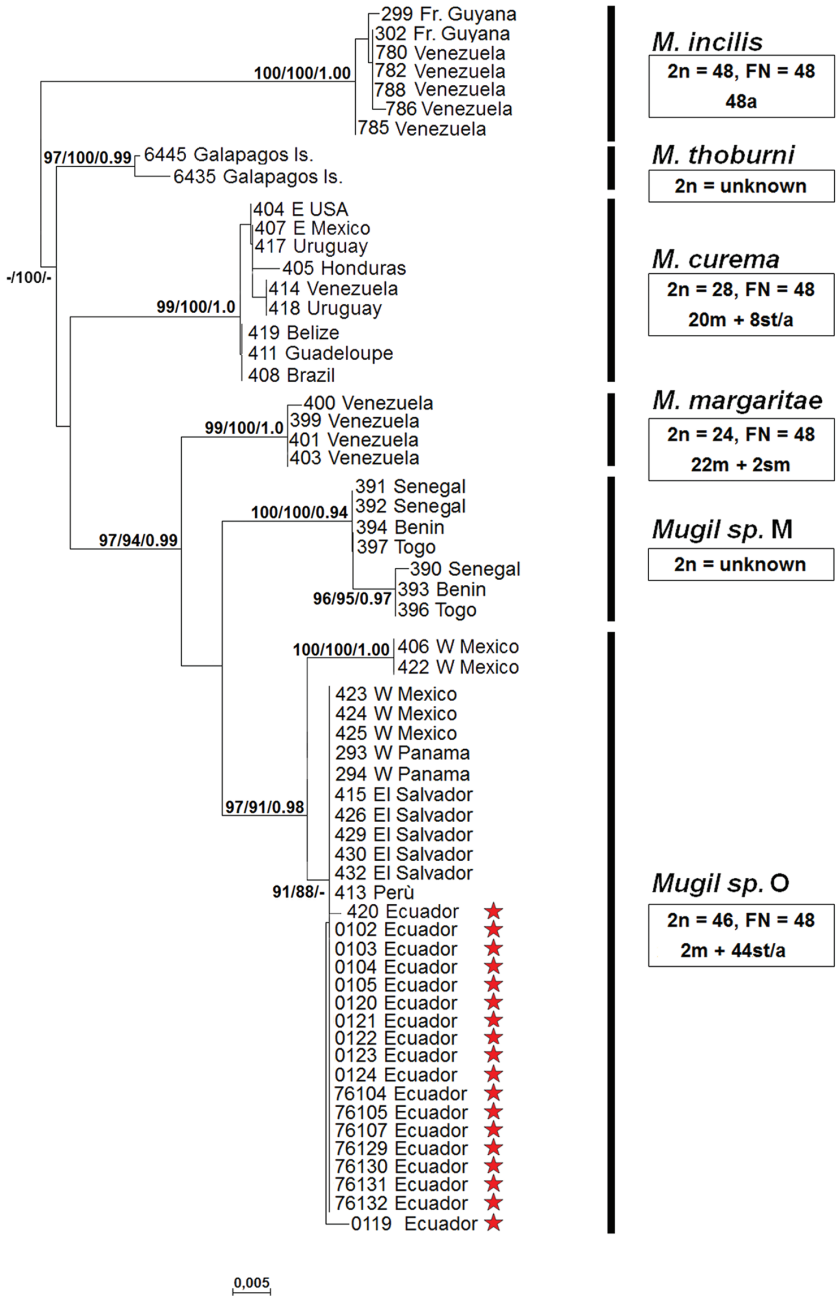


Figure 1. Neighbor-joining tree based on COI sequences. At each node, bootstrap values > 70% (NJ and ML) and posterior probabilities > 0.9 (BI) are shown. Stars indicate sequences obtained in this study; the remaining sequences are from Durand et al. (2012), Durand and Borsa (2015) and from Hett et al. (2011) (see Table 1). For each lineage, the karyotype (2n), the fundamental number (FN) and the chromosome formula are indicated. m: metacentric chromosomes; sm: submetacentric chromosomes; st/a: subtelocentric/acrocentric chromosomes.

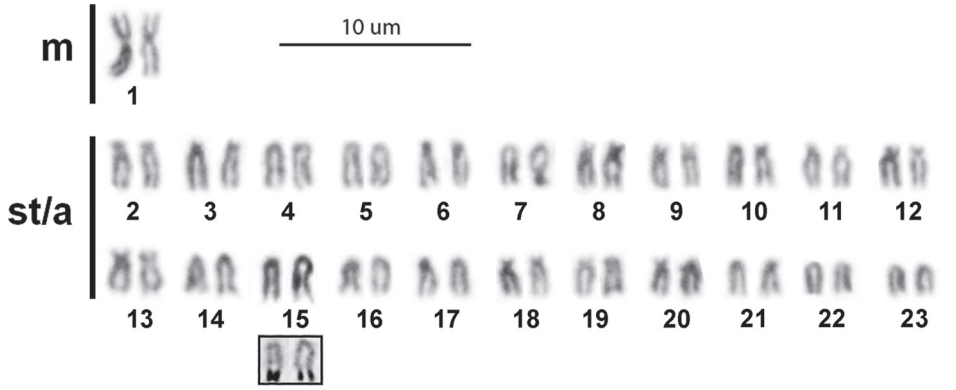


Figure 2. Conventional Giemsa-stained karyotype of the Pacific white mullet. In the inset, the acrocentric chromosome pair n. 15 sequentially Ag-stained; m: metacentric chromosomes; st/a: subtelocentric/acrocentric chromosomes. Scale bar: 10 μm.

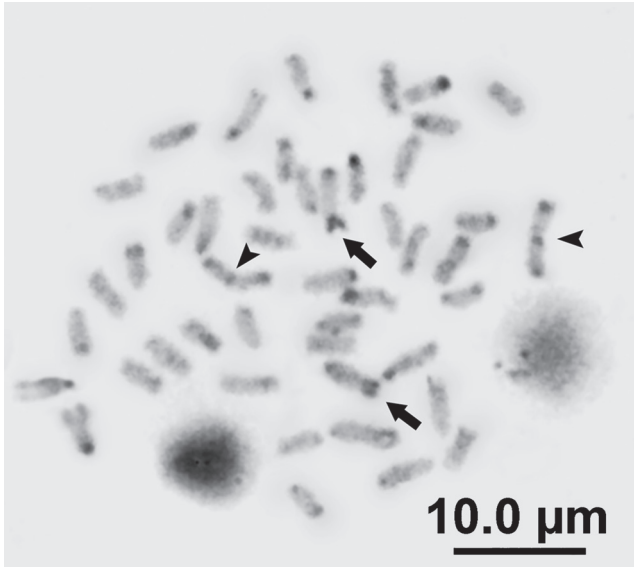


Figure 3. Somatic C-banded metaphases of the Pacific white mullets. Arrowheads indicate chromosome pair number one. Arrows indicate terminal heterochromatic blocks on chromosome pair 15. Scale bar: 10 μm.

ML and BI analyses (Fig. 1): all the sequences of white mullets from Ecuador collected in this study clustered within the Pacific *Mugil* sp. O.

In all the individuals, the karyotype is composed of 46 chromosomes, 2 metacentric and 44 subtelocentric/acrocentric, with a fundamental number (FN) of 48 (Fig. 2). The metacentric chromosome pair number 1 was clearly identifiable, whereas the homologues belonging to the subtelocentric and acrocentric series could not be unequivocally identified, due to their uniformly decreasing size. The only exception is

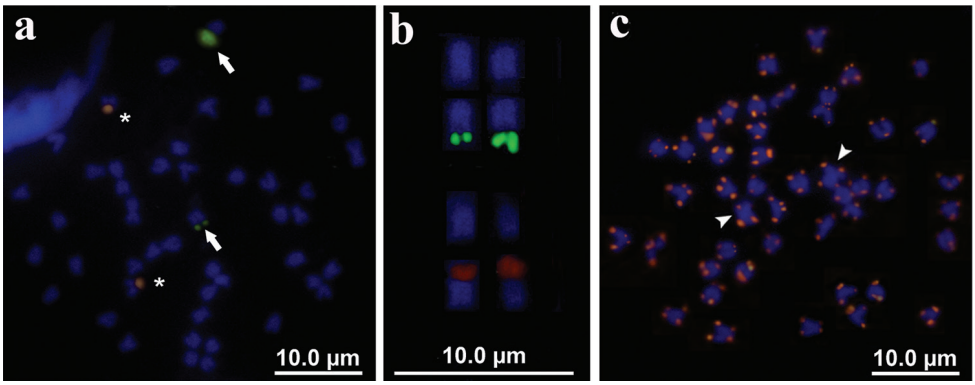


Figure 4. Somatic metaphases of the Pacific white mullet showing positive sites after FISH (**a**) with 18S rDNA (arrows) and 5S rDNA (asterisks) probes and (**c**) with telomeric repeats. Arrowheads indicate chromosome pair number one. In (**b**) enlargement of selected samples of chromosome pairs 15 and 20, after DAPI staining and FISH with rDNA probes, showing 18S (above) and 5S (below) positive sites, respectively. Scale bar: 10 µm.

the acrocentric chromosome pair classified as number 15 because its homologues show a more or less pronounced terminal achromatic region that is positively stained with $AgNO_3$ (Fig. 2, inset).

C-banding (Fig. 3) revealed the presence of constitutive heterochromatin at the centromeres of most chromosomes and at the telomeres of eight of them. The metacentric chromosome pair number 1 shows C-positive blocks both in the centromeric and in the terminal location; the acrocentric chromosome pair number 15 shows conspicuous heterochromatic blocks in the terminal region.

Dual FISH (Fig. 4a, b) revealed that the 18S rDNA probe yielded two hybridization signals on the same location detected by silver staining on chromosome pair number 15, whereas the 5S rDNA probes hybridized on one smaller medium-sized subtelo/acrocentric chromosome pair (likely number 20) proximal to the centromere.

Mapping of the (TTAGGG)n telomeric repeats showed the presence of positive signals on both telomeres of all chromosomes. No additional, interstitial or centromeric (TTAGGG)n positive signals were detected (Fig. 4c), even on metacentric chromosome pair number 1.

Discussion

Most of the approximately 20 species of Mugilidae cytogenetically investigated so far (see Rossi et al. 2016 for a review) show a conservative 48 uniarmed (subtelo-, acrocentric chromosomes) karyotype, as well as a conserved FN = 48. Even among the 15 cryptic species identified within the *Mugil cephalus* species complex (Durand and Borsa 2015), the six cytogenetically investigated lineages share not only the chromosome formula but also the major cytogenetic features (see Rossi et al. 2016

for references). The only exceptions to this picture of 2n = 48 are represented by *Liza* (currently *Planiliza) abu* (Heckel, 1843) (Değer et al. 2013) and the two "*M. curema*" lineages so far investigated (LeGrande and Fitzsimons 1976, Nirchio and Chequea 1998, Nirchio et al. 2005). *Planiliza abu* (Değer et al. 2013), which has a limited Asian distribution, shows a karyotype characterized by 2 large metacentric and 46 subtelo-, acrocentric chromosomes, a diploid number of 48 and FN = 50; a pericentric inversion is invoked to interpret the origin of the metacentric chromosome pair. The two cytogenetically known cytotypes of "*M. curema*", i.e., *M. curema sensu strictu* and *M. margaritae*, instead, are both characterized by FN = 48 and by a massive presence of biarmed chromosomes, likely derived from extensive Robertsonian centric fusions of subtelo- and acrocentric chromosomes. The *M. curema* karyotype is composed of 20 metacentric, 4 subtelo- and 4 acrocentric chromosomes, while the *M. margaritae* karyotype is composed of 22 metacentric and 2 submetacentric chromosomes.

The specimens analyzed in this study, molecularly assigned to the Pacific *Mugil* sp. O (Durand and Borsa 2015), show a still-undescribed karyotype in the family, i.e., a diploid number of 46 chromosomes, two of which are metacentrics and 44 of which are subtelo-acrocentrics. Most of the chromosomes of this karyotype are uniarmed, as in the other species belonging to the genus *Mugil*, as well as in different genera of Mugilidae (see Rossi et al. 2016). Nonetheless, the presence of biarmed chromosomes, of a reduced diploid number and of a conserved FN, which are shared with *M. curema sensu strictu* and *M. margaritae*, suggests that this karyotype originated by a limited number of centric fusions, i.e., only two uniarmed chromosome pairs were involved. These data confirm that a diploid number different from 48 characterizes all, and so far exclusively, the three investigated "*M. curema"* mitochondrial lineages, with a number of fusions covering the entire range of possibilities (Fig. 1). In *M. margaritae*, all uniarmed chromosomes underwent fusion, in *M. curema* most uniarmed chromosomes underwent fusion, and in the *Mugil* sp. lineage O, only two pairs. At present, it is not possible to discriminate whether the acquisition of the cytogenetic features and/or heterochromatin sequences that promote centric fusions occurred when the ancestor of the *M. curema* species complex split from the other *Mugil* species. Thus, it is not possible to ascertain whether these features were lost in some lineages, or, alternatively, were not acquired at that stage so that not all the molecular lineages were involved. In any case, as the lack of additional telomeric sequences is usually interpreted as a stabilizing factor for fusions (Slijepcevic 1998), the absence of telomeric sequences in a pericentromeric or interstitial position in all the cytogenetically studied "*M. curema"* lineages suggests that Robertsonian fusions are irreversible.

The *Mugil* sp. O described in this study shows the presence of NORs on a single chromosome pair, as well as minor ribosomal genes carried by a single chromosome pair. These features are common to most of the mugilids, including all the *Mugil* species (Rossi et al. 2016). Nevertheless, their location appears to be variable in different species/lineages of the *M. curema* species complex and does not allow any inference on the direction of chromosomal changes within the species complex.

Further analyses are required to draw a comprehensive picture of the chromosomal evolution within the *M. curema* species complex. Data on the karyotype of *M. thoburni*

and of the white mullet *Mugil* sp. M from the East Atlantic (Durand and Borsa 2015) are still missing, as well as data on the molecular analysis of the satellite DNA of the whole complex. It is worth noting that in the phylogenetic trees, the node separating "*M. curema"* lineages from *Mugil incilis* was not resolved (Fig. 4, Durand et al. 2012, Durand and Borsa 2015), and the latter species shows a karyotype (Hett et al. 2011) that is the closest to the "typical" all uniarmed mullet karyotype from which, presumably, the "*M. curema"* Robertsonian karyotypes derived. In a very recent paper (Xia et al. 2016) based both on molecular and diagnostic morphological characters, *M. incilis* appears to be the sister species to "*Mugil curema"* lineages, and *M. thoburni* is external to them. Unfortunately, only two of the "*Mugil curema"* lineages (Durand and Borsa 2015) were included in the analysis.

Data, although preliminary, strongly suggest that each of the "*Mugil curema"* lineages within the species complex has its own karyotype. This evidence, and the absence of intermediate karyotypes in the geographic area where different lineages/cytotypes are in sympatry, supports Durand and Borsa's hypothesis (2015) that chromosomal differences probably prevent interbreeding and indicate the actual reproductive isolation of cryptic species. In this context, a morphological analysis is now needed to assign a species name to the here-examined Pacific *Mugil* sp. O and possibly to the remaining allopatric East Atlantic *Mugil* sp. M.

Finally, it needs to be verified whether the karyotype observed in the specimens from Ecuador is also shared by specimens belonging to the *Mugil* sp. O. from other sampling sites along the American Pacific coast. In particular, a karyotypic analysis is needed for the western Mexican coast, because in the phylogenetic trees two individuals from this region are grouped in a subcluster that is highly divergent from the one that includes the remaining Pacific specimens.

Acknowledgments

This work was supported by Centro de Investigación, Universidad Técnica de Machala -UTMACH-, Ecuador (to MN); Sapienza University, Rome, Italy – Progetto di Università 2016 (to LS, VM, AR); Fundação de Amparo à Pesquisa do Estado de São Paulo -FAPESP-, Brazil and Conselho Nacional de DesenvolvimentoCientífico e Tecnológico -CNPq-, Brazil (to CO).

The authors wish to thank Dr Omar Rogerio Sánchez Romero (UTMACH) and Dr Ricardo Britzke (UTMACH) for their assistance in sampling.

References

Aljanabi SM, Martinez I (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Research 25(22): 4692–4693. https://doi.org/10.1093/nar/25.22.4692

AVMA (2013) Guidelines for the euthanasia of animals. https://www.avma.org/KB/Policies/Documents/euthanasia.pdf

Değer D, Ünlü E, Gaffaroğlu M (2013) Karyotype of mullet *Liza abu* Heckel, 1846 (Pisces: Mugilidae) from the Tigris River, Turkey. Journal of Applied Ichthyology 29(1): 234–236. https://doi.org/10.1111/j.1439-0426.2012.02028.x

Durand JD, Borsa P (2015) Mitochondrial phylogeny of grey mullets (Acanthopterygii: Mugilidae) suggests high proportion of cryptic species. Comptes Rendus Biologies 338(4): 266–277. https://doi.org/10.1016/j.crvi.2015.01.007

Durand JD, Shen KN, Chen WJ, Jamandre BW, Blel H, Diop K, Nirchio M, Garcia de León FJ, Whitfield AK, Chang CW, Borsai P(2012) Systematics of the Mugilidae (Teleostei: Mugiliformes): molecular phylogenetic evidence challenges two centuries of morphology-based taxonomy. Molecular Phylogenetics and Evolution 64(1): 73–92. https://doi.org/10.1016/j.ympev.2012.03.006

Eschmeyer WN, Fong JD (2016) Species by family/subfamily. http://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp [Accessed 6 March 2016]

Fraga E, Schneider H, Nirchio M, Santa-Brigida E, Rodrigues-Filho LF, Sampaio I (2007) Molecular phylogenetic analyses of mullets (Mugilidae, Mugiliformes) based on two mitochondrial genes. Journal of Applied Ichthyology 23(5): 598–604. https://doi.org/10.1111/j.1439-0426.2007.00911.x

Froese R, Pauly D (2016) FishBase. http://www.fishbase.org [Accessed 1 Apr 2016]

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology 59(3): 307–21. https://doi.org/10.1093/sysbio/syq010

Harrison IJ (1995) Mugilidae. In: Fischer W, Krupp F, Schneider W, Sommer C, Carpenter KE, Niem VH (Eds) Guia FAO para la identificacion de species para los fines de la pesca. Pacifico Centro-Oriental, vol III. FAO, Rome, 1293–1298. http://www.fao.org/docrep/010/v6250s/v6250s00.htm

Harrison IJ, Nirchio M, Oliveira C, Ron E, Gaviria J (2007) A new species of mullet (Teleostei: Mugilidae) from Venezuela, with a discussion on the taxonomy of *Mugil gaimardianus.* Journal of Fish Biology 71 (suppl. A): 76–97. https://doi.org/10.1111/j.1095-8649.2007.01520.x

Heras S, Gonzalez Castro M, Roldan MI (2006) *Mugil curema* in Argentinean waters: combined morphological and molecular approach. Aquaculture 261(2): 473–478. https://doi.org/10.1016/j.aquaculture.2006.07.003

Heras S, Roldán MI, Gonzalez Castro M (2009) Molecular phylogeny of Mugilidae fishes revised. Reviews in Fish Biology and Fisheries 19(2): 217–231. https://doi.org/10.1007/s11160-008-9100-3

Hett AK, Nirchio M, Oliveira C, Siccha ZR, Rossi AR, Sola L (2011) Karyotype characterization of *Mugil incilis* Hancock, 1830 (Mugiliformes: Mugilidae), including a description of an unusual co-localization of major and minor ribosomal genes in the family. Neotropical Ichthyology 9(1): 107–112. https://doi.org/10.1590/S1679-62252011005000005

Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36(8): 1014–1015. https://doi.org/10.1007/BF01953855

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8): 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Ijdo JW, Wells RA, Baldini A, Reeders ST (1991) Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. Nucleic Acids Research 19(17): 4780. https://doi.org/10.1093/nar/19.17.4780

LeGrande WH, Fitzsimons JM (1976)Karyology of the mullets *Mugil curema* and *Mugil cephalus* (Perciformes: Mugilidae) from Louisiana. Copeia 1976(2): 388–391. https://doi.org/10.2307/1443973

Menezes N, Nirchio M, Oliveira C, Siccha-Ramirez R (2015) Taxonomic review of the species of *Mugil* (Teleostei: Perciformes: Mugilidae) from the Atlantic South Caribbean and South America, with integration of the morphological, cytogenetic and molecular data. Zootaxa 3918(1): 1–38. https://doi.org/10.11646/zootaxa.3918.1.1

Milana V, Fusari A, Rossi AR, Sola L (2011) Molecular and morphological identification of an uncommon centrolophid fish. Central European Journal of Biology 6(3): 440–445. https://doi.org/10.2478/s11535-011-0016-x

Nirchio M, Cequea H (1998) Karyology of *Mugil liza* and *M. curema* from Venezuela. Boletín de Investigaciones Marinas y Costeras 27(1): 45–50.

Nirchio M, Oliveira C (2006) Citogenética de peces. Universidad de Oriente, Nueva Esparta, Porlamar,Venezuela, 203 pp.

Nirchio M, Cervigón F, Porto JIR, Perez JE, Gomez JA, Villalaz J (2003) Karyotype supporting *Mugil curem*a Valenciennes, 1836 and *Mugil gaimardianus* Desmarest, 1831 (Mugilidae: Teleostei) as two valid nominal species. Scientia Marina 67(1): 113– 115. https://doi.org/10.3989/scimar.2003.67n1113

Nirchio M, Cipriano RR, Cestari MM, Fenocchio AS (2005) Cytogenetical and morphological features reveal significant differences among Venezuelan and Brazilian samples of *Mugil curema*. Neotropical Ichthyology 3(1): 99–102. https://doi.org/10.1590/S1679-62252005000100006

Nylander JAA (2008) MrModeltest v2.3. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.

Pendás AM, Móran P, Freije JP, Garcia-Vásquez E (1994) Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenetics and Cell Genetics 67(1): 31–36. https://doi.org/10.1159/000133792

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences of the United States of America 83(9): 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14(9): 817–818. https://doi.org/10.1093/bioinformatics/14.9.817

Rossi AR, Crosetti D, Livi S (2016) Genetics of Mugilidae. In: Crosetti D, Blaber SJM (Eds) Biology, Ecology and Culture of Grey Mullet (Mugilidae). Taylor and Francis, Boca Raton, 349–397. https://doi.org/10.1201/b19927-16

Slijepcevic P (1998) Telomeres and mechanisms of Robertsonian fusion. Chromosoma 107(2): 136–140. https://doi.org/10.1007/s004120050289

Sumner AT (1972) A simple technique for demonstrating centromeric heterocromatin. Experimental Cell Research 75(1): 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28(10): 2731–2739. https://doi.org/10.1093/molbev/msr121

Thomson JM (1997) The Mugilidae of the world. Memoirs of the Queensland Museum 41: 457–562.

Ward RD, Zemlak TS, Innes BH, Last PR, Herbert PND (2005) DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society B - Biological Sciences 360(1462): 1847–1857. https://doi.org/10.1098/rstb.2005.1716

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds) PCR Protocols: A Guide to Methods and Applications. Academic Press Inc., London, 315–322. https://doi.org/10.1016/b978-0-12-372180-8.50042-1

Xia R, Durand JD, Fu C (2016) Multilocus resolution of Mugilidae phylogeny (Teleostei: Mugiliformes): implications for the family's taxonomy. Molecular Phylogenetics and Evolution 96: 161–177. https://doi.org/10.1016/j.ympev.2015.12.010

CompCytogen 11(2): 239–248 (2017)
doi: 10.3897/CompCytogen.v11i2.11683
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

A karyotype comparison between two species of bordered plant bugs (Hemiptera, Heteroptera, Largidae) by conventional chromosome staining, C-banding and rDNA-FISH

Lucila Belén Salanitro[1], Anabella Cecilia Massaccesi[1], Santiago Urbisaglia[1], María José Bressa[2,3], Mónica Gabriela Chirino[1,2,3]

1 *Laboratorio de Entomología Aplicada y Forense, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, Bernal (B1876BXD), Buenos Aires, Argentina* **2** *Grupo de Citogenética de Insectos, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, Ciudad Universitaria, Ciudad Autónoma de Buenos Aires (C1428EHA), Argentina* **3** *Consejo Nacional de Investigaciones Científicas y Técnicas, Godoy Cruz 2290, Ciudad Autónoma de Buenos Aires (C1425FQB), Argentina*

Corresponding author: *Mónica Gabriela Chirino* (mchirino@ege.fcen.uba.ar)

Academic editor: *S. Nokkala* | Received 2 January 2017 | Accepted 25 February 2017 | Published 13 April 2017

http://zoobank.org/761C7678-84D5-4250-8ACF-355D7DF3EDA9

Citation: Salanitro LB, Massaccesi AC, Urbisaglia S, Bressa MJ, Chirino MG (2017) A karyotype comparison between two species of bordered plant bugs (Hemiptera, Heteroptera, Largidae) by conventional chromosome staining, C-banding and rDNA-FISH. Comparative Cytogenetics 11(2): 239–248. https://doi.org/10.3897/CompCytogen.v11i2.11683

Abstract

A cytogenetic characterization, including heterochromatin content, and the analysis of the location of rDNA genes, was performed in *Largus fasciatus* Blanchard, 1843 and *L. rufipennis* Laporte, 1832. Mitotic and meiotic analyses revealed the same diploid chromosome number 2n = 12 + X0/XX (male/female). Heterochromatin content, very scarce in both species, revealed C-blocks at both ends of autosomes and X chromosome. The most remarkable cytological feature observed between both species was the different chromosome position of the NORs. This analysis allowed us to use the NORs as a cytological marker because two clusters of rDNA genes are located at one end of one pair of autosomes in *L. fasciatus*, whereas a single rDNA cluster is located at one terminal region of the X chromosome in *L. rufipennis*. Taking into account our results and previous data obtained in other heteropteran species, the conventional staining, chromosome bandings, and rDNA-FISH provide important chromosome markers for cytotaxonomy, karyotype evolution, and chromosome structure and organization studies.

Keywords

Largus, Heteroptera, C-banding, rDNA-FISH, holokinetic chromosomes, karyotype comparison

Introduction

All species of Hemiptera studied so far present holokinetic chromosomes (i.e. without a primary constriction). Kinetic activity is restricted to the chromosome ends and the chromosomes can be regarded as telokinetic during male meiosis, but holokinetic activity is recognized in mitosis. Meiotic behaviour is slightly different depending on whether we are dealing with autosomal bivalents, sex chromosomes, m chromosomes or autosomal univalents. As a rule, autosomal bivalents are chiasmatic and segregate reductionally, whereas sex and m chromosomes are achiasmatic and divide equationally at first male meiotic division. Besides, sex chromosomes do not present a defined position at metaphases I and II. Several reports on C-positive heterochromatin in true bugs showed that C-bands are terminally located (Ueshima 1979, Manna 1984, Papeschi and Bressa 2006).

At present, the seven species cytogenetically studied of Largidae possess a low diploid chromosome number, ranging between 11 and 17 autosomes, a X0/XX sex chromosome system (male/female), except for one species, and a pair of m chromosomes, excluding the genus *Largus* Hahn, 1831 (Ueshima 1979, Manna 1984, Manna et al. 1985, Mola and Papeschi 1993, Bressa et al. 2005).

The genus *Largus* comprises 61 taxonomically described species and most of them are distributed in America, where its geographic distribution ranges from the north of the United States to the south of Argentina. Although they are more diverse and abundant in tropical and subtropical areas, in Argentina there are only seven species recorded (Melo and Dellapé 2013, Rosas and Brailovsky 2016). At cytogenetic level, *Largus rufipennis* Laporte, 1832 is the only species analysed to this date, using only conventional methods (Mola and Papeschi 1993, Bressa et al. 1998, 2005). It possesses a male diploid number of 2n = 13 = 12 + X0 and very large chromosomes. The partial karyotype analyses allowed detecting several Argentinean populations with different number of autosomal univalents, variable chiasma frequency, and the presence/absence of B chromosomes.

The main aim of this study was to describe the karyotype of *L. fasciatus* Blanchard, 1843 and examine the structure of its holokinetic chromosomes by means of C- banding and fluorescent *in situ* hybridization (FISH) with 18S rDNA probes. Using these data we performed a detailed comparison of the content and distribution of constitutive heterochromatin and the location of rDNA gene clusters between *L. fasciatus* and *L. rufipennis* collected from several fields in Argentina.

Material and methods

Insects

Adults and nymphs of *L. fasciatus* (12 males and 2 females) and *L. rufipennis* (6 males) were collected from 1995 to 2009 in several fields from Buenos Aires and Entre Ríos in Argentina (Table 1). The collected adults were taxonomically determined by María del

Table 1. Species, locality, geographical coordinates, and number of adults' collected and examined of *Largus* for chromosomal analyses discriminated by gender.

Species	City and Province from Argentina	Coordinates (DMS)	N° of individuals
Largus fasciatus	Tornquist, Buenos Aires	38°05'45"S, 62°13'25"W	11 males, 2 females
Largus rufipennis	Isla Martín García, Buenos Aires	34°11'03"S, 58°14'58"W	1 male
	Sierra de los Padres, Buenos Aires	37°56'50"S, 57°46'40"W	3 males
	Santa Catalina, Buenos Aires	34°46'11"S, 58°27'19"W	1 male
	Ceibas, Entre Ríos	33°30'02"S, 58°48'16"W	1 male

Carmen Coscarón (Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata) and specimens were deposited in the Museo Argentino de Ciencias Naturales Bernardino Rivadavia (MACN, Buenos Aires, Argentina).

Chromosome preparations

The captured specimens were swollen in freshly prepared fixative (methanol: glacial acetic acid, 3:1). In the laboratory their gonads were dissected out in 70% ethanol. Cells of gonads were dissociated in a drop of 45% acetic acid, prepared by the squash technique, and stored at -20º C until use. Chromosome preparations were removed from freezer, dehydrated in an ethanol series, and air-dried. For mitotic and meiotic analyses, the chromosome preparations were stained with 5% Giemsa solution following conventional procedures. Heterochromatin content and distribution were analysed by means of C-bands according to Papeschi (1988), and the pre-treated slides were stained with 4'6-diamidino-2-phenylindole (DAPI; Fluka BioChemika, Sigma Aldrich Production GmbH, Buchs, Switzerland) for a better resolution (Poggio et al. 2011).

Fluorescence *in situ* hybridization

Spread chromosome preparations were made in a drop of 60% acetic acid with the help of tungsten needles and the spreading on the slide was performed using a heating plate at 45° C as described in Traut (1976). Unlabelled 18S ribosomal DNA (rDNA) probes were generated by polymerase chain reaction (PCR) using universal arthropod primers: forward 5′-CCTGAGAAACGGCTACCACATC-3′ and reverse 5′-GAGTCTCGTTCGTTATCGGA-3′ (Whiting 2002). Total genomic DNA of *Dysdercus albofasciatus* Berg, 1878, obtained by standard phenol-chloroform-isoamyl alcohol extraction, was used as a template. PCR was done following the profile described in Fuková et al. (2005). The PCR product showed a single band of about 1,000 bp on a 1% agarose gel. The band was excised from the gel and purified by using a QIAquick Gel Extraction Kit (Quiagen GmbH, Hilden, Germany). The 18S rDNA fragment was re-amplified by PCR and then labeled with biotin 14-dATP by nick translation using a BioNick Labeling System (Invitrogen, Life Technologies Inc.,

San Diego, CA, USA). FISH with a biotinylated 18S rDNA probe was carried out following the procedure described in Sahara et al. (1999) with several modifications described by Fuková et al. (2005) and Bressa et al. (2009).

Microscopy, photographs and image processing

Preparations were observed under high power magnification using a Leica DMLB epifluorescence microscope equipped with a Leica DFC350 FX CCD camera and Leica IM50 software, Version 4.0 (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Black-and-white images of chromosomes were recorded separately for each fluorescent dye with the CCD camera. Images were pseudo-coloured (light blue for DAPI and red for Cy3) and processed with Adobe Photoshop CS6 Version 6.1 (1999–2012) software.

Results

Based on the observation of metaphase I autosomal bivalents (AA) and the identification of the sex univalent we described the male karyotype of *L. rufipennis* as 2n = 6AA + X0 (see Mola and Papeschi 1993), and the chromosome complement of *L. fasciatus* as 2n = 6AA + X0/XX (male/female sex chromosomes) (Fig. 1). In both species, the autosomes decrease gradually in size and the X chromosome is the smallest of the complement having an equal or nearly equal diameter in all directions (Fig. 1). From diakinesis onwards, the X is negatively heteropycnotic (Fig. 1a, b, d, e). The X chromosome in *L. rufipennis* is slightly longer than in *L. fasciatus* (Fig. 1b, e). At metaphases I and II, the autosomes are arranged forming a ring and with the X located outside of it (Fig. 1b–e).

The C-banding pattern in *L. rufipennis* and *L. fasciatus* showed discrete C-positive bands terminally located in all autosomes and the X chromosomes, which were observed in all stages of mitosis and meiosis (Fig. 2).

FISH experiments with the 18S rDNA probe revealed differences in the location of the probe signals between both species analysed (Fig. 3). In *L. rufipennis* a cluster of rDNA genes was located at one end of the X chromosome (Fig. 3a–b), whereas in *L. fasciatus* the hybridization signals were located at a subterminal position in an autosomal bivalent (Fig. 3c–d).

Discussion

Largus rufipennis and *L. fasciatus*, the two species herein analysed, showed similar karyotypes composed of six pairs of autosomes, a simple sex chromosome system (X0/XX), and the same location and distribution of constitutive heterochromatin. The main cytogenetic difference between both species was detected in the location of the rDNA

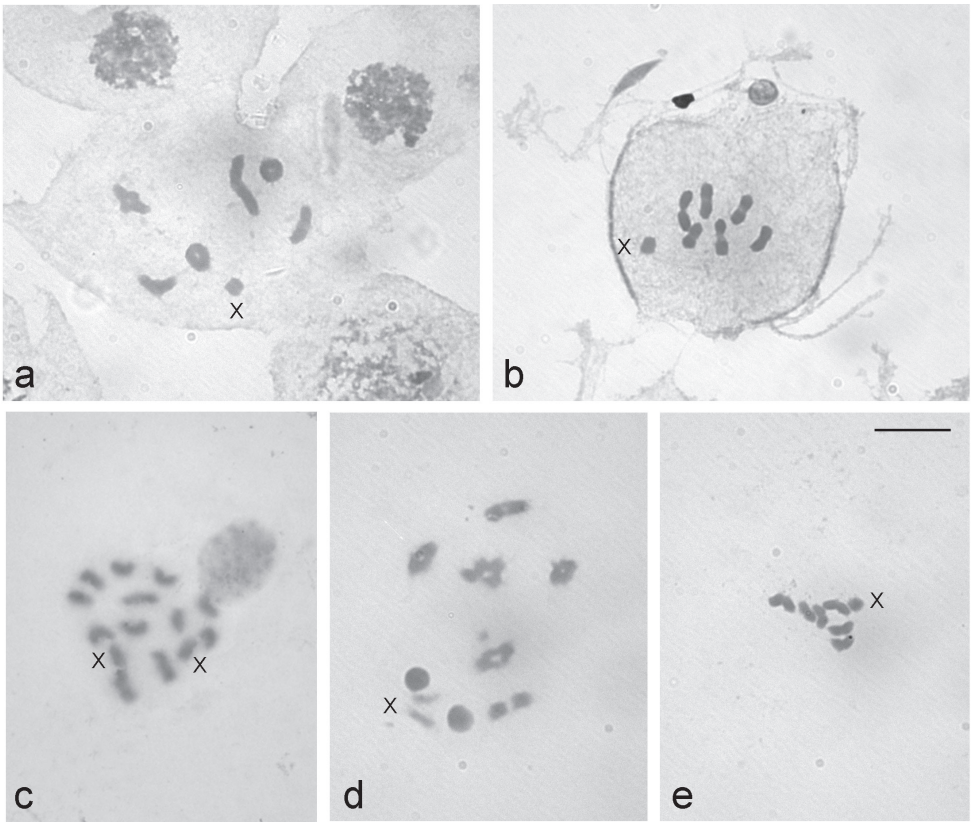


Figure 1. Karyotypes of *L. rufipennis* (**a–b**) and *L. fasciatus* (**c–e**) stained with 5% Giemsa. **a** diakinesis **b** metaphase I **c** oogonial metaphase (2n = 12 + XX) **d** diakinesis **e** metaphase I. X = sex chromosome. Scale bar: 10 μm.

clusters. Two signals were located at a subterminal position of an autosomal bivalent of *L. fasciatus* but only one signal was observed at one end of the X chromosome of *L. rufipennis*. Taking into account the data on the chromosomal location of rDNA clusters in other heteropteran species along with our results, the NORs chromosome location varies among several congeneric species, i.e. *Belostoma* Leach, 1815 (Nepomorpha), *Triatoma* Laporte, 1832, *Panstrongylus* Berg, 1879, *Rhodnius* Stål, 1859 (Cimicomorpha), and *Dysdercus* Guérin-Méneville 1831 (Pentatomomorpha) (Papeschi and Bressa 2006, Morielle-Souza and Azeredo-Oliveira 2007, Bressa et al. 2009, Chirino et al. 2013, Pita et al. 2013, Chirino and Bressa 2014, Grozeva et al. 2014, Panzera et al. 2014), and also among different species of Tingidae (Cimicomorpha) and several species belonging to different families of Pentatomomorpha (Bardella et al. 2013, 2016, Golub et al. 2015, 2016). The analysis in a wide number of species shows that 5S, 18S, and 45S rDNA remain mainly among the autosomes, although in some species the NORs are located in the sex and m chromosomes. This might be due to the fact that NORs can be easily translocated to other chromosomes changing their number and

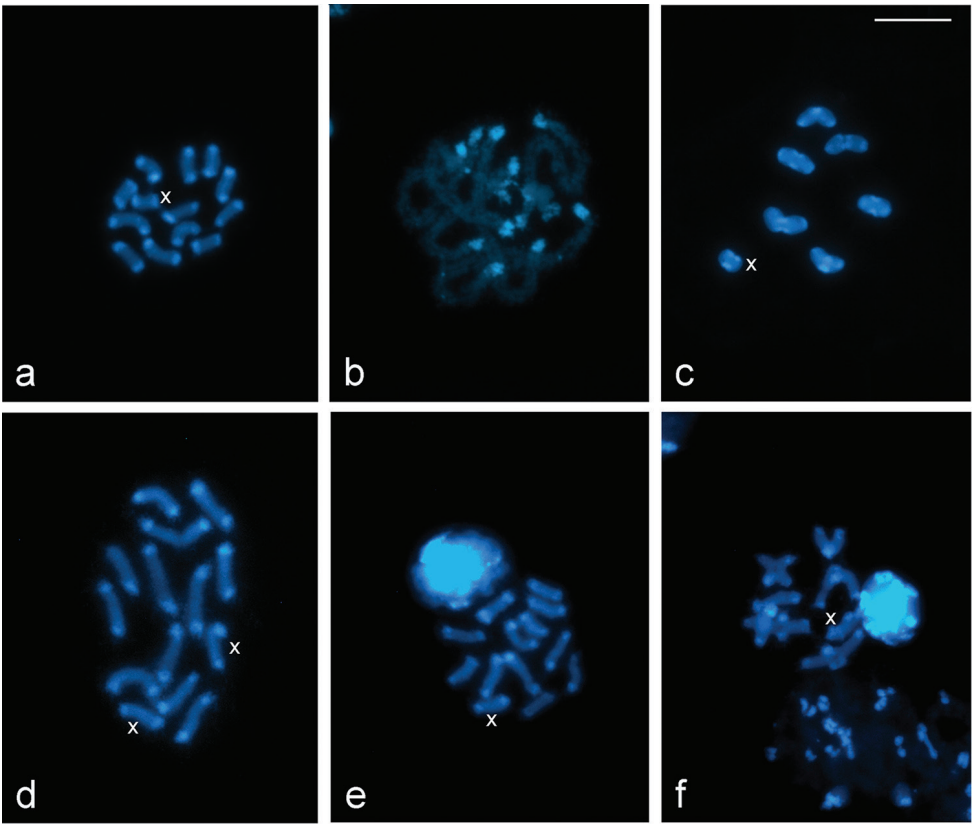


Figure 2. C-banding in chromosomes of *L. rufipennis* (**a–c**) and *L. fasciatus* (**d–f**) stained with DAPI. **a** spermatogonial metaphase **b** pachytene **c** metaphase I **d** oogonial promethaphase **e** spermatogonial metaphase **f** diakinesis. X = sex chromosome. Scale bar: 10 μm.

position. Consequently, the number and location of rDNA loci (determined by FISH and/or Ag-NOR banding) constitutes an important chromosome marker, which can be useful for studies on cytotaxonomy, karyotype evolution, and chromosome structure and organization for heteropteran species. Therefore, rearrangements involving rDNA-repositioning seem to be involved in the species´ evolutionary history, indicating a particular genome dynamics for this marker.

From the cytogenetic point of view, Largidae is an interesting heteropteran family because of its low diploid chromosome number and the large chromosome size observed in most of the species (Ueshima 1979, Manna 1984, Manna et al. 1985, this study). The six karyologically analysed species of the subfamily Larginae, *Largus* and *Macrochraia* Guérin-Méneville, 1835, are characterized by the absence of an m chromosome pair, the possession of an X0/XX sex chromosome mechanism, and a number of autosomes that varies between 10 and 14. Conversely, all the studied species belonging to Physopeltinae possess 12 autosomes, two m chromosomes, and different sex chro-

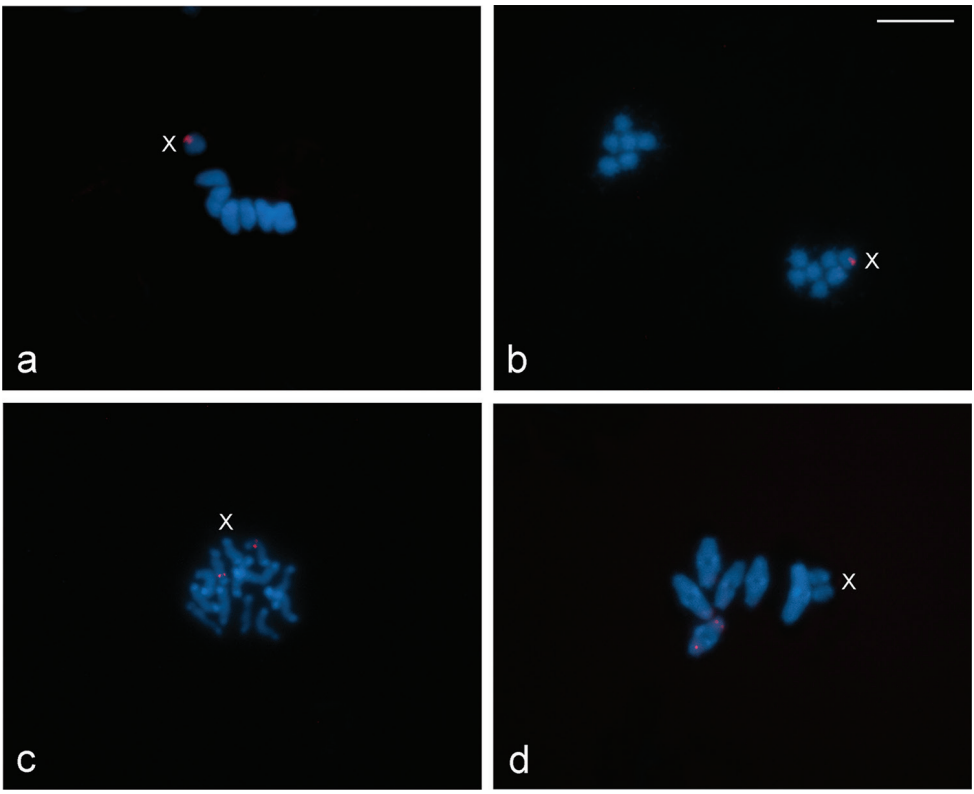


Figure 3. Location of rDNA genes in chromosomes of *L. rufipennis* (**a–b**) and *L. fasciatus* (**c–d**) by FISH with 18S rDNA probes (red signals). Chromosomes were counterstained with DAPI (blue). **a** metaphase I **b** telophase II **c** spermatogonial prometaphase **d** diakinesis. X = sex chromosome. Scale bar: 10 μm.

mosomes systems (X0 or X_1X_2Y) (see references in Papeschi and Bressa 2006). Based on the presence of a Y chromosome in very primitive heteropteran species, Nokkala and Nokkala (1983, 1984) and Grozeva and Nokkala (1996) suggested that the X0 system is a derived condition from the ancestral XY that is present in the majority of the species cytogenetically analysed. In Larginae, the X0 sex chromosome system most probably originated through the loss of the Y chromosome. The finding of a pair of m chromosomes in three species of Physopeltinae (Ueshima 1979, Manna et al. 1985) led us to suggest that this pair of chromosomes might be involved in the ancestral karyotype of the family Largidae. Then, the absence of m chromosomes and the presence of sex chromosome system X0 in species of Larginae could be considered as derived characters, which arose during karyotype evolution.

The use of different cytogenetic techniques will be very useful in further integrative studies because a group-level taxonomy followed by a reliable association among different data sets is fundamental to allow a more precise evaluation of the processes involved in the karyotype evolution and the interrelationships among different species.

Conclusions

Taking into account the data on the number and location of rDNA clusters in *L. rufipennis* and *L. fasciatus*, we can observe two different patterns of rDNA distribution. As a result, the rDNA clusters revealed by rDNA-FISH are very useful tools for the study of the karyotype structure and chromosome evolution in groups with holokinetic chromosomes due to it can contribute to understand the karyotype evolution and taxonomic relationships among several taxa.

Acknowledgements

This work was funded by grants PIP 2015 Nº 11220150100347 of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and PICT 0604 of Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) from Argentina.

References

Bardella VB, Fernandes T, Vanzela ALL (2013) The conservation of number and location of 18S sites indicates the relative stability of rDNA in species of Pentatomomorpha (Heteroptera). Genome 56(7): 425–429. https://doi.org/10.1139/gen-2013-0140

Bardella VB, Fernandes JAM, Cabral-de-Mello DC (2016) Chromosomal evolutionary dynamics of four multigene families in Coreidae and Pentatomidae (Heteroptera) true bugs. Molecular Genetics and Genomics 291(5): 1919–1925. https://doi.org/10.1007/s00438-016-1229-5

Bressa MJ, Papeschi AG, Mola LM, Larramendy ML (1998) Meiotic studies in *Largus rufipennis* (Castelnau) (Largidae, Heteroptera). II. Reciprocal traslocation heterizygosity. Caryologia 51: 253–264. doi:10.1080/00087114.1998.10797417

Bressa MJ, Larramendy ML, Papeschi AG (2005) Heterochromatin characterization in five species of Heteroptera. Genetica 124(2–3): 307–317. https://doi.org/10.1007/s10709-005-4524-3

Bressa MJ, Papeschi AG, Vítková M, Kubíčková S, Fuková I, Pigozzi MI, Marec F (2009) Sex chromosome evolution in cotton stainers of the genus *Dysdercus* (Heteroptera: Pyrrhocoridae). Cytogenetic and Genome Research 125(4): 292–305. https://doi.org/10.1159/000235936

Chirino MG, Papeschi AG, Bressa MJ (2013) The significance of cytogenetics for the study of karyotype evolution and taxonomy of water bugs (Heteroptera, Belostomatidae) native to Argentina. Comparative Cytogenetics 7(2): 9–27. https://doi.org/10.3897/CompCytogen.v7i2.4462

Chirino MG, Bressa MJ (2014) Karyotype evolution in progress: a new diploid number in *Belostoma candidulum* (Heteroptera: Belostomatidae) from Argentina leading to new insights

into its ecology and evolution. European Journal of Entomology 111(2): 165–174. https://doi.org/10.14411/eje.2014.027

Fuková I, Nguyen P, Marec F (2005) Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. Genome 48(6): 1083–1092. https://doi.org/10.1139/g05-063

Golub NV, Golub VB, Kuznetsova VG (2015) Variability of 18rDNA loci in four lace bug species (Hemiptera, Tingidae) with the same chromosome number. Comparative Cytogenetics 9(4): 513–522. https://doi.org/10.3897/CompCytogen.v9i4.5376

Golub NV, Golub VB, Kuznetsova VG (2016) Further evidence for the variability of the 18S rDNA loci in the family Tingidae (Hemiptera, Heteroptera). Comparative Cytogenetics 10(4): 517–528. https://doi.org/10.3897/CompCytogen.v10i4.9631

Grozeva S, Nokkala S (1996) Chromosomes and their meiotic behavior in two families of the primitive infraorden Dipsocoromorpha (Heteroptera). Hereditas 125(1): 31–36. https://doi.org/10.1111/j.1601-5223.1996.t01-1-00031.x

Grozeva S, Anokhin B, Kuznetsova VG (2014) Chapter 8. Recent advances in cytogenetics of bed bugs: FISH mapping of the 18S rDNA and TTAGG telomeric loci in *Cimex lectularius* Fabricius, 1803 (Hemiptera, Heteroptera, Cimicidae). In: Sharachov I (Ed.) Protocols for chromosome mapping of arthropod genomes, CRCPress, Taylor & Francis, Boca Raton, Florida, 283–322.

Manna GK, (1984) Chromosomes in evolution in Heteroptera. In: Sharma AK, Sharma A (Eds) Chromosomes in evolution of eukaryotic groups. CRCPress, Boca Raton, Florida, 189–225.

Manna GK, Ueshima N, Dey SK, Ded-Mallick S (1985) Market Sex chromosomal variations between Indian and a Japanese species of *Physopelta* (Largidae, Heteroptera). Cytologia 50: 621–630. https://doi.org/10.1508/cytologia.50.621

Melo MC, Dellapé PM (2013) Catalogue of the Pyrrhocoroidea (Hemiptera: Heteroptera) from Argentina. Revista de la Sociedad Entomológica Argentina 72(1–2): 55–74. http://www.scielo.org.ar/pdf/rsea/v72n1-2/v72n1-2a06.pdf [In Spanish]

Mola LM, Papeschi AG (1993) Meiotic studies in *Largus rufipennis* (Castelnau) (Largidae, Heteroptera): frequency and behaviour of ring bivalents, univalents and B chromosomes. Heredity 71: 33–40. doi:10.1038/hdy.1993.104

Morielle-Souza A, Azeredo-Oliveira MTV (2007) Differential characterization of holocentric chromosomes in triatomines (Heteroptera, Triatominae) using different staining techniques and fluorescent *in situ* hybridization. Genetics and molecular research 6(3): 713–720. https://science.report/pub/16044094

Nokkala S, Nokkala C (1983) Achiasmatic male meiosis in two species of *Saldula* (Saldidae, Hemiptera). Hereditas 99(1): 131–134. https://doi.org/10.1111/j.1601-5223.1983.tb00737.x

Nokkala S, Nokkala C (1984) The occurrence of the X0 sex chromosome system in *Dictyonota tricornis* (Schr.) (Tingidae, Hemiptera) and its significance for concepts of sex chromosome system evolution in Heteroptera. Hereditas 100(2): 299–301. https://doi.org/10.1111/j.1601-5223.1984.tb00130.x

Panzera F, Ferreiro MJ, Pita S, Calleros L, Pérez R, Basmadjián Y, Guevara Y, Frédérique Brenière S, Panzera Y (2014) Evolutionary and dispersal history of *Triatoma infestans*, main vector of Chagas disease, by chromosomal markers. Infection, Genetics and Evolution 27: 105-113. https://doi.org/10.1016/j.meegid.2014.07.006

Papeschi AG (1988) C-banding and DNA content in three species of *Belostoma* (Heteroptera) with large differences in chromosome size and number. Genetica 76: 43–51. https://doi.org/10.1007/BF00126009

Papeschi AG, Bressa MJ (2006) Evolutionary cytogenetics in Heteroptera. Journal of Biological Research 5: 3–21. http://www.jbr.gr/papers20061/01-Alba.pdf

Pita S, Panzera F, Ferrandis I, Galvao C, Gomez-Palacio A, Panzera Y (2013) Chromosomal divergence and evolutionary inferences in Rhodniini based on the chromosomal location of ribosomal genes. Memórias do Instituto Oswaldo Cruz 108(3): 376–382 https://doi.org/10.1590/S0074-02762013000300017

Poggio MG, Bressa MJ, Papeschi AG (2011) Male meiosis, heterochromatin characterization and chromosomal location of rDNA in *Microtomus lunifer* (Berg, 1900) (Hemiptera: Reduviidae: Hammacerinae). Compative Cytogenetics 5(1): 1–22. https://doi.org/10.3897/compcytogen.v5i1.1143

Rosas C, Brailovsky H (2016) Revision of the genus *Largus* (Hemiptera: Heteroptera: Largidae) from Mexico. Revista Mexicana de Biodiversidad 87: 347–375. https://doi.org/10.1016/j.rmb.2016.05.001

Sahara K, Marec F, Traut W (1999) TTAGG telomeric repeats in chromosomes of some insects and other arthropods. Chromosome Research 7: 449–460. https://doi.org/10.1023/A:1009297729547

Traut W (1976) Pachytene mapping in the female silkworm *Bombyx mori* L. (Lepidoptera). Chromosoma 58: 275–284. https://doi.org/10.1007/BF00292094

Ueshima N (1979) Hemiptera II: Heteroptera. Animal Cytogenetics 3 Insecta 6. Gebrüder Borntraeger, Stuttgart, 117 pp.

Whiting MF (2002) Phylogeny of the holometabolous insect orders: molecular evidence. Zoologica Scripta 31(1): 3–15. doi:10.1046/j.0300-3256.2001.00093.x

CompCytogen 11(2): 249–266 (2017)
doi: 10.3897/CompCytogen.v11i2.10804
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Variability of NOR patterns in European water frogs of different genome composition and ploidy level

Anna Zaleśna[1], Maria Florek[1], Mariusz Rybacki[2], Maria Ogielska[1]

1 *Department of Evolutionary Biology and Conservation of Vertebrates, Institute of Environmental Biology, University of Wrocław, Poland* **2** *Department of Zoology, Kazimierz Wielki University, Al. Ossolińskich 12, 85–067, Poland*

Corresponding author: *Maria Ogielska* (maria.ogielska@uwr.edu.pl)

Academic editor: *L. Kupriyanova* | Received 14 October 2016 | Accepted 14 February 2017 | Published 18 April 2017

http://zoobank.org/C821C505-DCF1-4B0C-9BE0-582DE28CEEE3

Citation: Zaleśna A, Florek M, Rybacki M, Ogielska M (2017) Variability of NOR patterns in European water frogs of different genome composition and ploidy level. Comparative Cytogenetics 11(2): 249–266. https://doi.org/10.3897/CompCytogen.v11i2.10804

Abstract

We studied water frogs from a complex composed of two species: *Pelophylax lessonae* (Camerano, 1882) (genome LL, 2n = 26) and *P. ridibundus* (Pallas, 1771) (RR, 2 = 26), and their natural hybrid *P. esculentus* (Fitzinger, 1843) of various ploidy and genome composition (RL, 2n = 26, and RRL or RLL, 3n = 39). Tetraploids RRLL were found (4n = 52) in juveniles. We applied cytogenetic techniques: $AgNO_3$, chromomycin A_3, PI and fluorescent *in situ* hybridization with a 28S rDNA probe. Results obtained by silver staining corresponded well with those stained with CMA_3, PI and FISH. As a rule, NORs are situated on chromosomes 10. The number of Ag-NORs visible on metaphase plates was the same as the number of Ag-nucleoli present in interphase nuclei of the same individual. In all analyzed metaphases, NORs exhibited variations in size after $AgNO_3$ and CMA_3 stainings. Sixty-six individuals (out of 407 analyzed) were polymorphic for the localization and number of NORs. Fifty-one diploids had NORs only on one chromosome of pair 10. Three triploids (LLR and RRL) displayed two NORs, and two other triploid RRL individuals displayed one, instead of expected three NORs. In ten individuals extra NORs were detected on chromosomes other than 10 (chromosomes 2 and 9).

Keywords

Pelophylax esculentus complex, hybridogenesis, triploidy, NOR inheritance

Introduction

Nucleolus organizer regions (NORs) are sites of nucleoli formation owing to the presence of genes (rDNA) coding for 18S rRNA, 5.8S rRNA and 28S rRNA. They are the only genes that can be recognized in genomes on the basis of chromosome structure and thereby are useful as cytogenetic markers. These chromosomes differ from others by the presence of secondary constrictions where NORs are situated. The number and position of NORs are species specific, although inter-individual variability of these regions has also been observed within species. In anuran amphibians, NORs detected by silver staining (Ag-NOR) revealed that most species, both from primitive and derived families, have only one pair of NOR-bearing chromosomes in their diploid karyotypes (Schmid 1982, King et al. 1990, Birstein 1984, Vitelli et al. 1982, Bruschi et al. 2012, Carvalho et al. 2014). The localization of NORs is conservative, *i.e.* they are almost always located intercalary or proximally to the centromeres or close to telomeres or related to other regions rich in heterochromatin (Iizuka et al. 2013). Exceptions to this rule may suggest chromosomal rearrangements that have occurred in NOR-carrying chromosome segments during evolution (Schmid 1982). Variations of Ag-NORs may be connected with different expression of rRNA genes during the preceding interphase (Reeder 1990). Using silver staining, only active NORs are identified and thereby the actual sites of rDNA transcription should be verified by more specific methods, such as fluorescence *in situ* hybridization (FISH) with use of 18S or 28S rDNA probes that unequivocally indicate the rDNA loci (Iizuka et al. 2013). The variability of NORs can also reflect geographical karyotypic variations. In the endemic frog *Proceratophrys boiei*, NORs were located on different chromosome pairs, depending on geographical region of its distribution (Amaro et al. 2012). Similar results were described in *Physalaemu olfersii* (Silva et al. 2000), *Hypsiboas* (Carvalho et al. 2014), *Physalaemus cuvieri* (Quinderé et al. 2009), and in the Jefferson salamanders *Ambystoma jeffersonianum* (Bi et al. 2009).

In water frogs, NORs are located in secondary constrictions on long arms of chromosome pair 10, as observed after $AgNO_3$ staining (Schmid 1982, Koref-Santibanez and Günther 1980, Heppich et al. 1982, Vitelli et al. 1982), chromomycin $A_{3,}$ and FISH with 18S+28S probes (Spasič-Bošković et al. 1999, Martirosyan and Stepanyan 2009).

Central European water frogs form a complex composed of two species: *Pelophylax lessonae* (Camerano, 1882) (genome LL, 2n = 26) and *P. ridibundus* (Pallas, 1771) (RR, 2 =26), and their natural hybrid *P. esculentus* (Fitzinger, 1843) of various ploidy and genome composition (RL, 2n = 26, and RRL or RLL, 3n = 39) (Ogielska et al. 2004, Zaleśna et al. 2011). Hybrids are sympatric with one of the parental species and live in mixed populations (called also genetic systems) composed of one of the parental species and hybrids (*P. lessonae- P.esculentus*, *P. ridibundus- P. esculentus*) or all-hybrid (*P. esculentus- P. esculentus*) composed of diploid and triploid individuals (Berger 1983, 1988, Graf and Polls-Pelaz 1989, Rybacki and Berger 2001, Plötner 2005). Reproduction and maintenance of the hybrid *P. esculentus* is the result of hybridogenesis, a unique way of hybrid reproduction (Schultz 1969). During this process, one of the

parental sets of chromosomes of a hybrid (in this case R or L) is discarded from the germ line before meiosis and the other one is duplicated and clonally transmitted into gametes (Graf and Polls-Pelaz 1979). Some hybrids produce diploid gametes which give rise to triploid progeny (Uzzell et al. 1975, Rybacki 1994) and the combinations of genomes provided by the mother and by the father result in two types of triploids, namely RRL and RLL. Progeny of higher ploidy (4n and 5n) were also recorded but such individuals died before completion of metamorphosis or soon after (Christiansen 2009, Hermaniuk et al. 2013).

We applied cytogenetic techniques, *i.e.* silver, chromomycin A_3 and Propidium Iodide (PI) staining, and fluorescent *in situ* hybridization with 28S rDNA probe commonly used in comparative studies of cold-blooded vertebrates: fish (Sola et al. 1992, Ráb et al. 1996, Boroń 1999, Rábová et al. 2001, 2003, Kirtiklis et al. 2010) and amphibians (Schmid 1982, Schmid et al. 1995). The aim of our study was to investigate the variability of NOR distribution patterns in metaphase chromosomes and interphase nuclei in water frogs, especially hybrid *P. esculentus* of various ploidy and genome composition. The results provide not only new data on distribution and/or polymorphism of NORs, but also may help to better understand inheritance of their patterns as the result of clonal genome transmission by hybrids during hybridogenetic gametogenesis.

Materials and methods

Animal sampling

In total, 407 individuals were analyzed: 272 adults and 135 newly metamorphosed juveniles. Juveniles were analyzed separately, as we expected a higher frequency of polyploids than in adults because tetraplois and pentaploids do not survive until adulthood (Hermaniuk et al. 2013, Christiansen 2009). Adult individuals (79 *P. lessonae*, 82 *P. ridibundus* and 111 *P. esculentus*) were collected from 11 natural populations in Central Europe (Table 1). Juveniles (15 *P. lessonae*, 52 *P. ridibundus* and 45 diploid, 18 triploid and 5 tetraploid *P. esculentus*) were obtained from *in vitro* artificial crosses. Their parents (*P. lessonae* LL, *P. ridibundus* RR, and *P. esculentus* RL, RRL, RLL) were collected from natural populations and served for 28 crosses resulting from various parental combinations (Table 2). Artificial crosses and rearing of progeny were done according to standard protocol for water frogs (Berger et al. 1994). Taxonomic identity of individuals was determined by morphology (Berger 1983, Plötner 2005, Kierzkowski et al. 2011) and at least one of the following methods: actinomycin D (AMD)-DAPI staining (Schweitzer 1976, Heppich et al. 1982, Ogielska et al. 2004), LDH electrophoresis and 17 microsatellite loci (both methods described in Hauswaldt et al. 2012).

Frogs were collected in years 2002–2009. All specimens used in this study were collected according to legal regulations concerning wild species protection under the following permits: Agency for Nature Conservation and Landscape Protection of

Table 1. Number, collection site, sex, taxonomic status and genotype of adult individuals.

Population				Number of individuals									
nr	Name	type	Coordinates of sampling sites	*lessonae*		*esculentus*						*ridibundus*	
				LL		RL		RLL		RRL		RR	
				F	M	F	M	F	M	F	M	F	M
1	Baczysław	EE	53°49'53"N, 14°51'53"E							1			
2	Barycz River valley	RE	51°25'42"N, 16°56'49"E 51°31'03"N, 17°02'04"E 51°31'05"N, 17°02'30"E 51°31'42"N, 17°49'54"E 51°34'23"N, 17°47'14"E		1	11	43				4	23	54
3	Golczewo	EE	53°49'16"N, 14°58'10"E					1					
4	Horni Budlovice	LE	49°44'54"N, 18°26'30"E			2	1						
5	Mewia Rewa	LE	54°38'54"N, 18°27'57"E	1	8		1						
6	Piła	LE	53°01'30"N, 17°16'20"E	3	13								
7	Poznań	LE	52°03'40"N, 16°13'24"E	5									
8	Pruszowice	LE	51°11'01"N, 17°08'11"E			4	5						
9	Raków	LE	51°10'21"N, 17°16'36"E	12	17	8	5					3	2
10	Urwitałt	LE	53°48'19"N, 21°38'38"E	7	12								
11	Wysoka	EE	53°48'56"N, 14°50'53"E 53°48'53"N, 14°51'56"E				1	2	8	8	6		
			Females/Males	**28**	**51**	**25**	**56**	**3**	**8**	**9**	**10**	**26**	**56**
			Total	**79**		**81**		**11**		**19**		**82**	

Table 2. Number, sex, taxonomic status and genotype of juvenile progeny of artificial crosses.

	Number of individuals											
	lessonae		*esculentus*								*ridibundus*	
	LL		RL		RRL		RLL		RRLL		RR	
	F	M	F	M	F	M	F	M	F	M	F	M
	7	8	32	13	14	1	1	2	4	1	44	8
Total	**15**		**45**		**15**		**3**		**5**		**52**	

the Czech Republic, Poodří Protected Landscape Area, c.j. 0926/PO/2008/AOPK, permit S/0673/PO/2008/AOPK, and Polish Ministry of Environment Protection and Forestry for performing studies on protected species OP 4072/218 /96, OP 4072/218/98/4501, OP 4201/144/99, and II Local Commission for Ethics in Experiments on Animals 13/02.

Chromosome preparation

Twenty-four hours before dissection, adults were injected intraperitoneally with 1 ml, and juveniles with 0.5 ml of 0.3% colchicine (Sigma- Aldrich, St. Louis, Mo.,

USA). Shortly before tissue preparation, the frogs were anesthetized with 0.25% water solution of 3-aminobenzoic acid ethyl ester (MS 222, Sigma-Aldrich). The intestine was dissected, hypotonized in distilled water (20 min for adults and 10 min for juveniles), and fixed in fresh-made fixative ethanol:glacial acetic acid (3:1) according to Heppich et al. (1982). Samples were stored in the fixative at –20°C until use. To obtain chromosome preparations, small pieces of the intestine epithelium were gently pressed against a slide to make tissue 'prints' that were immediately squashed under a coverslip in a drop of 70% acetic acid. The slides were placed on dry ice until frozen and then the coverslips were mechanically removed. The squashes were air-dried overnight and stored at –20°C. A minimum of 10 metaphases were analyzed for each individual.

Chromosome preparations of three *P. esculentus* individuals were made in a different way. The animals were injected intraperitoneally with 0.1% colchicine (10µl/g body weight) 2.5 h before dissection, femur bones were removed from euthanized frogs, epiphyses were clipped off, and bone marrow cavities were immediately flushed with 0.075 M KCl solution applied with a syringe at 37°C. Bone marrow tissue was pressed through a small-mesh gauze, flushed out with 0.075 M KCl solution, then placed in a centrifuge tube filled up to 7 ml with 0.075 M KCl solution, and incubated at 37°C for 20 min. Hypotonic treatment was stopped by fixation in absolute methanol:glacial acetic acid (3:1), and cell suspension was centrifuged at 1,500 rpm for 10 min. The supernatant containing fat droplets was discarded with a Pasteur pipette and fresh fixative was added up to 5 ml. The pellet was re-suspended by agitation and kept in a freezer for 20 min, then centrifuged again at 1,500 rpm for 10 min. The procedure (centrifugation, fixative exchange, and cooling) was repeated 3 times. The suspension was then transferred to a 1-ml syringe, dropped onto slides (previously cleaned in HCl:ethanol, 3:1), and finally air-dried. Chromosome preparations obtained with either protocol were suitable for all staining methods used in this study. However, chromosomes prepared from bone marrow cells were more uniformly condensed and thus more suitable for FISH than the chromosomes obtained from gut epithelium prints that varied in the degree of condensation (Zaleśna et al. 2011).

Chromosome banding

The nucleolus organizer regions (NORs) were stained in all individuals by the silver nitrate technique (Ag-NOR). Chromosome slides of 15 randomly selected individuals were sequentially stained with chromomycin A_3 (CMA_3) and $AgNO_3$. All individuals that displayed another number of NORs than one per a haploid set after Ag-NOR staining were examined by PI or CMA_3. For 22 individual (9 RR, 3 LL, 5RL, 1 RRL, 3 LLR, 1 RRLL) we applied the FISH method with 28S rDNA as a probe.

Silver staining. We followed the protocol of Howell and Black (1980). A few drops of freshly prepared silver nitrate buffer (0.5g $AgNO_3$/1ml H_2O/0.5ml gelatin

solution, *i.e.*1g gelatin/0.5ml formic acid/50ml bi-distilled water) were applied to each preparation. Slides were covered with a nylon mesh and incubated in a humid chamber at 60°C for 1min, washed in distilled water and air-dried. If the chromosomes were poorly visible, they were counterstained with DAPI. The number of active rDNA loci was documented by simultaneous use of fluorescence and incandescent light.

Chromomycin A_3 (CMA_3). The method was used according to Schweitzer (1976). Slides were incubated in McIlvain buffer pH 7.0 with 2.5mM $MgCl_2$ for 10 min and stained with CMA_3 solution (0.5 mg/mL buffer and 2.5mM $MgCl_2$) for 15 min in the dark, briefly rinsed in buffer and counterstained with methyl green for 15 min (0.175g methyl green/50ml buffer). After washing in buffer, slides were stained with DAPI solution (0.5µg/ml) for 10 min in the dark and briefly rinsed with buffer. Tissue prints were mounted in 50% glycerol and analyzed using fluorescence illumination. After CMA_3 staining, preparations were faded and cleaned in xylene and benzene, each bath for 2 min, and then stained with $AgN0_3$.

Propidium Iodode staining after denaturation of chromosomes (PI). Tissue prints were dehydrated in 70%, 85% and 95% ethanol (30 sec each wash), air-dried at room temperature and denatured in 70% deionized formamide in 2xSSC at 70°C for 3.5 min. Slides were immediately dehydrated in chilled 70% ethanol from a freezer for 2 min, then in 85% and 95% at room temperature for 30 sec. Chromosomes were stained with PI (200ng/ml).

Extraction and labeling of 28S rDNA

We applied the FISH method with 28S rDNA as a probe, according to the protocol of Traut et al. (1999). Isolation of 28S rDNA was carried out with use of commercial GeneMATRIX Bio-Trace Purification Kit (Eurix). DNA amplification was performed using PCR reaction with suitable mixture: 2.5µl PCR buffer in 15 mM MgCl2 (Eurix), 1µl 5mM dNTP (Eurix), 1µl 10mM primer A (5'-TCC GTG TTT CAA GAC GGG - 3') and 1 µl 10mM primer B (3'-ACC CGC TGA ATT TAA GCA T -5'), 1µl 1U/µl Polymerase (OptiTaq-Eurix), 1µl matrix DNA and 17.5µl water. PCR conditions included initial denaturation in 94°C for 5 min, followed by 35 cycles: 30 s denaturation (94°C), 30 s annealing (58°C), 1 min elongation (72°C), and 5 min of final elongation (72°C). The 28S rDNA probe was labelled with tetramethyl-rhodamine-6-dUTP (Roche, 11093088910) using a Nick Translation Mix according to the manufacturer's protocol (Roche, 10976776001). Then, DNA was precipitated by ethanol for purification and concentrated by adding one-tenth volume of 3M sodium acetate and 2.5 volume of chilled 96% ethanol from a freezer. The mixture was incubated for at least 15 min at –80°C. The precipitated DNA was spun at 15,000 rpm for 15 min at 4°C and the supernatant was discarded. The pellet was washed with 50–100 ml of chilled 70% ethanol and spun at 15,000 rpm for 10 min at 4°C. DNA was dried at 37°C for 15–20 min.

Fluorescent in situ hybridization (FISH)

Slides were incubated in 100 μg/ml DNase-free RNase A in 2x SSC for 1 h at 37° C in a humid chamber and then washed twice in 2xSSC at room temperature for 5 min, dehydrated in ethanol series (70%, 85%, and 95%, 30 s each) and air-dried. Chromosome preparations were denatured in 70% deionized formamide, 2x SSC at 70° C for 3.5 min, dehydrated immediately for 2 min in ice-cold 70% ethanol, then in 85% and 95% ethanol for 30s at room temperature, and finally air-dried. The 28S rDNA probe was dissolved in hybridization mixture consisted of 100% deionized formamide and 20% dextran sulfate in proportion 1:1. The mixture was denatured at 90° C for 5 min and then immediately placed on ice for 3 min. 20 μl of the probe was applied to each slide and covered with a coverslip. Hybridization lasted overnight in a dark humid chamber at 37° C. After hybridization, the coverslip was removed by rinsing with 50% formamide in 2xSSC at 42°C twice for 7 min and the slide was washed 3 times (7 min each) in 1xSSC at 42°C and then in 2xSSC at room temperature for 30 s. Chromosomes were counterstained with DAPI in Vectashield antifade buffer (Cambio, Cambridge, UK).

Image processing

Chromosomes were analyzed in Olympus Provis AX 70 or Carl Zeiss Axioskop 20 microscopes equipped with fluorescence lamp HBO50 and appropriate filters. Images were recorded with Olympus DP30BW CCD and cooled Carl Zeiss AxioCam HRc CCD cameras and processed using AxioVision and Lucia ver. 2.0 (Laboratory Imaging) softwares.

Results

The number of NORs was visualized for all (407) studied individuals (see Tables 1 and 2), *i.e.* 354 diploids (242 adults and 112 juveniles RR, LL, RL), 48 triploids (30 adults and 18 juveniles RRL and LLR), and 5 tetraploids RRLL. The results are summarized in Table 3. After $AgNO_3$ staining, one NOR per a haploid set was identified in secondary constrictions on long arms of chromosomes 10 in 341 individuals (83.8%), and in these individuals the number of NORs reflected the ploidy level. Two NORs were detected in 302 (85.3%) diploid individuals belonging to all studied taxa (232, *i.e.* 96% adults and 70, *i.e.* 62.5% juveniles), regardless of their capture site and taxonomic status. In all analyzed metaphases of the species (*P. lessonae* and *P. ridibundus*) and hybrids (*P. esculentus* 2n and 3n) , NORs exhibited variations in size after $AgNO_3$ and CMA_3 stainings, bands at one of the homologs were slightly thicker than at the other one (Fig. 1A). The number of Ag-NORs visible on metaphase plates was the same as the number of $AgNO_3$ stained nucleoli in interphase nuclei of the same individual (Fig. 1B–F). Three NORs were detected in 34 (70.8%) triploid hybrids, both RRL and RLL (26,

Table 3. Number of NORs in individuals of various ploidy level. The number of NORs that is in agreement with ploidy level is in bold.

Ploidy and genome composition	Number of individuals (adults and juveniles)				
		Number of NORs			
		1	**2**	**3**	**4**
2n (LL, RR, RL)	354	51	**302**	1	
3n (RRL, LLR)	48	2	3	**34**	9
4n (RRLL)	5				**5**
Total	407				

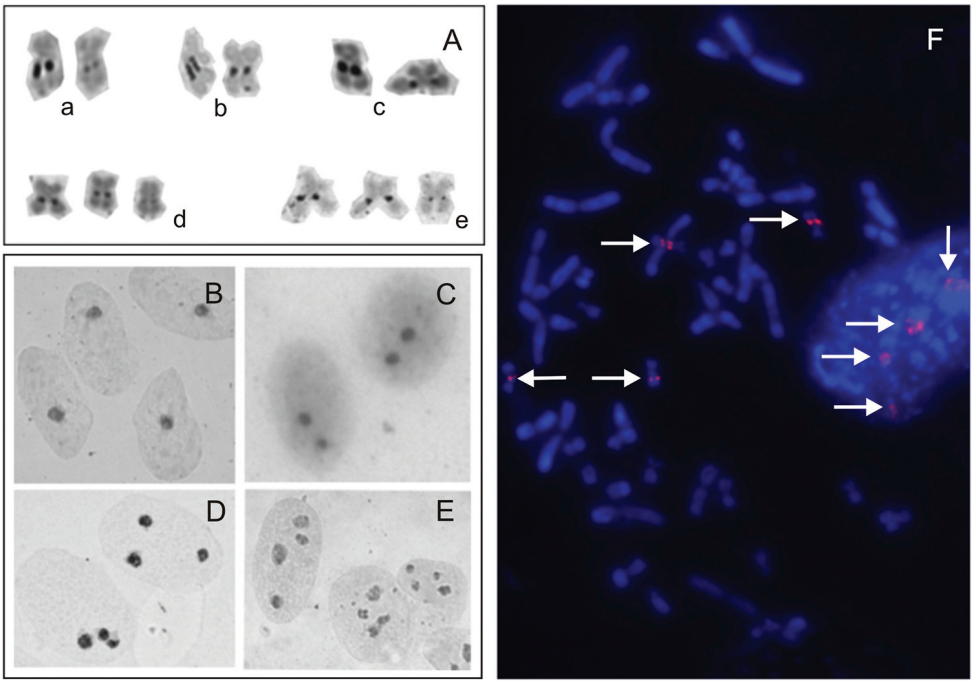


Figure 1. A The variability of size of AgNORs (black bands) on chromosomes 10 **a** *Pelophylax ridibundus* **b** *P. lessonae* **c** *P. esculentus* RL **d** *P. esculentus* RRL **e** *P. esculentus* LLR **B–E** Interphase nuclei of *P. esculentus* with Ag-nucleoli visualized as black dots by $AgNO_3$ **B** diploid RL with 1 AgNOR **C** diploid RL with 2 AgNORs **D** triploid RRL with 3 AgNORs **E** triploid RRL with 4 AgNORs **F** metaphase chromosomes and interphase nuclei of the same tetraploid P. esculentus RRLL with 4 sites of hybridization with 28S rDNA probe (FISH) (arrows).

i.e. 86.7% adults and 8, *i.e.* 44.4% juveniles), and four Ag-NORs were recorded in all metaphase plates of five tetraploids (RRLL).

Results obtained by silver staining corresponded well with those stained with CMA_3, PI or FISH (Fig. 2A–G). Only one individual *P. ridibundus* displayed one active NOR after $AgNO_3$ although two GC-rich chromatin blocks located in both

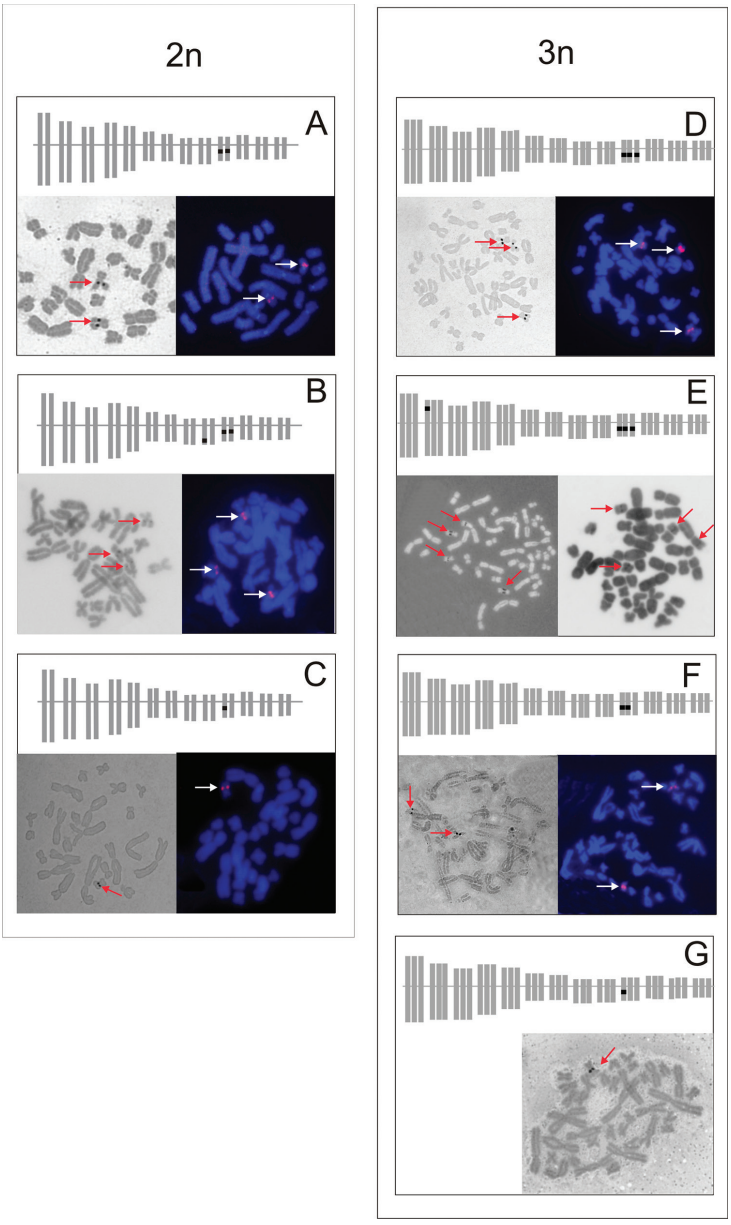


Figure 2. Localization and number of NORs (arrows) in diploid and triploid water frogs. Left column represent diploids (**A–C**) and right column represent triploids (**D–G**). Each picture is composed of a diagram of karyotype with black dots representing NORs and metaphase plates stained with silver (Ag-NOR), chromamycin A_3 (CMA_3), propidium iodide after denaturation (DPI) or after fluorescent *in situ* hybridization with 28S rDNA probe (FISH). **A** *P. lessonae* LL with 2 NORs, AgNORs (left) and FISH (right) **B** *P. esculentus* RL with 4 NORs on chromosome 9, CMA_3 (left) and FISH (right) **C** *P. esculentus* RL with 1 NOR, AgNORs (left) and FISH (right) **D** *P. esculentus* LLR with 3 NORs, AgNORs (left) and FISH (right) **E** *P. esculentus* RRL with 4 NORs, AgNOR (left) and DPI (right) **F** *P. esculentus* RRL with 2 NORs, AgNORs (left) and FISH (right) **G** *P. esculentus* RRL with 1 NOR, CMA_3.

chromosomes 10 were visulized after PI and CMA_3. All other individuals displayed the same number of PI and CMA_3 positive sites or 28S rDNA hybridizing signals as visualized by silver staining.

NOR polymorphism

In 66 individuals (16.2%, 13 adult and 53 juveniles), the number of NORs differed from the expected values. Lack of NORs was relatively more frequent (13.8%, 56 out of 407 individuals) than extra NORs (2.46%, 10 out of 407 individuals), regardless of genomic compositions (RR, LL, RRL and LLR) and origin of individuals. Eight diploid adult individuals (3 diploid *P. esculentus*, 3 *P. lessonae* and 2 *P. ridibundus*) and 43 diploid juveniles (11 *P. esculentus*, 6 *P. lessonae*, and 26 *P. ridibundus*) had NORs only on one chromosome of pair 10 (Fig. 2C). Two triploid adults (one LLR and one RRL) and one juvenile displayed two, instead of three NORs (Fig. 2F). Two other triploid RRL individuals (1 adult and 1 juvenile) displayed one, instead of expected three NORs in chromosomes 10 (Fig. 2G).

Additional NORs were detected in ten individuals. The extra NORs were located within additional secondary constrictions on one of the homologs of chromosomes other than 10. Extra NORs were found in one adult *P. ridibundus* in the distal position of the long arm of chromosome 9 (Fig. 2 B). In interphase nuclei of this individual we found the corresponding number of three Ag-nucleoli (Fig. 1D). Extra NORs were also observed on short arms of chromosomes 2 in nine triploid *P. esculentus* RRL: one adult and eight juveniles (Fig. 1E).

Inheritance of NOR patterns

The lack of one NOR was also detected in diploid progeny of two triploid RRL females and one LLR female. After AMD/DAPI staining we discovered that in the case of the RRL females the lack of NORs was inherited together with the haploid set of the *ridibundus* chromosomes, whereas in the case of the LLR female it was inherited with the haploid set of the *lessonae* chromosomes. Among diploid progeny of these females (altogether 37 juveniles) we observed 16 individuals with two NORs and 21 individuals with only one NOR. Each individual was stained with AgNOR and seven of them were confirmed by FISH. Thus, despite that the genomes of ova were the same (R in the case of RRL and L in the case of LLR females), the females produced in fact two types of gametes – with and without NORs (Fig. 3).

Discussion

The majority of individuals displayed one NOR per a haploid set of chromosomes (n = 13) located within the secondary constriction on the long arm of chromosome

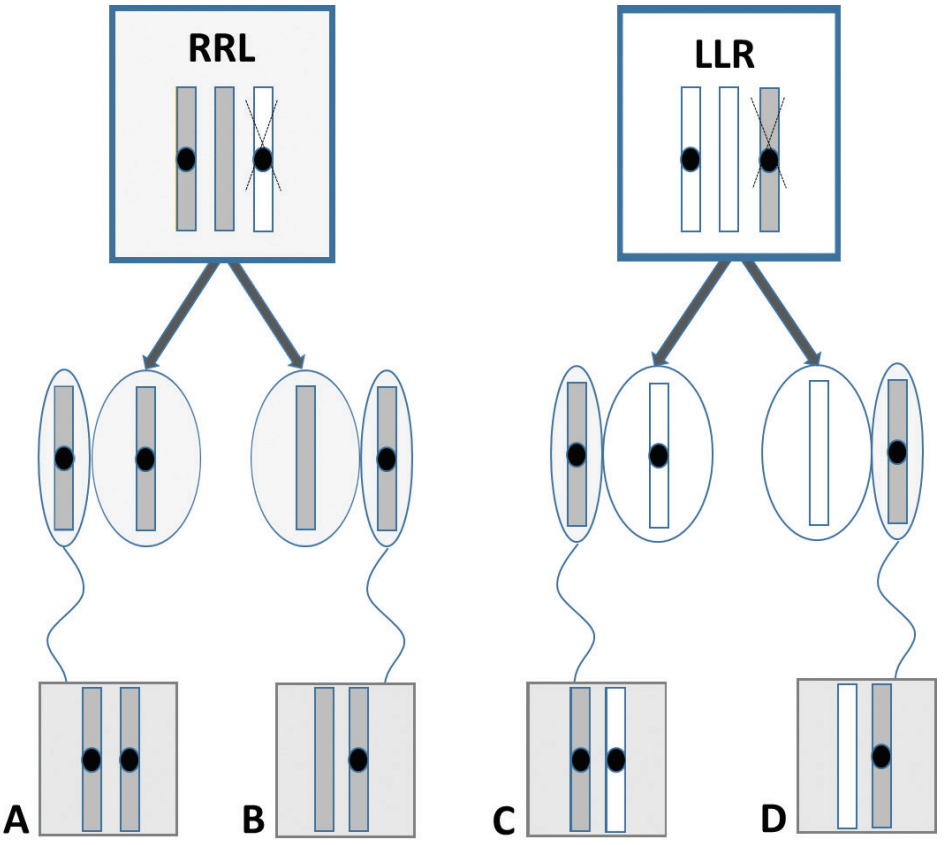


Figure 3. Inheritance of NORs by progeny of triploid females RRL (right) and RLL (left). The chromosome set represented by one copy is eliminated before oogenesis (marked by X) and the double one is segregated into eggs (represented by ovals). After fertilization (in this case by *ridibundus* sperm), two types of progeny arises: with two (**A** and **C**) and with one (**B** and **D**) NOR. The lack of NORs are transmitted either by *lessonae* (white) or by *ridibundus* (dark grey) chromosome sets. NORs are represented by black dots.

10 (named 9 in Biriuk et al. 2015). This result is in accordance with other reports on water frogs (Koref-Santibanez and Günther 1980, Schmid 1982, Heppich et al. 1982, Vitelli et al. 1982, Spasič-Boškovič et al. 1999, Ogielska et al 2004, Martirosyan and Stepanyan 2009, Zaleśna et al. 2011). Localization of NORs on chromosomes 10 is a conservative trait in the ranid frogs (Birstein 1984).

Ag-NORs observed in metaphase chromosomes in water frogs were also recognized in interphase nuclei, as was reported by Schmid (1982) and Biriuk et al (2015). Similar results were also observed in the lungless salamander *Onychodaxtylus fischeri* (Iizuka et al 2013), caecilians *Ichthyophis*, *Uraeotyphlus* and *Gegeneophis* (Venu 2014), and the frog *Physalaemus petersi* (Lourenço et al. 1998).

The number of visualized NORs corresponded well with the ploidy level, as expected for individuals with only one pair of NOR-bearing chromosomes. Thus, diploids had

two NORs, triploids - three NORs, and tetraploid - four NORs. However, number of NORs in interphase nuclei may be misleading in specific cases. As we demonstrated in this study, interphase nuclei in a diploid individual with an extra NOR on chromosome 9 displayed the same pattern as in a triploid individual with three NORs on the homologous chromosomes 10.

Polymorphism of NORs

The variability of AgNOR sizes in individuals from the same populations may reflect different amount of rDNA (Miller and Brown 1969 for *Bufo marinus* and Macgreor et al. 1977 for *Plethodon cinereus*). Various sizes of AgNORs in frogs were described by Schempp and Schmid (1981). Schmid (1982) found a correlation between sizes of bands in secondary constrictions of $AgNO_3$ stained chromosomes and the intensity of CMA_3 signals which were proportional to the amount of rDNA in NORs. We also found variability of NOR sizes in chromosomes 10, however Biriuk et al. (2015) reported that the differences measured as a relative length were not significant in 65 investigated individuals of diploid and triploid green frogs. The polymorphism of NORs can also reflect geographical karyotypic variations (Amaro et al. 2012, Silva et al. 2000, Quinderé et al. 2009, Bi et al. 2009). However, we found no variations of NOR number and size in water frogs deriving from different populations.

Extra NORs were also reported in *Rana catesbeiana* (*Lithobates catesbeianus*) that displayed from two to seven Ag-positive small NORs per haploid karyotype, apart from one "standard" NOR on the chromosome 10 (Schmid 1978a,b). In the same species, Vitelli et al. (1982) revealed the standard NORs on the chromosomes 10 by FISH with 18S+28S rDNA probe, whereas 5S rDNA sequences were clustered near the centromere on the short arm of chromosome 12 and corresponded to small Ag-positive bands. Other small NORs were not confirmed neither by FISH with the 18S + 28S rDNA nor by 5S rDNA probes. FISH with 18S+28S rDNA probe in *Pelophylax esculentus* (Vitelli et al. 1982) displayed signals in chromosome 10, and with 5S rDNA probe at telomere of short arm of chromosome 5. Moreover, FISH with 28S rDNA probe, as well as CMA_3 and PI stainings revealed that extra NORs corresponded with the extra Ag-positive signals on the chromosomes 9. Neither of these locations corresponded to extra NORs that were observed in our study.

More common was the lack of NORs (13.8% of all animal studied herein). We observed a lack of one NOR (2n and 3n) or two NORs (only in 3n), both in metaphase plates and interphase nuclei. Lack of NORs in amphibians was also reported by Schimd (1982) in *Bombina variegata*, *Xenopus laevis,* and *Bufo fowleri*. According to Schmid (1982) and Motovali-Bashi et al. (2004), the causes of deletion or amplification of NORs were usually unequal meiotic divisions, sister chromatid exchanges or disruption of DNA replication.

Intraspecific polymorphism of the location of extra NORs was also reported in *Hyla chrysoscelis* and *H. versicolor* (Wiley et al. 1989, Wiley and Little 2000), *H. nana*

(Medeiros et al. 2003), *Bufo terrestris* (Foote et al. 1991), *Agalychnis callidryas* (Schmid et al. 1995), *Physalaemus petersi* (Lourenço et al. 1998), *P. cuvieri* (Quinderé et al. 2009), *Phyllomedus arhodei* and *P. nordestina* (Barth et al. 2013). Possible mechanisms of the dispersion of NOR sites were translocations involving chromosomal segments containing NORs, however such chromosome rearrangements were connected with change in morphology of the NOR chromosomes (Lourenço et al. 1998). Other possible mechanisms involved in dispersion of NORs in anuran genomes could be inversions, transpositions by mobile genetic elements containing NORs (Wiley et al. 1989, Foote et al. 1991, Lourenço et al. 1998, Schmid et al. 1995, King et al. 1990, Bruschi et al. 2012, Carvalho et al. 2014) or rDNA amplification (Barth et al. 2013). However, in the case of water frogs presented here, translocations involving chromosomal segments containing NORs on chromosomes 2 and 9 seems unlikely because chromosomes with extra NORs did not vary in length and shape from relative chromosomes in other individuals.

Inheritance of NOR patterns

The lack of one NOR was detected in both RRL and LLR triploid females and was associated with the *ridibundus* and *lessonae* chromosome sets, respectively. Triploid water frogs produce haploid gametes and transmit the chromosome set that is represented by two copies (R in RRL and L in LLR) (Christiansen 2009). Because one of the doubled sets lacked NOR, we were able to trace this character and we found it in about a half of progeny whereas the other half inherited NORs (Fig. 3). This intriguing result deserves more attention and further studies because it may serve as an indicator of clonal versus recombined inheritance of the doubled chromosome sets, as was possible in triploid green toads *Bufo baturae* (Stöck et al. 2002, 2012). This all-triploid population is represented by males and females that carry NORs in two chromosome sets whereas the third one lacks NORs. This character, known also from other amphibian species (Schmid 1982, Motovali-Bashii et al. 2004, this study), was probably conserved in an isolated population in the Karakoram Mountains in Pakistan. Triploid *Bufo baturae* females produce diploid ova containing one (recombined) set with NORs whereas the set without NOR is transmitted without recombination (clonally).

Finally, we conclude that polymorphism concerning the number and localization of NORs in water frogs was characteristic of both *lessonae* and *ridibundus* genomes, and – as expected – was observed in individuals regardless of their taxonomic position (*P. lessonae*, *P. ridibundus* and *P. esculentus*), ploidy level (2n, 3n, 4n), genomic constitution (RR, LL, RRL, RLL and LLRR), and collection site. The number of active silver-stained NORs reflected ploidy levels: two in diploids, three in triploids, and four in tetraploids, and therefore we believe that there is no diploidization of polyploids in water frogs, as has been observed in some of polyploid amphibians (for review see Schmid et al. 2015). The variability of NORs may be hereditary, what is best represented by inheritance of lack of NORs by the progeny of triploid females.

Acknowledgements

The study was financially supported by grants 3490/B/P01/2007/33 and 3946/B/P01/2009/36 provided by the Polish Ministry of Science and Higher Education. We are grateful to Katarzyna Lipiec-Sidor for technical help with figures arrangement and setup.

References

Amaro RC, Rodrigues MT, Yonenaga-Yassuda Y, Carnaval AC (2012) Demographic processes in the montane Atlantic rainforest: Molecular and cytogenetic evidence from the endemic frog *Proceratophrys boiei*. Molecular Phylogenetics and Evolution 62: 880–888. https://doi.org/10.1016/j.ympev.2011.11.004

Barth A, Souza VA, Solé M and Costa MA (2013) Molecular cytogenetics of nucleolar organizer regions in *Phyllomedsua* and *Phasmahyla* species (Hylidae. Phyllomedusinae): a cytotaxonomic contribution. Genetics and Molecular Research 12(3): 2400–2408. https://doi.org/10.4238/2013.July.15.3

Berger L (1983) Western Palearctic water frogs (Amphibia, Ranidae), Systematics, genetics and population compositions. Experiential 39: 127–130. https://doi.org/10.1007/BF01958859

Berger L (1988) On the origin of genetic systems in European water frog hybrids. Zoologica Poloniae 35: 5–32.

Berger L, Rybacki M, Hotz H (1994) Artificial fertilization of water frogs. Amphibia-Reptilia 15: 408–413. https://doi.org/10.1163/156853894X00452

Bi K, Bogart JP, Fu J (2009) A populational survey of 45S rDNA polymorphism in the Jefferson Salamander using FISH. Current Zoology 55: 145–149.

Biriuk OV, Rosanov JM, Litvinchuk SN, Pasynkova RA (2015) Variability of nucleolus organizer region sizes in green frogs karyotypes. The Journal of V.N. Karazin Kharkiv National University Series Biology 25: 145–155.

Birstein VJ (1984) Localization of NORs in karyotypes of four *Rana* species. Genetica 64: 149–154. https://doi.org/10.1007/BF00115338

Boroń A (1999) Banded karyotype of spined loach *Cobitis taenia* and triploid Cobitis from Poland. Genetica 3: 293–300. https://doi.org/10.1023/A:1003939813878

Bruschi DP, Busin CS, Siqueira S, Recco-Pimentel SM (2012) Cytogenetic analysis of two species in the *Phyllomedusa hypochondrialis* group (Anura, Hylidae). Hereditas 149: 34–40. https://doi.org/10.1111/j.1601-5223.2010.02236.x

Carvalho MA, Rodrigues MT, Siqueira S, Garcia C (2014) Dynamics of chromosomal evolution in the genus *Hypsiboas* (Anura: Hylidae). Genetics and Molecular Research 13(3): 7826–7838. https://doi.org/10.4238/2014.September.26.21

Christiansen DG (2009) Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). BMC Evolutionary Biology 9: 1–16. https://doi.org/10.1186/1471-2148-9-135

Foote DL, Wiley JE, Little ML, Meyne (1991) Ribosomal RNA gene site polymorphism in *Bufo terrestris*. Cytogenet Cell Genet 57(4): 196–9. https://doi.org/10.1159/000133145

Graf JD,Polls-Pelaz M (1989) Evolutionary genetics of the *Rana esculenta* complex. In: Dawley RM, Bogart JP (Eds) Evolution and Ecology of Unisexual Vertebrates The New York State Museum, Albany, 289–302.

Hauswaldt SJ, Höer M, Ogielska M, Christiansen DG, Dziewulska-Szwajkowska D, Czernicka E, Vences M (2012) A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (*Pelophylax*). Molecular Ecology Resources 12(5): 797–805. https://doi.org/10.1111/j.1755-0998.2012.03160.x

Heppich S, Tunner HG, Greilhuber J (1982) Premeiotic chromosome doubling after genome elimination during spermatogenesis of the species hybrid *Rana esculenta*. Theoretical and Applied Genetics 61: 101–104. https://doi.org/10.1007/BF00273874

Hermaniuk A, Pruvost NBM, Kierzkowski P, Ogielska M (2013) Genetic and cytogenetic characteristics of pentaploidy in water frogs. Herpetologica 69: 36–45. https://doi.org/10.1655/HERPETOLOGICA-D-12-00037

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. https://doi.org/10.1007/BF01953855

Iizuka K, Matsuda Y, Yamada T, Nakazato T, Sessions SK (2013) Chromosomal localization of the 18S and 28S ribosomal RNA genes using FISH and $AgNO_3$ banding in *Hynobius quelpaertensis*, *H. tsuensis* and *Onychodactylus koreanus* (Urodela: Hynobiidae). Current Herpetology 32(2): 89–101. https://doi.org/10.5358/hsj.32.89

Kierzkowski P, Paśko Ł, Rybacki M, Socha M, Ogielska M (2011) Genome dosage effect and hybrid morphology - the case of the hybridogenetic water frogs of the *Pelophylax esculentus* complex. Annales Zoologici Fennici 48: 56–66. https://doi.org/10.5735/086.048.0106

King M, Contreras N, Honeycutt RL (1990) Variation within and between nucleolar organizer regions in Australian hylid frogs (Anura) shown by 18S 1 28S *in situ* hybridization. Genetica 80: 17–29. https://doi.org/10.1007/BF00120116

Kirtiklis L, Porycka K, Boroń A, Coutanceau J-P, Dettai A (2010) Use of the Chromosomal Co-location of the Minor 5S and the Major 28S rDNA as a Cytogenetic Marker within the Genus *Leuciscus* (Pisces, Cyprinidae). Folia biologica (Kraków) 58: 245–249. https://doi.org/10.3409/fb58_3-4.245-249

Koref-Santibanez S, Günther R (1980) Karyological and serological studies in *Rana lessonae*, *R. ridibunda* and their hybrid "*R. esculenta*" (Amphibia, Anura). Genetica 52/53: 195–207. https://doi.org/10.1007/BF00121828

Lourenço LB, Recco-Pimentel SM, Cardoso AJ (1998) Polymorphism of the nucleolus organizer regions (NORs) in *Physalaemus petersi* (Amphibia, Anura, Leptodactylidae) detected by silver staining and fluorescence in situ hybridization. Chromosome Research 6: 621–628. https://doi.org/10.1023/A:1009253410553

Macgreor HC, Vlad M, Barnett L (1977) An investigation of some problems concerning nucleolus organizers in salamanders. Chromosoma 59(4): 283–299. https://doi.org/10.1007/BF00327970

Martirosyan A, Stepanyan I (2009) Features of the karyotypes of *Pelophylax ridibundus* Pallas, 1771 and *Rana macrocnemis* Boulenger, 1885 (Amphibia: Ranidae) from Armenia. Comparative Cytogenetics 3(1): 11–24. https://doi.org/10.3897/compcytogen.v3i1.4

Medeiros LR, Rossa-Feres DC, Recco-Pimentel SM (2003) Chromosomal differentiation of *Hyla nana* and *Hyla sanborni* (Anura, Hylidae) with a description of NOR polymorphism in *H. nana*. Journal of Heredity 94(2): 149–154. https://doi.org/10.1093/jhered/esg019

Miller L, Brown DD (1969) Variation in the activity of nucleolar organizers and their ribosomal gene content. Chromosoma 28: 430–444. https://doi.org/10.1007/BF00284259

Motovali-Bashii M, Hojatia Z, Walmsleyb RM (2004) Unequal sister chromatid exchange in the rDNA array of *Saccharomyces cerevisiae*. Mutation Research 564: 129–137. https://doi.org/10.1016/j.mrgentox.2004.08.004

Ogielska M, Kierzkowski P, Rybacki M (2004) DNA content and genome composition of diploid and triploid water frogs belonging to the *Rana esculenta* complex (Amphibia, Anura). Canadian Journal of Zoology 82: 1894–1901. https://doi.org/10.1139/z04-188

Plötner J (2005) Die westpaläarktischen Wasserfrösche – Von Märtyrern der Wissenschaft zur biologischen Sensation. Laurenti Verlag, Bielefeld, 160 pp.

Quinderé YRSD, Lourenço LB, Andrade GV, Tomatis C, Baldo D, Recco-Pimentel SM (2009) Polytypic and polymorphic cytogenetic variations in the widespread anuran *Physalaemus cuvieri* (Anura, Leiuperidae) with emphasis on nucleolar organizing regions. Biological Research 42: 79–92. https://doi.org/10.4067/s0716-97602009000100008

Ráb P, Reed KM, Ponce de Leon FA, Phillips RB (1996) A new method for detecting nucleolus organizer regions in fish chromosomes using denaturation and propidium iodide staining. Biotechnic and Histochemistry 71(3): 157–162. https://doi.org/10.3109/10520299609117153

Rábová M, Ráb P, Ozouf-Costaz C (2001) Extensive polymorphism and chromosomal characteristics of ribosomal DNA in a loach fish, *Cobitis vardarensis* (Ostariophysi, Cobitidae) detected by different banding techniques and fluorescence in situ hybridization (FISH). Genetica 111(1–3): 413–22. https://doi.org/10.1023/A:1013763903513

Rábová M, Ráb P, Ozouf-Costaz C, Ene C, Wanzeböck J (2003) Comparative cytogenetics and chromosomal characteristics of ribosomal DNA in the fish genus Vimba (Cyprinidae). Genetica 118(1): 83–91. https://doi.org/10.1023/A:1022965302614

Reeder RH (1990) rRNA synthesis in the nucleolus. Trends in Genetics 6: 390–395. https://doi.org/10.1016/0168-9525(90)90298-K

Rybacki M (1994) Diploid males of *Rana esculenta* from natural populations in Poland producing diploid spermatozoa. Zoologica Poloniae 39: 518–518.

Rybacki M, Berger L (2001) Types of water frog populations (*Rana esculenta* complex) in Poland. Mitteilungen aus dem Museum für Naturkunde in Berlin (Zoologische Reihe) 77: 51–57. https://doi.org/10.1002/mmnz.20010770109

Schempp W, Schmid M (1981) Chomosome banding in Amphibia. VI. BrdU replication patterns in Anura and demonstration of XX/XY chromosomes in *Rana esculenta*. Chromosoma 83: 697–710. https://doi.org/10.1007/BF00328528

Schmid M (1978a) Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. Chromosoma 66: 361–388. https://doi.org/10.1007/BF00328536

Schmid M (1978b) Chromosome banding in Amphibia. II. Constitutive heterochromatin and nucleolus organizer regions in Ranidae, Microhylidae and Rhacophoridae. Chromosoma 68: 131–148. https://doi.org/10.1007/BF00287145

Schmid M (1982) Chromosome Banding in Amphibia VII. Analysis of the Structure and Variability of NORs in Anura Chromosoma 87: 327–344.

Schmid M, Feichtinger W, Weimer R, Mais C, Bolaños F, Leon P (1995) Chromosome banding in Amphibia. XXI. Inversion polymorphism and nucleolus organizer regions in *Agalychnis callidryas* (Anura, Hylidae). Cytogenetetics and Cell Genetics 69: 8–26. https://doi.org/10.1159/000431388

Schmid M, Evans BJ, Bogart JP (2015) Polyplody in Amphibia. Cytogenetic and Genome Research 145: 315–330.

Schultz RJ (1969) Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. The American Naturalist 103: 605–619. https://doi.org/10.1086/282629

Schweitzer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI Chromosoma 58: 307–324.

Silva APZ, Junior FAB, Haddad CFB, Kasahara S (2000) Karyotypes and nucleolus organizer regions in four species of the genus *Physalaemus* (Anura, Leptodactylidae). Iheringia, Série Zoologia 88: 159–164.

Sola L, Rossi AR, Iaselli V, Rasch EM, Monaco PJ (1992) Cytogenetics of bisexual/unisexual species of *Poecilia*. II. Analysis of heterochromatin and nucleolar organizer regions in *Poecilia mexicana mexicana* by C-banding and DAPI, quinacrine, chromomycin A_3, and silver staining. Cytogenetics and Cell Genetics 60(3–4): 229–35. https://doi.org/10.1159/000133346

Spasič-Bošković O, Krizmanić I, Vujošević M (1999) Population composition and genetic variation of water frogs (Anura: Ranidae) from Yugoslavia. Caryologia 52: 9–20. https://doi.org/10.1080/00087114.1998.10589148

Stöck M, Lamatsch DK, Steinlein C, Epplen JT, Grosse WR, Hock R, Klapperstück T, Lampert KP, Scheer U, Schmid M, Schartl M (2002) A bisexually reproducing all-triploid vertebrate. Nature Genetics 30: 325–328. https://doi.org/10.1038/ng839

Stöck M, Ustinova J, Betto-Colliard C, Schartl M, Moritz C, Perrin N (2012) Simultaneous Mendelian and clonal genome transmission in a sexually reproducing, all-triploid vertebrate. Proceedings of the Royal Society B Biological Sciences 279: 1293–1299. https://doi.org/10.1098/rspb.2011.1738

Traut W, Sahara K, Otto TD, Mařec F (1999) Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. Chromosoma 108: 173–180. https://doi.org/10.1007/s004120050366

Uzzell T, Berger L, and Günther R (1975) Diploid and triploid progeny from a diploid female of *Rana esculenta* (Amphibia, Salientia). Proceedings of the Academy of Natural Sciences of Philadelphia 127: 81–91.

Venu G (2014) Studies on silver staining of chromosomes of caecilians (Amphibia:Gymnophiona) of Western Ghats of India. International Journal of Advanced Research 2(3): 63–72.

Vitelli L, Batistoni R, Andronico F, Nardi I, Barsacchi-Pilone G (1982) Chromosomal localization of 18S + 28S and 5S ribosomal RNA genes in evolutionarily diverse anuran amphibians. Chromosoma 84: 475–491. https://doi.org/10.1007/BF00292849

Wiley JE, Little ML (2000) Replication banding patterns of the diploid-tetraploid treefrogs *Hyla chrysoscelis* and *H. versicolor.* Cytogenetics and Cell Genetics 88: 11–14. https://doi.org/10.1159/000015475

Wiley JE, Little ML, Romano MA, Blount DA, Cline GR (1989) Polymorphism in the location of the 18S and 28S rRNA genes on the chromosomes of the diploid-tetraploid tree frogs *Hyla chrysoscelis* and *H. versicolor*. Chromosoma 97: 481–487. https://doi.org/10.1007/BF00295033

Zaleśna A, Choleva L, Ogielska M, Rábová M, Marec F, Ráb P (2011) Evidence for integirty of parental genomes in the diploid hybridogenetic water frog *Pelophylax esculentus* by genomic in situ hybridization. Cytogenetic and Genome Research 134(3): 206–212.

CompCytogen 11(2): 267–283 (2017)
doi: 10.3897/CompCytogen.v11i2.11524
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Comparative analysis based on replication banding reveals the mechanism responsible for the difference in the karyotype constitution of treefrogs *Ololygon* and *Scinax* (Arboranae, Hylidae, Scinaxinae)

Simone Lilian Gruber[1], Gabriela Isabela Gomes de Oliveira[1], Ana Paula Zampieri Silva[1], Hideki Narimatsu[1], Célio Fernando Baptista Haddad[2], Sanae Kasahara[1]

1 *UNESP, Universidade Estadual Paulista, Instituto de Biociências, Departamento de Biologia, Av. 24A, 1515, 13506-900, Rio Claro, SP, Brazil* **2** *UNESP, Universidade Estadual Paulista, Instituto de Biociências, Departamento de Zoologia, Av. 24A, 1515, 13506-900, Rio Claro, SP, Brazil*

Corresponding author: *Sanae Kasahara* (kasahara@rc.unesp.br)

Academic editor: *T. Capriglione* | Received 18 November 2016 | Accepted 12 March 2017 | Published 18 April 2017

http://zoobank.org/F89B2541-7485-41B8-B09A-6423B887A65F

Citation: Gruber SL, de Oliveira GIG, Silva APZ, Narimatsu H, Haddad CFB, Kasahara S (2017) Comparative analysis based on replication banding reveals the mechanism responsible for the difference in the karyotype constitution of treefrogs *Ololygon* and *Scinax* (Arboranae, Hylidae, Scinaxinae). Comparative Cytogenetics 11(2): 267–283. https://doi.org/10.3897/CompCytogen.11(2).11254

Abstract

According to the recent taxonomic and phylogenetic revision of the family Hylidae, species of the former *Scinax catharinae* (Boulenger, 1888) clade were included in the resurrected genus *Ololygon* Fitzinger, 1843, while species of the *Scinax ruber* (Laurenti, 1768) clade were mostly included in the genus *Scinax* Wagler, 1830, and two were allocated to the newly created genus *Julianus* Duellman et al., 2016. Although all the species of the former *Scinax* genus shared a diploid number of 2n = 24 and the same fundamental number of chromosome arms of FN = 48, two karyotypic constitutions were unequivocally recognized, related mainly to the distinct size and morphology of the first two chromosome pairs. Some possible mechanisms for these differences had been suggested, but without any experimental evidence. In this paper, a comparison was carried out based on replication chromosome banding, obtained after DNA incorporation of 5-bromodeoxiuridine in chromosomes of *Ololygon* and *Scinax*. The obtained results revealed that the loss of repetitive segments in chromosome pairs 1 and 2 was the mechanism responsible for karyotype difference. The distinct localization of the nucleolus organizer regions in the species of both genera also differentiates the two karyotypic constitutions.

Keywords
Anura, Hylidae, 5-bromodeoxiuridine, comparative cytogenetics, NOR, karyotype evolution

Introduction

The family Hylidae was recently revised by Duellman et al. (2016), who proposed a new phylogenetic tree based on 503 species and 16, 128 aligned sites of 19 genes. According to this phylogeny, the families Hylidae, Pelodryadidae, and Phyllomedusidae were placed in an unranked taxon named Arboranae, a new subfamily Scinaxcinae and a new genus *Julianus* Duellman, Marion, Hedges, 2016 were created, and the genus *Ololygon* Fitzinger, 1843 was resurrected, among other modifications. The hylids *Scinax* Wagler, 1830 and *Ololygon* are included in Scinaxinae and form a cluster along with *Julianus* and *Sphaenorhynchus* Tschudi, 1838. The support value for the cluster of the *Julianus*, *Ololygon*, and *Scinax* species was high (97%), but the placement of *Sphaenorhynchus* was low (49%), thus, according to Duellman et al. (2016), this relationship is still uncertain and needs to be reviewed.

Over the years, the hylid frogs of the former *Scinax* genus have been subjected to several taxonomic and phylogenetic reviews (Faivovich 2002, Faivovich et al. 2005, Wiens et al. 2010), with a large sample of specimens based mainly on molecular sequencing of mitochondrial and nuclear genes. In the classification proposed by Duellman et al. (2016), the newly constituted genus *Scinax* includes the species of the *Scinax ruber* (Laurenti, 1768) clade, with the exception of *S. uruguayus* (Schmidt, 1944) and *S. pinima* (Bokermann & Sazima, 1973) (from the former *S. uruguayus* group), both assigned to the genus *Julianus*, along with *Sphaenorhynchus*, whereas *Ololygon* comprises species of the former *Scinax catharinae* (Boulenger, 1888) clade.

The *Ololygon* and *Scinax* species are distributed across the Americas from Mexico to Argentina and Uruguay, and the islands of Tobago, Trinidad, and Saint Lucia (Duellman et al. 2016, Frost 2016). They are abundant in Brazil, with great diversity and endemism in the Atlantic Forest and Cerrado domains (Araújo-Vieira et al. 2016, Lourenço et al. 2016). They are considered complex groups due to great morphological similarity between individuals, which hinders proper species identification and determination of clear synapomorphies for taxonomic description (Pombal Jr. et al. 1995, Lourenço et al. 2016).

Early researches on the cytogenetics of the former *Scinax* species determined the chromosome number by observing mitotic or meiotic cells. All of the species exhibited a diploid number of 2n = 24 bi-armed chromosomes, corresponding to the fundamental number of FN = 48 chromosome arms (revisions in Catroli and Kasahara 2009 and in Peixoto et al. 2015). The work of Cardozo et al. (2011) stands out for its inclusion of chromosomal data obtained from a large sample of 28 representatives, and because the results were analyzed for the first time from an evolutionary point of view provided by available phylogenies. This work highlighted a karyotype differentiation

between the *S. catharinae* and the *S. ruber* clades, regarding the morphology and size of the first two chromosome pairs, the position of Ag-NORs, and the amount of C-banded heterochromatin. Additionally, the combined analysis with phylogeny allowed these authors to establish that the characteristic Ag-NORs marker in small pair 11, as exhibited by the species in the *S. ruber* clade, was considered a plesiomorphy, whereas the location in pair 6 in the species of the *S. catharinae* clade is a derivative condition. Only recently, a small number of species of *Scinax* (*lato sensu*) have had their karyotypic analyses extended through the use of more advanced cytogenetic techniques, such as investigation of the nature of repetitive regions using fluorochrome staining or fluorescent *in situ* hybridization (FISH) with microsatellite probes, and the location of ribosomal or telomeric sequences using the FISH technique (Oliveira 2011, Nogueira et al. 2015a, Nogueira et al. 2015b, Peixoto et al. 2015, Peixoto et al. 2016).

In the present work our efforts were the use of replication banding after 5-bromodeoxiuridine treatment, an useful approach to identify homeologous chromosomes (Kasahara et al. 2003, Silva et al. 2004, Gruber et al. 2007, Gazoni et al. 2012, Gruber et al. 2012, Gruber et al. 2013). This methodology is very effective for comparative analyses in fishes, amphibians, and reptiles due to the difficulty in producing classical Q, G, and R banding patterns in the chromosomes of the ectothermic vertebrates (Sumner 2003).

Replication banding was fundamental to provide evidence of the structural rearrangement responsible for the difference in the karyotypes between *Ololygon* and *Scinax* species, thereby contributing for clarify the phylogenetic relationships between these two genera.

Material and methods

Five species currently included in the genus *Ololygon*, *O. albicans* (Bokermann, 1967), *O. argyreornata* (Miranda-Ribeiro, 1926), *O. hiemalis* (Haddad & Pombal, 1987), *O. littoralis* (Pombal & Gordo, 1991), and *O. obtriangulata* (Lutz, 1973), and six species of genus *Scinax*, *S. caldarum* (Lutz, 1968), *S. crospedospilus* (Lutz, 1925), *S. eurydice* (Bokermann, 1968), *S. fuscovarius* (Lutz, 1925), *S. hayii* (Barbour, 1909), and *S. similis* (Cochran, 1952) were collected from Brazilian locations (Table 1) with the consent and approval of Instituto Chico Mendes de Conservação da Biodiversidade (38827-2). The classification adopted in the present work is based on the Amphibian Species of the World electronic database (Frost 2016).

The sample includes species whose chromosomes are described for the first time in this paper: *Scinax caldarum* and *S. crospedospilus*; specimens belonging to species already described in Cardozo et al. (2011) but collected in non studied populations: *Ololygon hiemalis*, *O. littoralis*, *O. obtriangulata*, *S. fuscovarius*, and *S. hayii*; additional banding data of specimens from the analyzed sample in Cardozo et al. (2011): *O. albicans*, *O. argyreornata*, *O.littoralis, S. eurydice*, and *S. similis*; and specimen CFBH 28730 of *S. fuscovarius*, the cytogenetic data of which were previously described in

Table 1. Analyzed species, voucher number (CFBH), sample size, sex, and collection locality.

Species	Voucher Number (CFBH)	Sample size	Sex	Collection locality
Ololygon albicans	10178[3]	1	Male	Petrópolis, RJ 22°30'17"S; 43°10'56"W
Ololygon argyreornata	17289[3], 17290[3]	2	Males	Ilha do Cardoso, SP 25°07'31"S; 47°58'06"W
Ololygon hiemalis	28591[2], 36231[2], 36233[2]	3	Males	Mogi Guaçu, SP 22°21'37"S; 57°07'07"W
Ololygon littoralis	40631[2]	1	Male	Bertioga, SP 23°56'27"S; 45°19'48"W
	22378[3]	1	Male	Ubatuba, SP 23°26'19"S; 45°05'25"W
Ololygon obtriangulata	CFBHT*20329[2]	1	Female	Biritiba Mirim, SP 23°34'17"S; 46°02'15"W
Scinax caldarum	22552[1]	1	Male	Poços de Caldas, MG 21°47'18"S; 46°33'45"W
Scinax crospedospilus	36201[1], 36202[1]	2	Males	Mogi das Cruzes, SP 23°31'29"S; 46°11'14"W
Scinax eurydice	16736[3]	1	Male	Serra do Japi, Jundiaí, SP 37°25'818"S; 122°05'36"W
Scinax fuscovarius	22415[2]	1	Female	Biritiba Mirim, SP 23°34'17"S; 46°02'15"W
	28730[4]	1	Male	Socorro, SP 22°35'29"S; 46°31'44"O
Scinax hayii	24216[2]	1	Male	Biritiba Mirim, SP 23°34'17"S; 46°02'15"W
	28588[2]	1	Male	Mogi das Cruzes, SP 23°31'29"S; 46°11'14"W
Scinax similis	5933[3], 5932[3]	2	Females	Três Marias, MG 18°12'43.3"S; 45°13'19"W

[1]-species with first karyotype description; [2]-specimens belonging to species already described in Cardozo et al. (2011) but collected in non studied populations; [3]-additional banding data of specimens from the analyzed sample in Cardozo et al. (2011); [4]-specimen reanalyzed from Kasahara et al. (2003) sample; * identification based on the COI sequencing from tissue sample.

Kasahara et al. (2003): the replication bands of this specimen were reanalyzed herein because its high resolution banding was more appropriate for comparative analysis.

The animals were identified by Dr. Célio F. B. Haddad and the vouchers were fixed in formalin (10%), preserved in 70% ethanol and deposited in the amphibian collection CFBH of the Departamento de Zoologia, Instituto de Biociências, UNESP, Rio Claro, SP, Brazil. Identification of the specimen CFBHT 20329 was confirmed by COI sequencing from muscle sample collected soon after the animal euthanasia and preserved in 70% ethanol. Direct cytological preparations were obtained from bone marrow, liver, and also the testes of male samples (Baldissera Jr et al. 1993). Spleen was eventually used depending on its size and also dissociated in hypotonic solution. For some animals, cytological preparations were made from the intestinal epithelium

(Schmid 1978) with modifications. For direct preparation, the animals received a previous injection of 0.1% colchicine during 2 to 6 hours to improve the quality and number of metaphases. Some specimens were submitted to *in vivo* treatment for 13 to 21 hours after injection of 5-bromodeoxiuridine (BrdU) plus 5-fluorodeoxyuridine (FudR) (10mg BrdU and 0.5mg FudR in 2mL 0.9% NaCl solution), in the proportion of 0.1mL/10g body weight (Silva et al. 2000). Briefly, the steps in this process are incorporation of BrdU during DNA replication, after *in vivo* injection or *in vitro* treatment during lymphocytes or fibroblast cultures, followed by differentiation of replication banding patterns with Hoechst 33258 staining, black light irradiation, and Giemsa staining (Dutrillaux and Couturier 1981, Sumner 2003).

Mitotic and meiotic preparations were analyzed after Giemsa staining. Mitotic chromosomes were also submitted to the techniques of silver impregnation (Ag-NOR) (Howell and Black 1980), C-banding (Sumner 1972), and fluorochrome staining with chromomycin A_3 (CMA_3) and 4',6-diamidino-2-phenylindole (DAPI) (Christian et al. 1998). The differentiation of replication bands after BrdU incorporation was achieved using Fluorochrome Plus Giemsa (FPG) staining as described by Dutrillaux and Couturier (1981) and modified by Kasahara et al. (1998). FISH was performed with an HM123 ribosomal probe (Meunier-Rotival et al. 1979, Pinkel et al. 1986), and a telomeric probe (DAKO Cytomation Denmark A/S), following manufacturer's instructions.

Chromosomes were analyzed under standard and UV light. The best mitotic and meiotic cells were photographed using an Olympus BX51 microscope and digital capture system DP71. Copies of the material were obtained digitally. Karyograms were assembled according to the morphology of the chromosomes in decreasing order of size (Table 2). The bi-armed chromosomes were classified as metacentric, submetacentric or subtelocentric (Green and Sessions 1991, 2007).

Results

All investigated specimens presented 24 bi-armed chromosomes. In *Ololygon* species, chromosome pairs 1 and 2 were submetacentric with a slight size difference between them, while in the species of *Scinax* genus chromosome pairs 1 and 2 were metacentric with great size difference. For illustration, the Giemsa-stained karyotypes of one of the species of *Ololygon* (*O. hiemalis*) from a population not studied before and of the two species of *Scinax* (*S. caldarum* and *S. crospedospilus*) karyotyped for the first time are presented in Figure 1. The karyotype of one of the specimens of *S. similis* with heteromorphic chromosome pairs 3 and 4 is also shown. The remaining species had the standard stained karyotypes published previously in Cardozo et al. (2011).

Replication banding patterns for the chromosomes of *O. hiemalis*, *S. crospedospilus*, *S. eurydice*, *S. fuscovarius*, and for two specimens of *S. similis* are presented in Figure 2. Comparison of the banded chromosomes of species of *Ololygon* and *Scinax* genera revealed close correspondence for the majority of their chromosome pairs, except for the chromosomes of pairs 1 and 2 (Figure 3a, b). In species of *Ololygon,* the short arms

Table 2. Relative length (RL), centromeric index (CI), and nomenclature for centromeric position (CP) on mitotic chromosomes of *Ololygon* and *Scinax*, according to Green and Sessions (1991, 2007).

Species		Chromosome pair											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
O. albicans	RL	12.54	12.10	10.52	10.37	9.22	8.93	8.93	6.20	6.05	5.48	4.76	4.90
	CI	0.349	0.300	0.314	0.222	0.235	0.250	0.250	0.455	0.382	0.500	0.500	0.500
	CP	**sm**	**sm**	**sm**	**st**	**sm**	**st**	**sm**	**m**	**m**	**m**	**m**	**m**
O. argyreornata	RL	11.65	10.67	11.59	10.45	9.53	8.87	8.17	7.35	6.37	6.31	4.84	4.19
	CI	0.363	0.340	0.274	0.348	0.311	0.265	0.250	0.429	0.500	0.500	0.444	0.429
	CP	**sm**	**sm**	**sm**	**sm**	**sm**	**sm**	**sm**	**m**	**m**	**m**	**m**	**m**
O. hiemalis	RL	13.33	11.16	10.23	9.92	8.99	9.61	8.84	6.20	6.20	5.89	4.96	4.65
	CI	0.300	0.333	0.294	0.290	0.286	0.290	0.310	0.500	0.400	0.500	0.500	0.500
	CP	**sm**	**sm**	**sm**	**sm**	**sm**	**sm**	**sm**	**m**	**m**	**m**	**m**	**m**
O. littoralis	RL	12.30	11.50	10.70	10.70	9.36	8.02	8.56	7.09	6.15	5.75	5.35	4.55
	CI	0.292	0.364	0.282	0.250	0.333	0.310	0.313	0.462	0.455	0.435	0.500	0.500
	CP	**sm**	**sm**	**sm**	**sm**	**sm**	**sm**	**sm**	**m**	**m**	**m**	**m**	**m**
O. obtriangulata	RL	11.71	11.31	10.50	9.96	9.96	9.42	7.81	6.73	6.19	5.92	5.25	5.25
	CI	0.349	0.333	0.300	0.211	0.263	0.257	0.267	0.500	0.417	0.455	0.500	0.474
	CP	**sm**	**sm**	**sm**	**st**	**sm**	**sm**	**sm**	**m**	**m**	**m**	**m**	**m**
S. caldarum	RL	13.87	12.14	10.40	9.25	9.25	8.67	8.09	7.80	6.07	5.20	4.62	4.62
	CI	05.00	0.455	0.444	0.250	0.375	0.333	0.429	0.467	0.400	0.500	0.500	0.375
	CP	**m**	**m**	**M**	**sm**	**sm**	**sm**	**m**	**m**	**m**	**m**	**m**	**m**
S. crospedospilus	RL	14.53	12.50	10.47	10.81	8.28	8.28	6.59	6.59	5.91	5.74	5.24	5.07
	CI	0.476	0.444	0.344	0.281	0.320	0.333	0.400	0.474	0.474	0.471	0.500	0.500
	CP	**m**	**m**	**sm**	**sm**	**sm**	**sm**	**m**	**m**	**m**	**m**	**m**	**m**
S. eurydice	RL	15.51	11.57	10.16	10.01	9.31	8.60	7.05	6.35	5.50	5.64	5.22	5.08
	CI	0.500	0.455	0.333	0.316	0.250	0.333	0.407	0.478	0.500	0.450	0.444	0.444
	CP	**m**	**m**	**sm**	**sm**	**sm**	**sm**	**m**	**m**	**m**	**m**	**m**	**m**
S. fuscovarius	RL	16.05	12.23	10.39	10.39	8.56	7.73	6.70	6.11	5.81	5.50	5.47	5.04
	CI	0.481	0.429	0.353	0.294	0.286	0.308	0.385	0.500	0.444	0.500	0.444	0.471
	CP	**m**	**m**	**sm**	**sm**	**sm**	**sm**	**sm**	**m**	**m**	**m**	**m**	**m**
S. hayii	RL	13.73	11.16	10.94	10.30	9.23	8.15	7.94	7.08	6.22	5.36	5.36	4.51
	CI	0.500	0.462	0.370	0.417	0.381	0.500	0.444	0.500	0.429	0.500	0.462	0.400
	CP	**m**	**m**	**sm**	**m**	**sm**	**m**	**m**	**m**	**m**	**m**	**m**	**m**
S. similis	RL	15,20	12.94	12.01	10.39	7.45	7.89	7.06	6.13	5.98	5.59	4.95	4.41
	CI	0.500	0.419	0.458	0.227	0.301	0.354	0.400	0.462	0.385	0.483	0.404	0.500
	CP	**m**	**m**	**M**	**st**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**	**m**

m = metacentric; sm = submetacentric; st = subtelocentric.

of chromosomes 1 and 2 are respectively smaller than the short arms of chromosomes 1 and 2 of *Scinax* species, whereas the long arms of both chromosomes have the same size and corresponding bands.

Nucleolus organizer regions were located on pair 6 for the *Ololygon* species and in pair 11 for the *Scinax* species. Figure 4 shows Ag-NOR, CMA_3 staining, and FISH us-

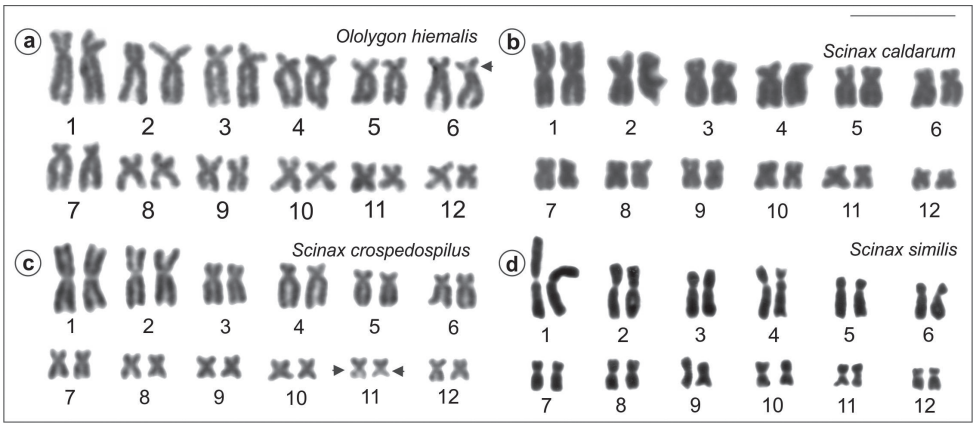


Figure 1. Giemsa stained karyotypes of *Ololygon* and *Scinax* species. **a** *O. hiemalis*, male **b** *S. caldarum*, male **c** *S. crospedospilus*, male **d** *S. similis*, female, with heteromorphic chromosomes pairs 3 and 4. Secondary constrictions (arrowhead) are visible on chromosome 6 in **a** and on chromosomes 11 in **c**. Bar = 10 μm.

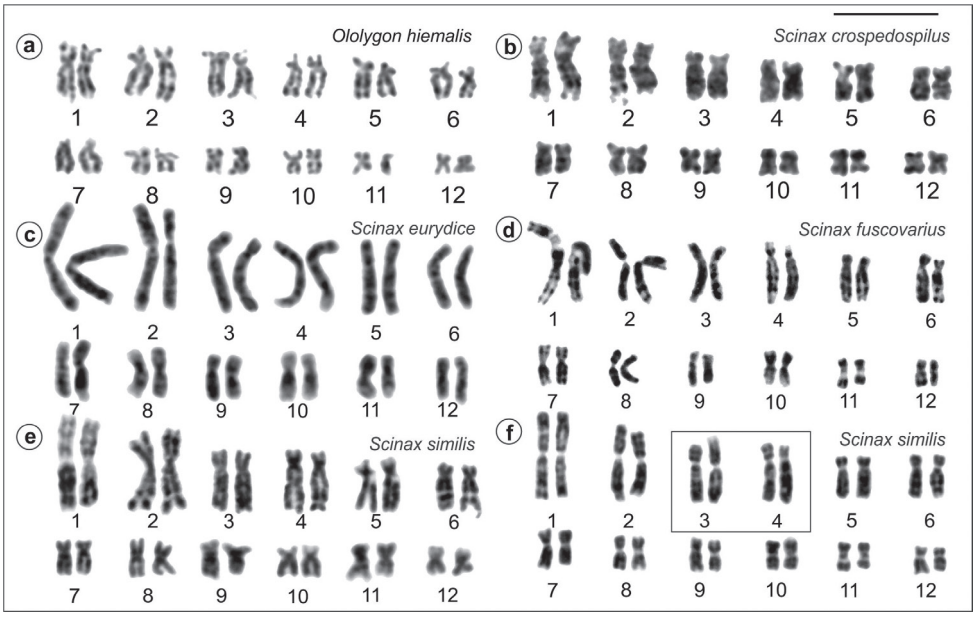


Figure 2. Replication banding in *Ololygon* and *Scinax* species. **a** *O. hiemalis* **b** *S. crospedospilus* **c** *S. eurydice* **d** *S. fuscovarius* **e–f** *S. similis*. Note heteromorphic chromosome pairs 3 and 4 in inset. Bar = 10 μm.

ing an rDNA probe in *O. obtriangulata* and *S. fuscovarius* CFBH 22415. In the CFBH 22415 specimen an additional Ag-NOR site was observed in one of the chromosomes 12, and this was confirmed after both CMA_3 fluorochrome staining and FISH labeling.

Chromosome preparations of *O. hiemalis*, *O. littoralis*, *O. obtriangulata*, *S. crospedospilus*, and *S. hayii* were submitted to hybridization with a telomeric probe and

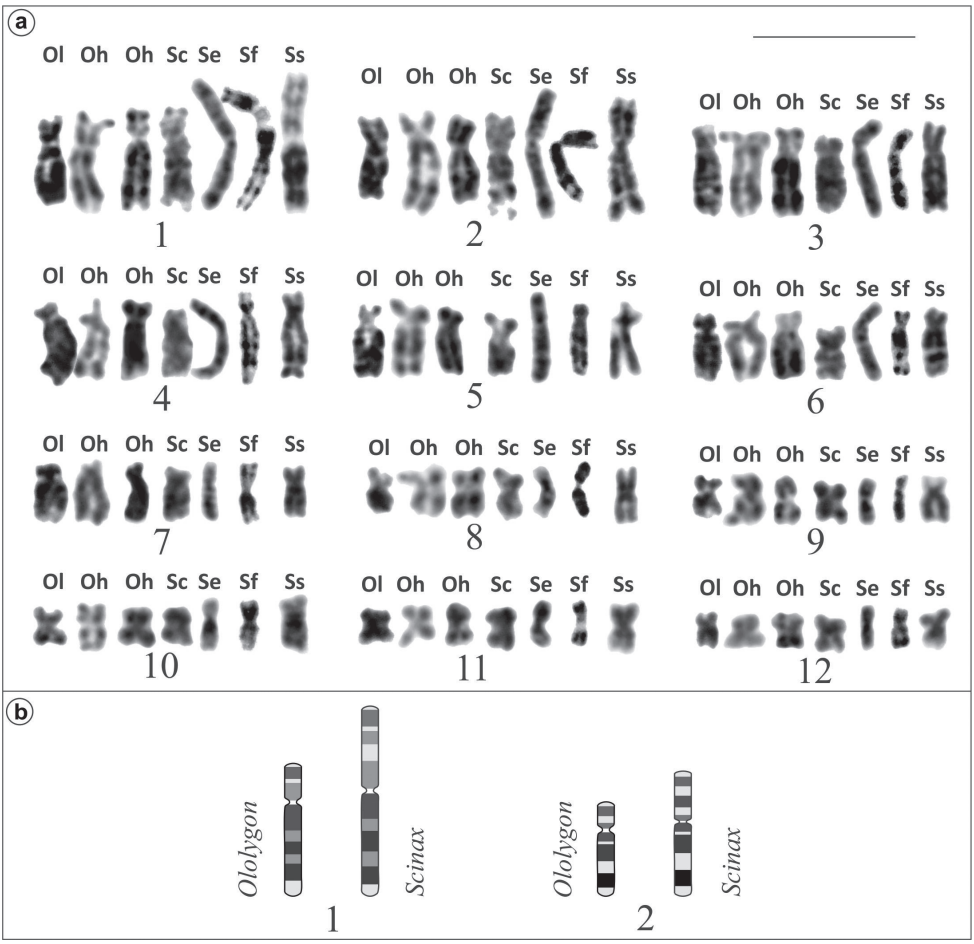


Figure 3. a Comparison of replication bands of the chromosomes of: **Ol** = *O. littoralis*; **Oh** = *O. hiemalis* (two distinct metaphases of the same specimen); **Sc** = *S. crospedospilus*; **Se** = *S. eurydice*; **Sf** = *S. fuscovarius*; **Ss** = *S. similis*. **b** Ideogram of chromosomes 1 and 2 of *Ololygon* and *Scinax* evidencing loss of segment in the short arms of both chromosomes in *Ololygon*. Bar = 10 μm.

only the terminal regions of all chromosomes in these species were labeled (Figure 5). No probe hybridization was observed outside the telomeres in any of these species. The C-banding technique conducted on the chromosome preparations of *O. albicans*, *O. argyreornata*, *O. littoralis*, *S. crospedospilus*, *S. hayii*, and *S. fuscovarius* revealed poorly defined C-bands, which were faintly stained, mainly in the centromeric region of some chromosomes of the species (Figure 6). Fluorochrome staining using CMA_3 showed bright labeling of the NORs in the species of the sample and in some of them, such as *O. littoralis, S. crospedospilus*, and *S. hayii*, the centromeric regions also appeared less brightly stained (Figure 7). DAPI fluorochrome staining revealed that the chromosomes of the species presented a homogeneously stained appearance, with no particularly fluorescent region.

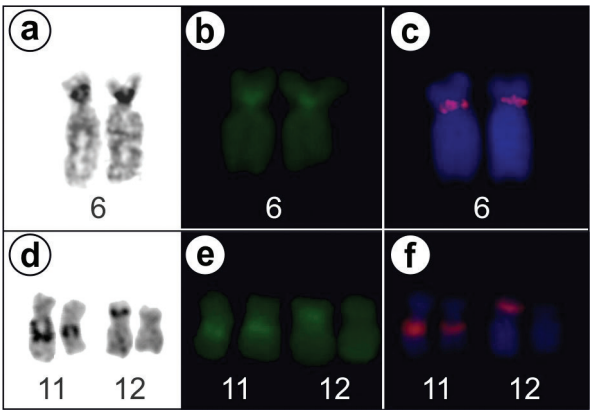


Figure 4. Nucleolus organizer regions in *Ololygon obtriangulata* (**a, b, c**) and *Scinax fuscovarius* (**d, e, f**) identified by **a, d** silver impregnation **b, e** CMA_3 staining, and **c, f** FISH with rDNA probe.

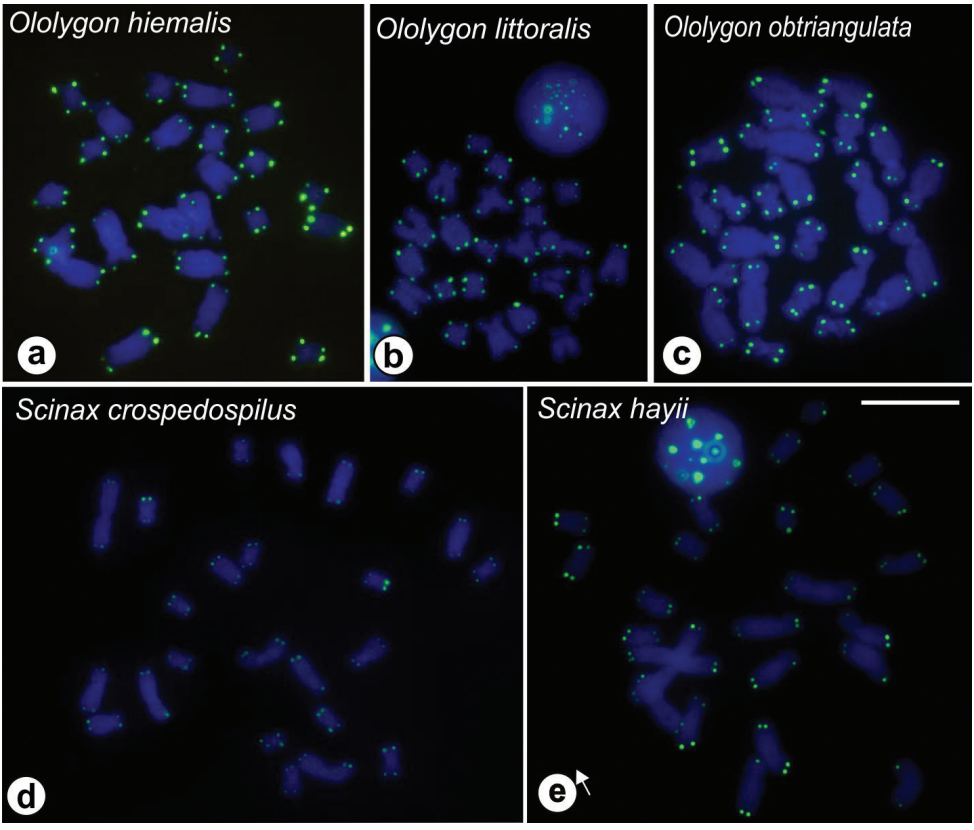


Figure 5. FISH with telomeric probes in *Ololygon* and *Scinax* species. **a** *O. hiemalis* **b** *O. littoralis* **c** *O. obtriangulata* **d** *S. crospedospilus* **e** *S. hayii.* Bar = 10 μm.

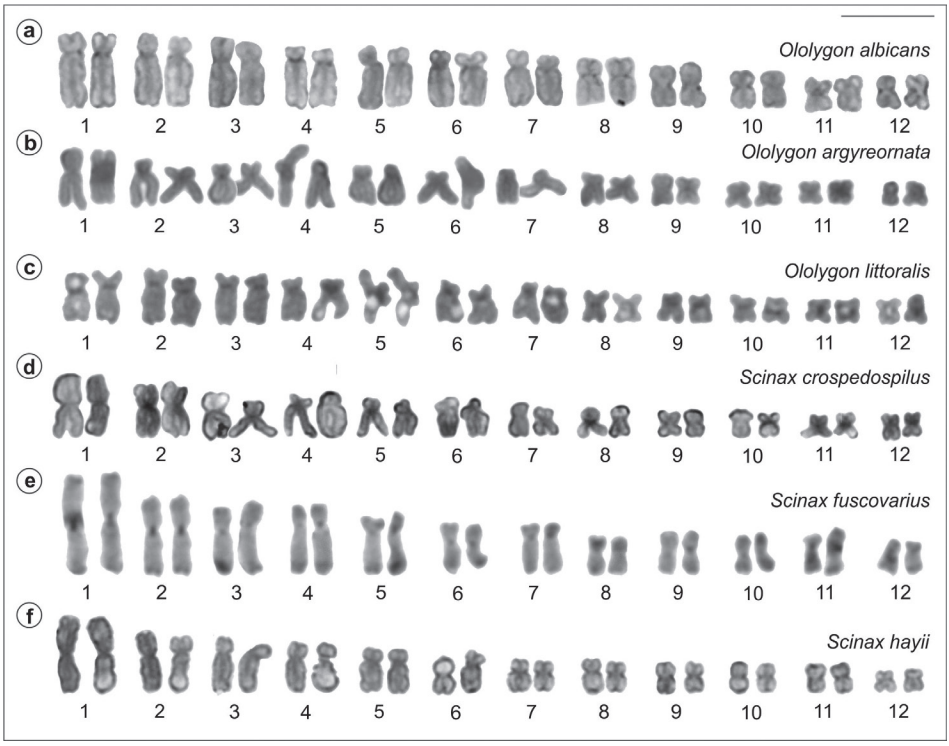


Figure 6. C-banding of *Ololygon* and *Scinax* species. **a** *O. albicans* **b** *O. argyreornata* **c** *O. littoralis* **d** *S. crospedospilus* **e** *S. fuscovarius* **f** *S. hayii*. Bar = 10 μm.

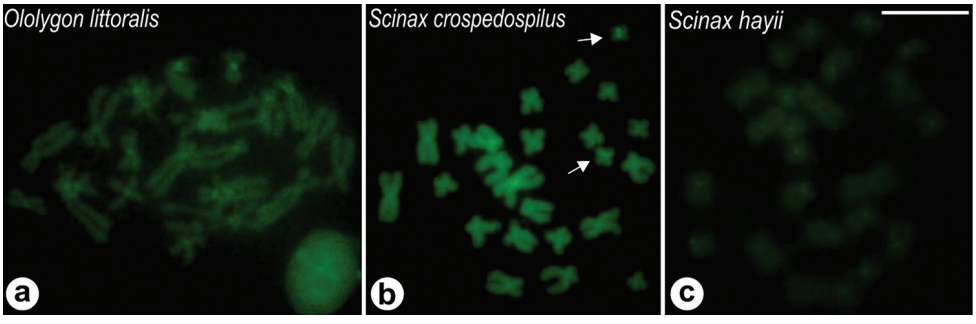


Figure 7. Cromomycin A_3 staining in *Ololygon* and *Scinax* species. **a** *O. littoralis* **b** *S. crospedospilus* **c** *S. hayii*. NOR-bearing chromosomes in **b** (arrow). Bar = 10 μm.

Testis preparations were obtained from male specimens of *O. argyreornata*, *O. hiemalis*, *O. littoralis*, *S. caldarum*, *S. crospedospilus*, *S. eurydice*, and *S. hayii*. In all of them, metaphase I cells presented 12 bivalents, whereas metaphase II cells presented 12 chromosomes, as shown in Figure 8 for one species of each analyzed genus, namely *O. hiemalis* and *S. crospedospilus*. No heteromorphic bivalent that could suggest the presence of differentiated sex chromosomes of the XY type was observed in any of the analyzed species.

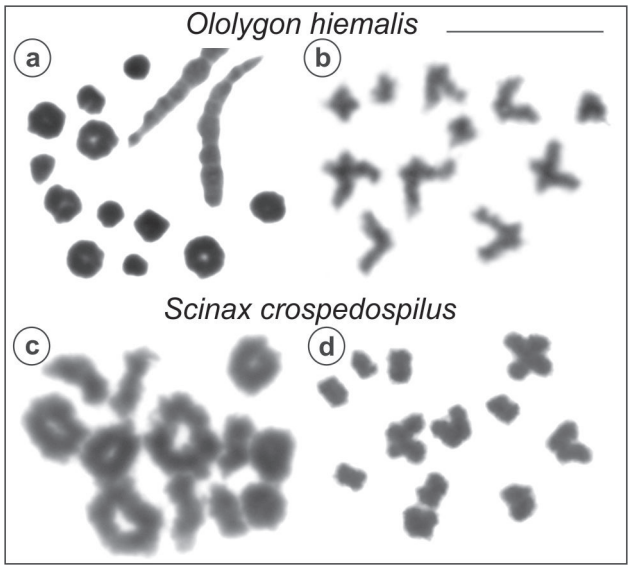


Figure 8. Meiotic cells in males of *Ololygon hiemalis* **(a, b)** and *Scinax crospedospilus* **(c, d)**. **a, c** Metaphase I with 12 bivalents **b, d** Metaphase II with 12 chromosomes. Bar = 10 μm.

Discussion

Although *Ololygon* and *Scinax* species share the same diploid and fundamental numbers, two karyotypic constitutions were unequivocally distinguished by visual inspection of standard stained chromosomes as well as comparative measurements. One of these karyotypes is characteristic for all five species, currently recognized as belonging to the genus *Ololygon* (Duellman et al. 2016, Frost 2016), but formerly assigned to the *Scinax catharinae* clade. The other karyotypic constitution is exhibited by the six remaining species of the sample, currently recognized as belonging to the genus *Scinax* but previously included in the *Scinax ruber* clade.

The difference between the two karyotypes, which is related to the morphology and size of chromosome pairs 1 and 2, had already been indicated in one of the first cytogenetic studies performed in the 1970's by Bogart (1973) when the species of both taxa were assigned to the genus *Hyla*. Nevertheless, a possible third karyotypic constitution that had been suggested by that author was not confirmed here or by Cardozo et al. (2011) who studied a large sample of species of *Scinax* (*lato sensu*). Subsequent studies (Oliveira 2011, Nogueira et al. 2015a, Nogueira et al. 2015b, Peixoto et al. 2015, Lourenço et al. 2016, and Peixoto et al. 2016) confirmed that the two chromosome constitutions remained invariably the same, indicating that the karyotypes are relatively conserved within each of the two taxonomic groups.

Although without experimental evidence, Cardozo et al. (2011) suggested possible mechanisms responsible for the morphological and size differences of pairs 1 and 2 in the two karyotypic constitutions, such as pericentric inversion, addition/loss of repetitive sequences or unidentified structural chromosome alterations. Reproducible band-

ing patterns obtained by BrdU incorporation in the present sample were sufficient for precise pairing of the homologous chromosomes. It was also useful in interspecific comparative analysis for identifying homeologous sets of chromosomes, mainly those which were large or medium sized. Pericentric inversion could be ruled out, but not the addition or loss of repetitive chromosome segments, due to the different size of the short arms of chromosomes 1 and 2 in the two compared karyotypic constitutions. This is quite evident in the case of chromosome 1 and less noticeable in the case of chromosome 2. Nevertheless, it is important to remark that although the poor quality of our results these repetitive regions were not positively stained after C-banding data, a finding also described by Cardozo et al. (2011) analyses.

The probable direction of chromosome evolution, in other words, the loss or gain of repetitive segments in chromosome pairs 1 and 2, is suggested in light of the cytogenetic data of *Ololygon* and *Scinax* species, and the phylogeny of Faivovich et al. (2005). Similar integrative taxonomic approach was carried out by Lourenço et al. (2015) for amphibians and Mezzasalma et al. (2015) for reptiles. As already pointed out by Cardozo et al. (2011) and subsequently confirmed in other works, the species of *Ololygon* (the former *Scinax catharinae* clade) have NORs in chromosome pair 6, whereas the species of *Scinax* (the former *Scinax ruber* clade) generally have this marker in the chromosome pair 11, which also indicates the conservation of this character to cytogenetically differentiate species of both groups. It is important to note that a chromosome constitution with 2n = 24, including the position of NORs, which is characteristic of currently recognized *Scinax* species, is observed in the vast majority of representatives of hylinaes sharing a 2n = 24 (Kasahara et al. 2003, Cardozo et al. 2011). It can consequently be assumed that the chromosome constitution characteristic of the *Scinax* genus (the former *Scinax ruber* clade) would be the ancestor, while that presented by the species of *Ololygon* (the former *Scinax catharinae* clade) would be the derived condition, as it was previously pointed out by Cardozo et al. (2011) In this case, the process of differentiating the karyotype of both groups was probably the deletion and not the addition of repetitive DNA in the short arms of chromosomes 1 and 2, in an ancestral karyotypic form similar to that exhibited by the current *Scinax* species and other Hylinae. Not only do the present data provided the probable direction of the chromosome evolution, they also support the subdivision of the former *Scinax* into two genera, as previously suggested by Pombal Jr et al. (1995), Faivovich (2002), based on morphology, bioacoustics, among others characters and effectively established by Duellman et al. (2016). In this case, the most derived group is actually represented by the species of *Ololygon*.

Obtaining replication bands along chromosomes was helpful to clarify the heteromorphism in pairs 3 and 4 in one of the two specimens of the sample of *Scinax similis* described in Cardozo et al. (2011). Although the Giemsa-stained karyotypes strongly suggested that this heteromorphism resulted from reciprocal translocation, in our study it was definitely confirmed by comparative replication banding analysis. The structural rearrangement is most likely a sporadic finding that can occur in natural populations (Siqueira et al. 2004, Schmid et al. 2010). The results obtained so far us-

ing silver impregnation revealed that this technique is widely useful for characterizing the species of *Ololygon* and *Scinax* (the former *S. catharinae* and *S. ruber* clades, respectively). In a few cases, there were exceptions to the localization of Ag-NORs, which were found to be discrepancies in ordering the chromosomes in the karyograms or due to true changes in the position of this chromosome marker which can eventually occur in natural populations (Cardozo et al. 2011). Another finding reported by these authors refers to the occurrence of multiple Ag-NORs in some species of the sample. In the specimen CBFH 22415 of *S. fuscovarius* herein analyzed three Ag-NORs per metaphase (two in chromosome pair 11 and one in a single homologue of pair 12) were observed but FISH using an rDNA probe confirmed the additional labeling in chromosome 12 as true NOR. This variant pattern of NOR may be a sporadic derived condition possibly characteristic for the population ascertained for the first time in the present work.

Based on C-banding data of species of the former *Scinax* clades, Cardozo et al. (2011) suggested that the amount of centromeric C-banded heterochromatin would also represent a difference between the karyotypes of the analyzed species. Nevertheless, the data obtained herein for species of *Ololygon* and *Scinax* and those of Nogueira et al. (2015a), Nogueira et al. (2015b), and Peixoto et al. (2015) for the species of the former *Scinax catharinae* and *Scinax ruber* clades are inconsistent for a clear-cut differentiation of the two karyotypic constitutions with this chromosome marker.

The FISH technique using telomeric probes tested in the chromosome preparations of *Ololygon* and *Scinax* of our sample, which could potentially clarify the occurrence of structural rearrangement during the chromosome evolution, as demonstrated by Fagundes and Yonenaga-Yassuda (1998) in Akodont rodents, did not provide any evidence.

Conclusions

Our cytogenetic data confirm the loss of repetitive sequence in the short arms of chromosomes 1 and 2 as the mechanism responsible for the difference in the karyotypic constitutions of *Ololygon* and *Scinax.* Nevertheless, relationships in Scinaxcinae species are not easy to interpret. Despite the taxonomic difficulties, increasing the number of analyzed species and using more cytological markers based on molecular cytogenetics methodologies will eventually help to clarify phylogenetic questions within *Ololygon*, *Scinax*, and related genera.

Acknowledgements

This work was supported by grants #2013/50741-7, #2014/50342-8, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors thank to Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for providing the collec-

tion permits. The authors are grateful to Daniela Moraes Leme and Glaucilene Catrolli for help during chromosome preparation and analysis, to Dr. Juliana Zina for collecting the specimen CFBH16736 in Serra do Japí, Jundiaí, SP, and to Mariana Lyra for sequencing COI gene from tissue sample of one specimen.

References

Araújo-Vieira K, Valdujo PH, Faivovich J (2016) A new species of *Scinax* Wagler (Anura: Hylidae) from Mato Grosso, Brazil. Zootaxa 4061(3): 261–273. https://doi.org/10.11646/zootaxa.4061.3.4

Baldissera FA Jr, Oliveira PSL, Kasahara S (1993) Cytogenetics of four Brazilian *Hyla* species (Amphibia-Anura) and description of a case with a supernumerary chromosome. Revista Brasileira de Genética 16: 335–345.

Bogart JP (1973) Evolution of anuran karyotypes. In: Vial JL (Ed.) Evolutionary Biology of Anurans. University of Missouri Press, Columbia, 337–349.

Cardozo DE, Leme DM, Bortoleto JF, Catroli GF, Baldo D, Faivovich J, Kolenc F, Silva APZ, Borteiro C, Haddad CFB, Kasahara S (2011) Karyotypic data on 28 species of *Scinax* (Amphibia: Anura: Hylidae): diversity and informative variation. Copeia 2: 251–263. https://doi.org/10.1643/CH-09-105

Catroli GF, Kasahara S (2009) Cytogenetic data on species of the family Hylidae (Amphibia, Anura): results and perspectives. Publ UEPG Biology Health Science 15: 67–85.

Christian A, McNiel E, Robinson J, Drabek J, LaRue C, Wadren C, Bedford JA (1998) A versatile image analyses approach for simultaneous chromosome identification and localization of FISH probes. Cytogenetic and Cell Genetics 82: 172–179. https://doi.org/10.1159/000015093

Duellman WE, Marion AB, Hedges SB (2016) Phylogenetics, classification, and biogeography of tree frogs (Amphibia: Anura: Arboranae). Zootaxa 4104(1): 001–109. https://doi.org/10.11646/zootaxa.4104.1.1

Dutrillaux B, Couturier J (1981) La Pratique de l´Analyse Chromosomique. Masson, Paris.

Fagundes V, Yonenaga-Yassuda Y (1998) Evolutionary conservation of whole homeologous chromosome arms in the Akodont rodents *Bolomys* and *Akodon* (Muridae, Sigmodontinae): maintenance of interstitial telomeric segments (ITBs) in recent event of centric fusion. Chromosome Research 6: 643–648. https://doi.org/10.1023/A:1009213712370

Faivovich J (2002) A cladistic analysis of *Scinax* (Anura: Hylidae). Cladistics 18: 367–393. https://doi.org/10.1111/j.1096-0031.2002.tb00157.x

Faivovich J, Haddad, CFB, Garcia PCA, Frost DR, Campbell JA, Wheeler WC (2005) Systematic review of the frog family Hylidae, with special reference to Hylinae: Phylogenetic analysis and taxonomic revision. Bulletin of the American Museum of Natural History 294: 1–240. https://doi.org/10.1206/0003-0090(2005)294[0001:srotff]2.0.co;2

Frost DR (2016) Amphibian species of the world: an online reference**.** version 6.0 Electronic Database. http://research.amnh.org/vz/herpetology/amphibian/index.html [accessed 10. September 2016]

Gazoni T, Gruber SL, Silva APZ, Araújo OGS, Strüssmann C, Haddad CFB, Kasahara S (2012) Cytogenetic analyses of eight species in the genus *Leptodactylus* Fitzinger 1843 (Amphibia, Anura, Leptodactylidae), including a new diploid number and a karyotype of multiple translocations. BMC Genetics 13: 109. https://doi.org/10.1186/1471-2156-13-109

Green DM, Sessions SK (1991) Nomenclature for Chromosomes. In: Green DM, Sessions SK (Eds) Amphibian Cytogenetics and Evolution. Academic Press, San Diego, 431–432. https://doi.org/10.1016/B978-0-12-297880-7.50021-4

Green DM, Sessions SK (2007) Karyology and Cytogenetics. In: Heatwole H, Tyler M (Eds) Amphibian Biology 7. Surrey Beatty and Sons, Chipping Norton, Australia, 2756–2841.

Gruber SL, Haddad CFB Kasahara S (2007) Chromosome banding in three species of *Hypsiboas* (Hylidae, Hylinae), with special reference to a new case of B-chromosome in anuran frogs and to the reduction of the diploid number of 2n = 24 to 2n = 22 in the genus. Genetica 130: 281–291. https://doi.org/10.1007/s10709-006-9105-6

Gruber SL, Zina J, Narimatsu H, Haddad CFB Kasahara S (2012) Comparative karyotype analysis and chromosome evolution in the genus *Aplastodiscus* (Cophomantini, Hylinae, Hylidae). BMC Genetics,13: 28. https://doi.org/10.1186/1471-2156-13-28

Gruber SL, Silva APZ, Haddad CFB, Kasahara S (2013) Cytogenetic analysis of *Phyllomedusa distincta* Lutz, 1950 (2n = 2x = 26), *P. tetraploidea* Pombal and Haddad, 1992 (2n = 4x = 52), and their natural triploid hybrids (2n = 3x = 39) (Anura, Hylidae, Phyllomedusinae). BMC Genetics 14: 75. https://doi.org/10.1186/1471-2156-14-75

Haddad CFB, Pombal Jr JP (1987) *Hyla hiemalis,* nova espécie do grupo *rizibilis* do Estado de São Paulo. Revista Brasileira de Biologia 47: 127–132.

Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. https://doi.org/10.1007/BF01953855

Kasahara S, Silva APZ, Gruber SL (1998) Use of lymphocyte cultures for BrdU replication banding patterns in anuran species (Amphibia). Genetics and Molecular Biology 21: 471–476. https://doi.org/10.1590/S1415-475711998000400011

Kasahara S, Silva APZ, Gruber SL, Haddad CFB (2003) Comparative cytogenetic analysis on four tree frog species (Anura, Hylidae, Hylinae) from Brazil. Cytogenetic and Genome Research 103: 155–162. https://doi.org/10.1159/000076304

Lourenço ACC, Zina J, Catroli GF, Kasahara S, Faivovich J, Haddad CFB (2016) A new species of the Scinax catharinae group (Anura: Hylidae) from southeastern Brazil. Zootaxa 4154(4):415–435. https://doi.org/10.11646/zootaxa.4154.4.3

Lourenço LB, Targueta CP, Baldo D, Nascimento J, Garcia PCA, Andrade GV, Haddad CFB, Recco-Pimentel SM (2015) Phylogeny of frogs from the genus *Physalaemus* (Anura, Leptodactylidae) inferred from mitochondrial and nuclear gene sequences. Molecular Phylogenetics and Evolution 92: 204–216. https://doi.org/10.1016/j.ympev.2015.06.011

Meunier-Rotival M, Cortadas J, Macaya G (1979) Isolation and organization of calf ribosomal DNA. Nucleic Acids Research 6: 2109–2123. https://doi.org/10.1093/nar/6.6.2109

Mezzasalma M, Dall'Asta A, Loy A, Cheylan M, Lymberakis P, Zuffi MAL, Tomovic L, Odierna G, Guarino FM (2015) A sisters' story: comparative phylogeography and taxonomy of *Hiero-*

phis viridiflavus and *H. gemonensis* (Serpentes, Colubridae). Zoologica Scripta 44: 495–508. https://doi.org/10.1111/zsc.12115

Nogueira L, Paim F, Diniz D, Solé M, Affonso P, Siqueira S, Sampaio I (2015a) Cytogenetic analysis of *Scinax auratus* and *Scinax eurydice* (Anura, Hylidae) with emphasis on cytotaxonomy. Comparative Cytogenetics 9: 227–236. https://doi.org/10.3897/CompCytogen.v9i2.4593

Nogueira L, Zanoni JB, Solé M, Affonso PRAM, Siqueira S, Sampaio I (2015b) Cytogenetic studies in six species of *Scinax* (Anura: Hylidae) clade *Scinax ruber* from northern and northeasthern Brazil. Genetics and Molecular Biology 38: 156–161. https://doi.org/10.1590/S1415-4757382220140280

Oliveira AKC (2011) Estudo de comunidade de anuros e citogenética do gênero *Scinax* em área de ecótone no Paraná. PhD Thesis, Universidade Federal do Paraná, Curitiba, Paraná 88 pp. [In Portuguese]

Peixoto MAA, Lacerda JVA, Coelho-Augusto C, Feio RN, Dergam JA (2015) The karyotypes of five species of the *Scinax perpusillus* group (Amphibia, Anura, Hylidae) of southeastern Brazil show high levels of chromosomal stabilization in this taxon. Genetica (online). https://doi.org/10.1007/s10709-015-9870-1

Peixoto MAA, Oliveira MPC, Feio RN, Dergan JA (2016) Karyological study of *Ololygon tripui* (Lourenço, Nascimento and Pires, 2009), (Anura, Hylidae) with comments on chromosomal traits among populations. Comparative Cytogenetics 10(4): 505–516. https://doi.org/10.3897/CompCytogen.v10i4.9176

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Science USA 83: 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Pombal Jr JP, Gordo M (1991) Duas novas espécies de *Hyla* da floresta atlântica no Estado de São Paulo. Memórias do Instituto Butantã 53(1): 135–144.

Pombal Jr JP, Bastos RB, Haddad CFB (1995) Vocalizações de algumas espécies do gênero *Scinax* (Anura: Hylidae) do sudeste do Brasil e comentários taxonômicos. Naturalia 20: 213–225.

Schmid M (1978) Chromosome banding in Amphibia I. Constitutive heterochromatin and nucleolus organizers regions in *Bufo* and *Hyla*. Chromosoma 66: 361–388. https://doi.org/10.1007/BF00328536

Schmid M, Steinlein C, Bogart JP, Feichtinger W, León P, La Marca E, Diaz LM, ·Sanz A, Chen S, Hedges SB (2010) The chromosomes of Terraranan frogs. Insights into vertebrate cytogenetics. Cytogenetic and Genome Research 129: 1–568. https://doi.org/10.1159/000301339

Siqueira S, Recco-Pimentel SM, Ananias F (2004) Cytogenetics of three Brazilian species of *Eleutherodactylus* (Anura, Leptodactylidae) with 22 chromosomes and re-analysis of multiple translocations in *E. binotatus.* Genetics and Molecular Biology 27: 363–372. https://doi.org/10.1590/S1415-47572004000300010

Silva APZ, Haddad CFB, Kasahara S (2000) Chromosomal studies on five species of the genus *Leptodactylus* Fitzinger, 1826 (Amphibia, Anura) using differential staining. Cytobios 103: 25–38.

Silva APZ, Garcia PCA, Martins VG, Bacci, M, Kasahara S (2004) Chromosomal and molecular analysis of *Leptodactylus gracilis gracilis*, *L. gracilis delattini*, and *L. plaumanni* (Anura, Leptodactylidae): taxonomic implication. Amphibia-Reptilia 25: 185–196. https://doi.org/10.1163/1568538041231193

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

Sumner AT (2003) Chromosomes. Organization and Function. Blackwell Publishing, Oxford.

Wiens JJ, Kuczynski CA, Hua X, Moen DS (2010) An expanded phylogeny of treefrogs (Hylidae) based on nuclear and mitochondrial sequence data. Molecular Phylogenetics and Evolution 55: 871–882. https://doi.org/10.1016/j.ympev.2010.03.013

CompCytogen 11(2): 285–297 (2017)
doi: 10.3897/CompCytogen.11i2.11395
http://compcytogen.pensoft.net

DATA PAPER

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

The Cerrado (Brazil) plant cytogenetics database

Fernando Roa[1], Mariana Pires de Campos Telles[1,2]

1 *Programa de pós-graduação em Genética e Melhoramento de Plantas, Escola de Agronomia, Universidade Federal de Goiás, 74001-970, Goiânia, GO, Brazil* **2** *Escola de Ciências Agrárias e Biológicas, Pontifícia Universidade Católica de Goiás, 74605-010, Goiânia, GO, Brazil*

Corresponding author: *Fernando Roa* (fernando.roa@ufpe.br)

Academic editor: *P. Kumar* | Received 3 December 2016 | Accepted 9 March 2017 | Published 25 April 2017

http://zoobank.org/90DE4485-0B73-4F72-AEA7-F5A938ABE07B

Citation: Roa F, Telles MPC (2017) The Cerrado (Brazil) plant cytogenetics database. Comparative Cytogenetics 11(2): 285–297. https://doi.org/10.3897/CompCytogen.11(2).11395

Abstract

Cerrado is a biodiversity hotspot that has lost ca. 50% of its original vegetation cover and hosts ca. 11,000 species belonging to 1,423 genera of phanerogams. For a fraction of those species some cytogenetic characteristics like chromosome numbers and C-value were available in databases, while other valuable information such as karyotype formula and banding patterns are missing. In order to integrate and share all cytogenetic information published for Cerrado species, including frequency of cytogenetic attributes and scientometrics aspects, Cerrado plant species were searched in bibliographic sources, including the 50 richest genera (with more than 45 taxa) and 273 genera with only one species in Cerrado. Determination of frequencies and the database website (http://cyto.shinyapps.io/cerrado) were developed in R. Studies were pooled by employed technique and decade, showing a rise in non-conventional cytogenetics since 2000. However, C-value estimation, heterochromatin staining and molecular cytogenetics are still not common for any family. For the richest and best sampled families, the following modal 2n counts were observed: Oxalidaceae 2n = 12, Lythraceae 2n = 30, Sapindaceae 2n = 24, Solanaceae 2n = 24, Cyperaceae 2n = 10, Poaceae 2n = 20, Asteraceae 2n = 18 and Fabaceae 2n = 26. Chromosome number information is available for only 16.1% of species, while there are genome size data for only 1.25%, being lower than the global percentages. In general, genome sizes were small, ranging from 2C = ca. 1.5 to ca. 3.5 pg. Intraspecific 2n number variation and higher 2n counts were mainly related to polyploidy, which relates to the prevalence of even haploid numbers above the mode of 2n in most major plant clades. Several orphan genera with almost no cytogenetic studies for Cerrado were identified. This effort represents a complete diagnosis for cytogenetic attributes of plants of Cerrado.

Keywords

Cerrado, cytogenetics, database, scientometrics, shiny-R

Introduction

Cerrado, a phytogeographic domain from Brazil, is the third largest biodiversity hotspot in the world considering species endemism and degree of threat (Myers et al. 2000; Forzza et al. 2012). In fact, it has suffered more anthropic impact than the Amazonian tropical forest (Spera et al. 2016). With originally 2m km^2 of wilderness area, of which ca. 50% are currently cultivated, this region hosts ca. 11,000 species belonging to 1,423 genera of 171 families of phanerogams (Sano et al. 2008; Ferreira et al. 2016). Classical cytogenetic studies, comprehending somatic or meiotic chromosome counts are known for a considerable portion of species occurring in Cerrado but in many cases they were not performed on plants from this region. Meanwhile, studies of genome size, differential staining and molecular cytogenetics are very rare.

Since the chromosome number is the most basic karyotype feature and it can be observed by conventional staining of meristem cells or pollen mother cells, most cytogenetic databases deal only with this attribute (Peruzzi and Bedini 2014; Rice et al. 2015). Estimates of genome size, which are currently addressed mostly by comparing the relative fluorescence of propidium iodide stained nuclei measured in a flow cytometer to that of known patterns, are compiled in the Kew C-value database (Doležel and Greilhuber 2010; Garcia et al. 2014a). Other valuable information such as: karyotype formula, dependent on the relative position of centromere along the chromosome and chromosome length (Guerra 1986), silver staining of nucleolar organizer regions (AgNOR) (Vieira et al. 1990), and heterochromatin staining after treatment with acids, bases or denaturation (Schwarzacher et al. 1980; Guerra 2000; Barros e Silva and Guerra 2010) are not included in databases. In contrast, fluorescence in situ hybridization (FISH), in which a DNA probe produced by molecular biology methods anneals with chromosome preparations and is detected by fluorescence has gained more attention (Roa and Guerra 2012; Garcia et al. 2014b; Roa and Guerra 2015).

The aim of this study was to assess the current cytogenetic knowledge of Cerrado plants, aiming to provide a consistent database that didn't exclude any attribute and diagnose recent progress in the field.

Methods

Data compilation

Plant names in the Cerrado plant list (Sano et al. 2008) and the cytogenetic literature were checked according to the Brazilian Flora 2020 site (REFLORA 2016) through the www.plantminer.com app (Carvalho et al. 2010). In order to get to the original cytological sources, websites in Table 1 were used. The information in the primary source was organized in the following fields: family (APG 2016), genus, original name as reported in the publication (field = name_on_source), accepted name following the online Brazilian Flora 2020 (field = accepted_name), place of sampling (field = provenance), dis-

tribution of C-, Chromomycin A_3, AgNOR-bands (fields = C-bands, CMA_bands, AgNOR), availability of images (field = images), karyotype formula (field = karyotype), FISH sites position (field = FISH), genome size (field = C-value), meiotic or gametophyte chromosome number (field = meiosis-n), sporophytic chromosome number-2*n* (field = 2*n*), level of ploidy (field = ploidy), average chromosome size (field = ACS), Total chromosome length (field = TCL), Total chromosome area (field = TCA), reference, and authors' affiliation (field = affiliation). Metadata were gathered partially by scripts from search engines as Scopus (Elsevier) and Crossref (PILA).

Data statistics

All statistics and the online database were performed in R (R Core Team 2016). Packages used were shiny (Chang et al. 2016), shinyjs (Attali 2016), shinydashboard (Chang 2015), ggplot2 (Wickham 2009), plyr (Wickham 2015a), dplyr (Wickham and Francois 2016), stringr (Wickham 2015b), data.table (Dowle et al. 2015), DT (Xie 2015), mongolite (Ooms 2016), gtools (Warnes et al. 2015), robustHD (Alfons 2016) and scales (Wickham 2016). Several frequencies were determined, such as: employed techniques or country of author affiliation per decade; chromosome number (2*n*), genome size, TCL and TCA per taxa. Where not available, TCL and TCA were measured from photos by ImageJ (Schneider et al. 2012). Due to the scarcity of genome size data, a correlation analysis between TCA/TCL and known genome sizes was performed in order to have a rough idea of genome size in pg based on TCL or TCA. In accessions without 2*n* number, they were calculated from meiosis or pollen observations (n).

Even n numbers are more common than odd ones because of the prevalence of even 2n that eventually undergo duplication (Otto and Whitton 2000; Rice et al. 2015). As expected, this trend is more pronounced for high 2n which are usually the result of whole genome duplication events, but may vary by dysploidy. Accessions were subdivided in two groups according to n under or over the mode (n) (low 2n, high 2n); and the even/odd ratio for each group was calculated in order to assess indirectly the degree of polyploidy/dysploidy in each major clade.

Results

Coverage

A subset of 38.9% (4,590 taxa) of the Cerrado Plant list was searched, including the 50 richest genera and 273 genera with only one species. Information of 1,431 accessions, from 366 available primary sources, were included in the database. 702 (16.8%) species of our searched sample (4182) had any cytogenetic data and about 500 of them had at least one accession collected in Brazil. Complete lack of information happens

for 70% of the single-species genera addressed (Suppl. material 1, 2, 3). The genera *Hyptis* Jacq., 1787 (Lamiaceae), *Stachytarpheta* Vahl, 1804 (Verbenaceae), *Microlicia* D.Don, 1823 (Melastomataceae), *Leiothrix* Ruhland, 1903 (Eriocaulaceae) and *Ditassa* R.Br., 1809 (Apocynaceae) may be considered orphan because they are among the 50 most diverse in Cerrado, but almost no chromosome numbers are known for them (Suppl. material 1). At the family level Eriocaulaceae (Monocots – Commelinids), Lamiaceae, Apocynaceae (Core eudicots-Superasterids-Asterids-Lamiids), Polygalaceae (Core eudicots-Superrosids-Fabids) and Lauraceae (Magnoliids) are also poorly studied (Suppl. material 2), while among the major clades (previously in parentheses), studies for Magnoliids are scarce. Every figure and table is interactive in the database website (http://cyto.shinyapps.io/cerrado).

For the richest and best sampled families, the following modal 2n counts were observed: Oxalidaceae 2n = 12, Lythraceae 2n = 30, Sapindaceae 2n = 24, Solanaceae 2n = 24, Cyperaceae 2n = 10, Poaceae 2n = 20, Asteraceae 2n = 18 and Fabaceae 2n = 26 (Suppl. material 2). At the genus level, *Paspalum* L., 1759 (Poaceae), *Cuphea* P.Browne, 1756 (Lythraceae) and *Rhynchospora* Vahl, 1805 (Cyperaceae) stand among the best studied with ca. 50% of coverage. For ca. 20% of species in most major clades, the chromosome number has been studied (Suppl. material 3).

Techniques chronology

Frequencies of use of karyological techniques show that the first cytogenetic analysis were mainly based on meiosis from 1928 on, and subsequently by 1990 the analysis of mitosis (sporophyte) gained more prevalence. Measurements of karyotype formula (morphology) achieved significance after the 1990's. A trend to include images in publications, only with a fall during the 2000's decade was observed. Sophisticated techniques that show a substantial rise from the 2000's decade are the estimation of the C-value, CMA banding (including C-CMA banding) and FISH. However, they have been applied to a limited number of species, ranging from 0.5 to 2.3% of the 1,431 entries in the database (Fig. 1).

Author affiliations

In order to track affiliation of authors over time, they were clustered by decade in a time-line. For accessions collected in Brazil [774 (54%)] the country of affiliation of the author showed an increase in the prevalence of Brazilian based research after the 1990's. Other significant contributions for Brazilian samples have been made by Argentinian based authors (Fig. 2). Most publishing authors for the 1935 – 1970 period were affiliated in the U.S.A, and afterwards, in Brazil. Among the authors with more entries, Coleman, Irwin and Turner studied in the 1935 – 1970 period mainly Asteraceae and Fabaceae. In the 1970 decade, Coleman studied Fabaceae and Nassar, Eu-

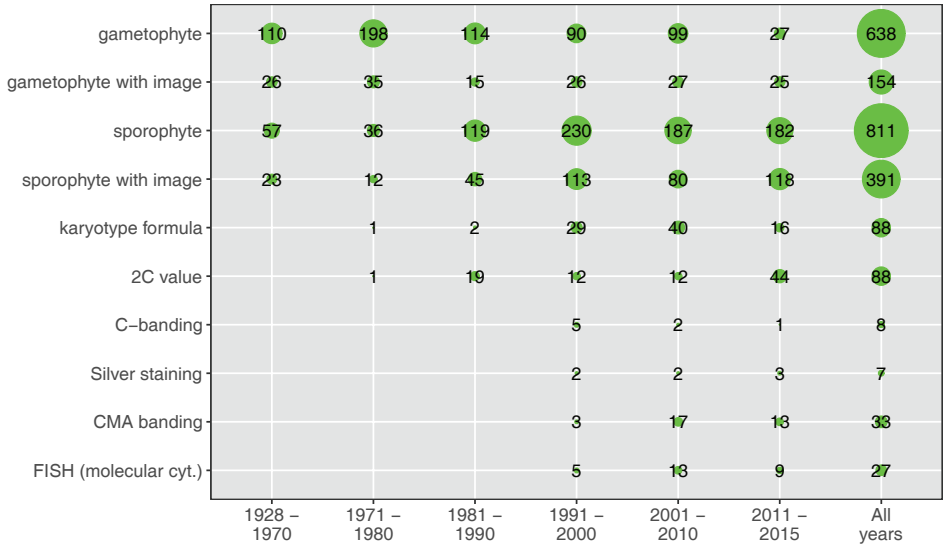


Figure 1. Chronology of cytogenetic techniques or parameters published for Cerrado plant species. Numbers of accessions in the database.

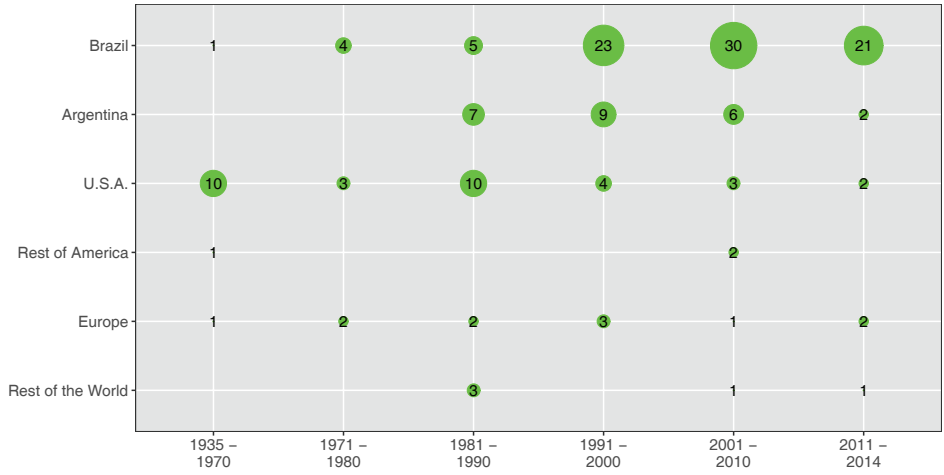


Figure 2. Chronology of author affiliation for Cerrado plant species collected in Brazil. Numbers of references in the database.

phorbiaceae. In the 80's and 2000's Graham studied Lythraceae, and in the 90's Guerra published mainly data on Cyperaceae, Orchidaceae and Velloziaceae. In the current decade, Félix published mainly about Euphorbiaceae and Fabaceae (Suppl. material 4). Accordingly, those authors correspond to the most important nodes in the co-author network (database website).

Chromosome numbers and genome size

699 (16.1%) species of the searched list have known chromosome numbers with a general median of 2*n* = 28. Fig. 3 shows two ploidy related prevalent chromosome numbers; neopolyploids in Commelinids, which corresponds to *Paspalum* counts (2n = 20 and 40), and several paleopolyploid genera as *Habenaria* with 2n = 42 (Monocots) and *Mikania* with 2n = 36 (Eudicots-Asterids-Campanulids). An unusual conspicuous high frequency of 2n = 36 for the whole sample, corresponds mainly to genera *Manihot* Mill., 1754 (Euphorbiaceae), Tibouchina Aubl., 1775 (Melastomataceae) and *Mikania* Willd., 1803 (Asteraceae). Intraspecific chromosome number variation occurs in 14.3% of species, being polyploidy the cause in 88% of them. At the genus level, most high chromosome numbers are multiples of one of the lowest modal numbers of the genus, i.e. base numbers (database website). The even/odd ratio of n is higher for the groups with "*n* greater than the mode" in almost every clade, as expected, except for Asterids-Campanulids (ratio 1.5), while Asterids-Lamiids shows the greatest bias towards even numbers (ratio 6.5) in karyotypes with n greater than the mode (Table 2).

Only 88 C-values were found for the taxa of Cerrado, most of them ranging from 2C = ca. 1.5 pg to ca. 3.5 pg with a median value of 2.38 pg (Fig. 4). That corresponds to 1.25% of the addressed species list. For a sample of known TCL (or TCA) and C-value, a correlation analysis, although significant, resulted in low r^2 values (weak to moderate). Applying a linear model to predict 2C-value based on TCL, resulted in prediction intervals of ca. 2pg. TCL and TCA values together with some predicted 2C- values are shown in Suppl. material 5, 6 and the database website.

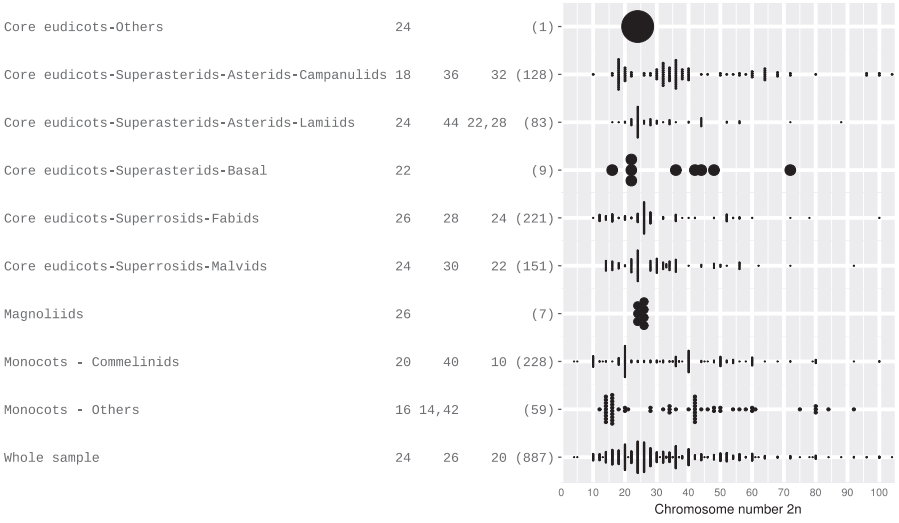


Figure 3. Dot-plot of observed and calculated 2n numbers for Cerrado plant species considering APG major clades. The three most common numbers (frequency ties separated by comma) and sample size in parentheses are indicated. Dot size varies depending on the maximum frequency of each group.

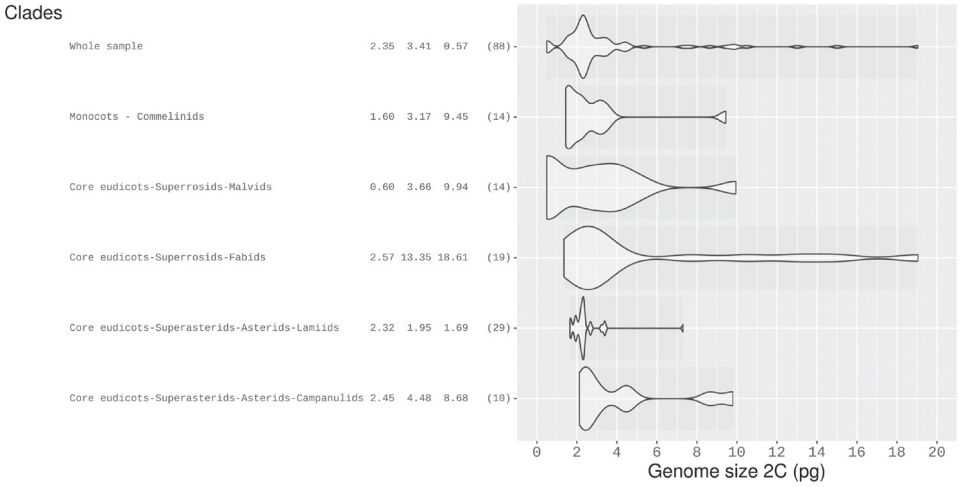


Figure 4. Violin-plots for genome sizes in pg for Cerrado angiosperms. Y-axis contains clades, three highest peaks of the Gaussian kernel density and sample size. Bandwidth of the density estimator customizable in http://cyto.shinyapps.io/cerrado

Table 1. Websites used for the search of literature.

Name	URL
Chromosome Counts Database*	ccdb.tau.ac.il
Plant DNA C-values Database*	data.kew.org/cvalues
International Organization of Plant Biosystematists	www.iopb.org
Scholar Google	scholar.google.com
Scopus	www.scopus.com
Biodiversity Heritage Library	biodiversitylibrary.org
JSTOR	www.jstor.org
Real Jardín Botánico CSIC	bibdigital.rjb.csic.es/spa/index.php
Naturalis Biodiversity Center	www.repository.naturalis.nl
Botanicus Digital Library	www.botanicus.org
Smithsonian Contributions to Botany	repository.si.edu/handle/10088/6943
Crossref	www.crossref.org

*citation in text, data taken from available primary sources.

Table 2. Even/odd ratio for haploid (n) numbers considering mode of n.

Clade or group	n even/odd ratio	
	Greater than n mode	Lesser
Monocots - Commelinids	3.47	0.40
Other monocots	4.00	0.12
Core eudicots-Superasterids-Asterids-Lamiids	6.50	1.00
Core eudicots-Superasterids-Asterids-Campanulids	1.50	1.57
Core eudicots-Superrosids-Malvids	3.22	0.50
Core eudicots- Superrosids-Fabids	3.40	0.56

Discussion

There is strong interest for genomics and genetics of Cerrado species (Souza et al. 2016). Knowledge of basic data as chromosome number, ploidy level and genome size is critical for the selection of model species (Kelly et al. 2012). Though recent cytogenetic studies tend to incorporate phylogenetic frameworks and correlate cytogenetic with ecological variables, this kind of studies are scarce for Cerrado (Glick and Mayrose 2014; Suda et al. 2015; Silveira et al. 2016). As noted by Guerra (1990), previous to the 1990 decade, cytogenetic studies of authors from U.S.A. prevailed. After that period, mitotic analysis increased compared to meiotic studies, which relate to the establishment of cytogenetic laboratories allowing the treatment with antimitotics of root meristems. Still, the amount of chromosome number data for species occurring in Cerrado is lower than the world total, 16.1 vs. 20% (Rice et al. 2015) and several "orphan" genera were identified, such as *Stachytarpheta* (Verbenaceae), *Microlicia* (Melastomataceae) and *Leiothrix* (Eriocaulaceae). Unlike other techniques, C-banding and silver staining studies did not increase in recent decades, which might be related to low repetitiveness of methods (Guerra 1990). Despite recent efforts, the percentage of known Cerrado C-values is still lower than the 2.1% for all angiosperms (Garcia et al. 2014a). Data of TCA, and TCL for a larger number of species were intended to be used as proxy for genome size after an analysis that resulted in weak to moderate significant correlation with 2C-value in pg. Though they can give a rough estimate of the magnitude of the genome, the prediction interval is high considering most genomes fall within a 2 pg range.

Discontinuities in chromosome numbers seen as multimodal distributions indicate that higher numbers are polyploids generated by genome duplication. A polyploid origin for most high n numbers is also evidenced by the high (> 1) even/odd ratios of *n* (Rice et al. 2015). In contrast, low even/odd value (for *n* greater than the mode) is rare and suggests increased dysploidy and/or low frequency of polyploidy, compared with groups that follow the opposite general trend.

Conclusion

The Database of Cytogenetics of Cerrado plants and its website is presented, making an important step in facilitating access to most known cytogenetic attributes (http://cyto.shinyapps.io/cerrado/). The amount of chromosome number data for Cerrado is lower than the world total. The chromosome number, is still lacking in several rich genera and families, and therefore, they can be considered "orphan". This complete lack of information also happens for 70% of the single-species genera addressed. Analysis of chromosome numbers at several taxonomical levels revealed a straightforward relationship between polyploidy and high chromosome numbers. Regarding other techniques, like heterochromatin staining, molecular cytogenetics and C-value estimation, they have been applied to a very small percentage of species, however, those studies have

been steadily increasing since 2000. Though cytogenetic data are the basis for some evolutionary and ecological studies, there is a lack of those kind of interdisciplinary studies for Cerrado.

Acknowledgements

FR wishes to thank CAPES for their financial support, through a PNPD fellowship. MPCT is supported by productivity grants CNPq/FAPEG/Pró-Centro-Oeste (563839/2010-4 and 201110267000125), Pronex – FAPEG/CNPq (Funding Announcement No. 07/2012) and CNPq (447754/2014-9). This publication was funded by the PPGS CAPES/FAPEG F. A. No. 08/2014. Current research is developed in the context of the National Institutes for Science and Technology (INCT) in Ecology, Evolution and Biodiversity Conservation, supported by MCTIC/CNpq (proc. 465610/2014-5) and FAPEG.

References

Alfons A (2016) robustHD: Robust Methods for High-Dimensional Data. https://cran.r-project.org/package=robustHD [accessed 1. June 2016]

APG (2016) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Botanical Journal of the Linnean Society 181(1): 1–20. https://doi.org/10.1111/boj.12385

Attali D (2016) shinyjs: Perform Common JavaScript Operations in Shiny Apps using Plain R Code. https://cran.r-project.org/package=shinyjs [accessed 1. June 2016]

Barros e Silva AE, Guerra M (2010) The meaning of DAPI bands observed after C-banding and FISH procedures. Biotechnic & Histochemistry 85(2): 115–125. https://doi.org/10.3109/10520290903149596

Carvalho GH, Cianciaruso MV, Batalha MA (2010) Plantminer: A web tool for checking and gathering plant species taxonomic information. Environmental Modelling & Software 25(6): 815–816. https://doi.org/10.1016/j.envsoft.2009.11.014

Chang W (2015) shinydashboard: Create Dashboards with "Shiny." https://cran.r-project.org/package=shinydashboard [accessed 1. June 2016]

Chang W, Cheng J, Allaire JJ, Xie Y, McPherson J (2016) shiny: Web Application Framework for R. https://cran.r-project.org/package=shiny [accessed 1. June 2016]

Doležel J, Greilhuber J (2010) Nuclear genome size: Are we getting closer? Cytometry Part A 77(7): 635–642. https://doi.org/10.1002/cyto.a.20915

Dowle M, Srinivasan A, Short T [with contributions from R Saporta SL, Antonyan E] (2015) data.table: Extension of Data.frame. https://cran.r-project.org/package=data.table [accessed 1. June 2016]

Ferreira ME, dos Anjos AF, Ferreira LG, Bustamante M, Fernandes GW, Machado RB (2016) Cerrado O fim da história ou uma nova história? Ciênciahoje 56(334): 24–29.

Forzza RC, Baumgratz JFA, Bicudo CEM, Canhos DAL, Carvalho AA, Coelho MAN, Costa AF, Costa DP, Hopkins MG, Leitman PM, Lohmann LG, Lughadha EN, Maia LC, Martinelli G, Menezes M, Morim MP, Peixoto AL, Pirani JR, Prado J, Queiroz LP, Souza S, Souza VC, Stehmann JR, Sylvestre LS, Walter BMT, Zappi DC (2012) New Brazilian Floristic List Highlights Conservation Challenges. BioScience 62(1): 39–45. https://doi.org/10.1525/bio.2012.62.1.8

Garcia S, Gálvez F, Gras A, Kovařík A, Garnatje T (2014b) Plant rDNA database: update and new features. Database: the journal of biological databases and curation 2014 bau063. https://doi.org/10.1093/database/bau063

Garcia S, Leitch IJ, Anadon-Rosell A, Canela MÁ, Gálvez F, Garnatje T, Gras A, Hidalgo O, Johnston E, Mas de Xaxars G, Pellicer J, Siljak-Yakovlev S, Vallès J, Vitales D, Bennett MD (2014a) Recent updates and developments to plant genome size databases. Nucleic Acids Research 42(Database issue): 1159–1166. https://doi.org/10.1093/nar/gkt1195

Glick L, Mayrose I (2014) ChromEvol: Assessing the pattern of chromosome number evolution and the inference of polyploidy along a phylogeny. Molecular Biology and Evolution 31(7): 1914–1922. https://doi.org/10.1093/molbev/msu122

Guerra M (1986) Reviewing the chromosome nomenclature of Levan et al. Brazilian Journal of Genetics 9(4): 741–743.

Guerra M (1990) A situação da citotaxonomia de angiospermas nos trópicos e, em particular, no Brasil. Acta Botanica Brasilica 4(2): 75–86. https://doi.org/10.1590/S0102-33061990000300009

Guerra M (2000) Patterns of heterochromatin distribution in plant chromosomes. Genetics and Molecular Biology 23(4): 1029–1041. https://doi.org/10.1590/S1415-47572000000400049

Kelly LJ, Leitch AR, Fay MF, Renny-Byfield S, Pellicer J, Macas JJ, Leitch IJ (2012) Why size really matters when sequencing plant genomes. Plant Ecology & Diversity 5(4): 415–425. https://doi.org/10.1080/17550874.2012.716868

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403(6772): 853–858. https://doi.org/10.1038/35002501

Ooms J (2016) mongolite: Fast and Simple MongoDB Client for R. https://cran.r-project.org/package=mongolite

Otto SP, Whitton J (2000) Polyploid Incidence and Evolution. Annual Review of Genetics 34(1): 401–437. https://doi.org/10.1146/annurev.genet.34.1.401

Peruzzi L, Bedini G (2014) Online resources for chromosome number databases. Caryologia 67(4): 292–295. https://doi.org/10.1080/0144235X.2014.974358

R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

REFLORA (2016) Flora do Brasil 2020. http://floradobrasil.jbrj.gov.br/ [accessed 22. April 2016]

Rice A, Glick L, Abadi S, Einhorn M, Kopelman NM, Salman-Minkov A, Mayzel J, Chay O, Mayrose I (2015) The Chromosome Counts Database (CCDB) - a community resource of plant chromosome numbers. New Phytologist 206(1): 19–26. https://doi.org/10.1111/nph.13191

Roa F, Guerra M (2012) Distribution of 45S rDNA sites in chromosomes of plants: structural and evolutionary implications. BMC Evolutionary Biology 12 225. https://doi.org/10.1186/1471-2148-12-225

Roa F, Guerra M (2015) Non-Random Distribution of 5S rDNA Sites and Its Association with 45S rDNA in Plant Chromosomes. Cytogenetic and Genome Research 146(3): 243–249. https://doi.org/10.1159/000440930

Sano SM, Pedrosa de Almeida S, Ribeiro J (2008) Cerrado: ecologia e flora. Embrapa Informação Tecnológica: Planaltina, DF: Embrapa Cerrados.

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9(7): 671–675. https://doi.org/10.1038/nmeth.2089

Schwarzacher T, Ambros P, Schweizer D (1980) Application of Giemsa banding to orchid karyotype analysis. Plant Systematics and Evolution 134(3–4): 293–297. https://doi.org/10.1007/BF00986805

Silveira RM, Machado RM, Forni-Martins ER, Verola CF, Costa IR (2016) Environmental variations drive polyploid evolution in neotropical Eugenia species (Myrtaceae). Genet Mol Res. https://doi.org/10.4238/gmr15048842

Souza UJB de, Telles MP de C, Diniz-Filho JAF (2016) Tendências da literatura científica sobre genética de populações de plantas do Cerrado. Hoehnea 43(3): 461–477. https://doi.org/10.1590/2236-8906-06/2016

Spera SA, Galford GL, Coe MT, Macedo MN, Mustard JF (2016) Land-use change affects water recycling in Brazil's last agricultural frontier. Global Change Biology 22(10): 3405–3413. https://doi.org/10.1111/gcb.13298

Suda J, Meyerson LA, Leitch IJ, Pyšek P (2015) The hidden side of plant invasions: The role of genome size. New Phytologist 205(3): 994–1007. https://doi.org/10.1111/nph.13107

Vieira R, Queiroz Á, Morais L, Barão A, Mello-Sampayo T, Viegas W (1990) Genetic control of 1R nucleolus organizer region expression in the presence of wheat genomes. Genome 33(5): 713–718. https://doi.org/10.1139/g90-107

Warnes GR, Bolker B, Lumley T (2015) gtools: Various R Programming Tools. https://cran.r-project.org/package=gtools [accessed 1. June 2016]

Wickham H (2009) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York

Wickham H (2015a) plyr: Tools for Splitting, Applying and Combining Data. https://cran.r-project.org/package=plyr [accessed 1. June 2016]

Wickham H (2015b) stringr: Simple, Consistent Wrappers for Common String Operations. https://cran.r-project.org/package=stringr [accessed 1. June 2016]

Wickham H (2016) scales: Scale Functions for Visualization. https://cran.r-project.org/package=scales [accessed 1. June 2016]

Wickham H, Francois R (2016) dplyr: A Grammar of Data Manipulation. https://cran.r-project.org/package=dplyr [accessed 1. June 2016]

Xie Y (2015) DT: A Wrapper of the JavaScript Library "DataTables." https://cran.r-project.org/package=DT [accessed 1. June 2016]

Supplementary material 1

Summary statistics for genera
Authors: Fernando Roa, Mariana Pires de Campos Telles
Data type: cytogenetic attributes
Explanation note: more details in http://cyto.shinyapps.io/cerrado

Supplementary material 2

Summary statistics for families
Authors: Fernando Roa, Mariana Pires de Campos Telles
Data type: cytogenetic attributes
Explanation note: more details in http://cyto.shinyapps.io/cerrado

Supplementary material 3

Summary statistics for major clades
Authors: Fernando Roa, Mariana Pires de Campos Telles
Data type: cytogenetic attributes
Explanation note: more details in http://cyto.shinyapps.io/cerrado

Supplementary material 4

Authors with more publications of cytogenetic data of *Cerrado* plants per decade
Authors: Fernando Roa, Mariana Pires de Campos Telles
Data type: affiliation and taxa – categorical data table
Explanation note: more details in http://cyto.shinyapps.io/cerrado

Supplementary material 5

Figure S1. Total chromosome length for *Cerrado angiosperms*
Authors: Fernando Roa, Mariana Pires de Campos Telles
Data type: Histogram
Explanation note: See text for details.

Supplementary material 6

Figure S2. Total chromosome area for *Cerrado angiosperms*
Authors: Fernando Roa, Mariana Pires de Campos Telles
Data type: Histogram
Explanation note: See text for details.

CompCytogen 11(2): 299–307 (2017)
doi: 10.3897/CompCytogen.11i2.11942
http://compcytogen.pensoft.net

SHORT COMMUNICATION

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Chromosomal stasis in distinct families of marine Percomorpharia from South Atlantic

Fabilene Gomes Paim[1], Leandro Aragão da Hora Almeida[1], Paulo Roberto Antunes de Mello Affonso[1], Patrícia Elda Sobrinho-Scudeler[2], Claudio Oliveira[2], Débora Diniz[1]

1 *Departamento de Ciências Biológicas, Universidade Estadual do Sudoeste da Bahia (UESB), Jequié, Bahia, Brasil* **2** *Laboratório de Biologia e Genética de Peixes, Instituto de Biociências de Botucatu, UNESP, Botucatu, SP, Brasil*

Corresponding author: *Fabilene Gomes Paim* (fabillene@yahoo.com.br)

Academic editor: *E. Krysanov* | Received 26 January 2017 | Accepted 28 March 2017 | Published 4 May 2017

http://zoobank.org/F4B2AB9C-FECD-49EF-9D74-1209F1DABACE

Citation: Paim FG, Almeida LAH, Affonso PRAM, Sobrinho-Scudeler PE, Oliveira C, Diniz D (2017) Chromosomal stasis in distinct families of marine Percomorpharia from South Atlantic. Comparative Cytogenetics 11(2): 299–307. https://doi.org/10.3897/CompCytogen.11(2).11942

Abstract

The weakness of physical barriers in the marine environment and the dispersal potential of fish populations have been invoked as explanations of the apparent karyotype stasis of marine Percomorpha, but several taxa remain poorly studied cytogenetically. To increase the chromosomal data in this fish group, we analyzed cytogenetically three widespread Atlantic species from distinct families: *Chaetodipterus faber* Broussonet, 1782 (Ephippidae), *Lutjanus synagris* Linnaeus, 1758 (Lutjanidae) and *Rypticus randalli* Courtenay, 1967 (Serranidae). The three species shared a karyotype composed of 2n=48 acrocentric chromosomes, single nucleolus organizer regions (NORs) and reduced amounts of centromeric heterochromatin. A single NOR-bearing pair was identified in all species by physical mapping of 18S rDNA while non-syntenic 5S rRNA genes were located at centromeric region of a single pair. The similar karyotypic macrostructure observed in unrelated groups of Percomorpharia reinforces the conservative karyoevolution of marine teleosteans. Nonetheless, the species could be differentiated based on the pair bearing ribosomal cistrons, revealing the importance of microstructural analyses in species with symmetric and stable karyotypes.

Keywords
Cytotaxonomy, Ephippiformes, ribosomal genes, South Atlantic

Introduction

Perciformes have long been regarded as the largest order of vertebrates with nearly 10.000 species, 1540 genera, and 160 families, most of them inhabiting the marine environment (Nelson 2006, Helfman et al. 2009). Recently, robust molecular studies resolved their phylogenetic uncertainties by placing this and other Percomorpha representatives into 11 orders within a new supraordinal group called Percomorpharia, even though Perciformes remained as the most species-rich order (Betancur et al. 2013). Nonetheless, in spite of their ecological and evolutionary relevance, the marine representatives from this large fish group remain poorly studied from a cytogenetic viewpoint (Galetti et al. 2006) when compared to typical freshwater families (Balen et al. 2013, Cardoso et al. 2013, Gouveia et al. 2013).

In general, chromosomal studies in marine Percomorpharia reveal stable karyotypes composed of 2n=48 and a predominance of acrocentric pairs. Indeed, the presence of 24 acrocentric pairs is shared by several species from distinct families of Perciformes (Affonso et al. 2001, Accioly and Molina 2007, 2008, Cipriano et al. 2008, Motta Neto et al. 2011a, 2011b, 2012, Molina et al. 2012, Molina et al. 2013, Costa et al. 2016). This pattern raises some intriguing questions: (1) how could such a morphologically diversified group evolve without significant chromosomal changes (Brum 1996, Galetti et al. 2000, Molina 2007)? (2) What are the advantages (if any) of maintaining stable karyotypes?

One of the hypotheses invoked to explain the conserved karyoevolution of this fish group refers to their biological traits, such as the absence or fragility of physical barriers in oceans that favor the connectivity among populations, wide range of most species and chromosomal or genomic peculiarities (Molina 2007). In fact, freshwater families of Percomorpha, like Cichlidae, are characterized by higher karyotype variation than marine groups, corroborating the role of allopatric evolution in the process of chromosome differentiation (Brum and Galetti 1997, Feldberg et al. 2003).

On the other hand, most families of marine Percomorpharia have been divided into two groups based on the rate of karyotype changes, comprising families of high karyotype stability or with moderate rates of karyoevolution (Molina et al. 2014). Nonetheless, several species and families lack basic cytogenetic information and refined analyses of chromosomal microstructure are particularly rare in marine fish, thus restraining evolutionary inferences and the extent of their conservative karyoevolution.

To test the corollary that the high dispersal and gene flow associated with the weakness of geographic barriers accounts for the chromosomal stasis in marine Percomorpharia groups, we analyzed cytogenetically three widespread Atlantic species from distinct families: *Chaetodipterus faber* (Ephippidae), *Lutjanus synagris* (Lutjanidae) and *Rypticus randalli* (Serranidae). Besides inferring their karyoevolutionary pathways, we provided the first cytogenetic report in *C. faber* and *R. randalli*.

Methods

The specimens of *Chaetodipterus faber* (N=7, 1♀, 6 unidentified sex), *Lutjanus synagris* (N=8, 4♀, 4 unidentified sex), and *Rypticus randalli* (N=10, 3♂, 2♀, 5 unidentified sex) were collected by gillnets and snorkeling in Camamu Bay and Boipeba Island, located on the coast of Bahia, northeastern Brazil, South Atlantic. The vouchers were deposited in the Laboratory of Genetics of Aquatic Organisms (LAGOA) from Universidade Estadual do Sudoeste da Bahia, in Jequié, Bahia.

After collection, the specimens were mitotically stimulated by inoculation of fungal antigens and kept in fish tanks for 24 to 72 h (Lee and Elder 1980). After euthanasia in iced water (Blessing et al. 2010), the anterior kidney was removed and used to obtain mitotic chromosomes (Netto et al. 2007, Blanco et al. 2012). These procedures were approved by the Committee of Animal Ethics (CEUA/UESB) from Universidade Estadual do Sudoeste da Bahia (71/2014).

The heterochromatin regions were visualized by C-banding (Sumner 1972) while active nucleolus organizer regions (NORs) were detected by silver nitrate staining (Howell and Black 1980). The sequences of 18S and 5S rRNA genes were mapped simultaneously onto chromosomes by double fluorescence *in situ* hybridization (FISH) with a stringency of 77% (Pinkel et al. 1986). The 18S and 5S ribosomal sequences were obtained via polymerase chain reaction (PCR) using samples of genomic DNA of *Moenkhausia sanctaefilomenae* and labeled with16-dUTP–biotin and digoxigenin-11-dUTP (Roche®), respectively. The signal detection was accomplished by using fluorescein isothiocyanate-avidin conjugate (Sigma-Aldrich®) for 18S and anti-digoxigenin-Rhodamine (Roche®) for 5S rDNA. The chromosomes were counterstained using 4'6-diamidino-2-phenylindole (DAPI) at 0.2 mg/mL in Vectashield Mounting Medium (Vector®).

The metaphase spreads were photographed using an epifluorescence microscope Olympus BX-51 equipped with the software ImagePro-Plus v. 6.2. (Media Cybernetics).The chromosomes were classified according to their arm ratio (Levan et al. 1964) and organized into pairs by decreasing size order in karyotypes.

Results

The species *C. faber*, *L. synagris*, and *R. randalli* share a modal diploid number of 2n = 48, composed exclusively of acrocentric chromosomes (Figure 1). The heterochromatin distribution is reduced, being located at centromeric or pericentromeric regions of most chromosomes in the three species (Figure 1A, B, C). Particularly, *C. faber* showed conspicuous heterochromatic blocks in pair 3, being coincident with NORs (Figure 1A, box).

Single NORs were invariably detected, but located at distinct pairs according to each species (Figure 1A, 1B, 1C, Box). The NORs in *C. faber* were located at interstitial position on long arms of pair 3 (Figure 1A, box). On the other hand, the NOR-bearing pair corresponds to the 23rd pair in *L. synagris,* with marks at interstitial region close to centromeres, in agreement with secondary constrictions revealed by Giemsa-staining (Figure 1B, box).

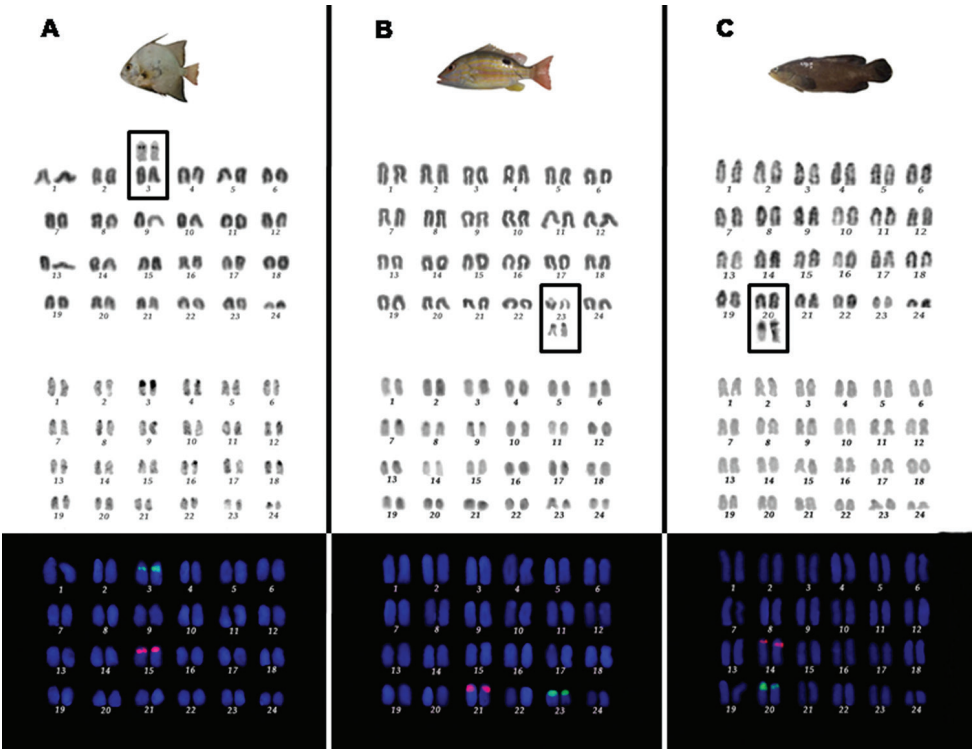


Figure 1. Karyotypes of *Chaetodipterus faber* (**A**), *Lutjanus synagris* (**B**), and *Rypticus randalli* (**C**) with 2n=48 acrocentric chromosomes after conventional Giemsa-staining (top), C-banding (center) and double FISH with 18S (green signals) and 5S (magenta signals) rDNA probes (bottom). In boxes, the pairs bearing nucleolus organizer regions after silver nitrate staining (Ag-NORs).

In *R. randalli*, the NORs were detected at pericentromeric region of pair 20, being characterized by size heteromorphism between homologous (Figure 1C, box).

The 18S rDNA sites were located at interstitial positions on the long arms of pairs 3, 20, and 23 in *C. faber*, *R. randalli*, and *L. synagris*, respectively. Size differences in the 18S rDNA clusters between homologs were observed in *C. faber* and *R. randalli*, as also revealed by silver nitrate staining (Figure 1A, 1B, 1C). The 5S rDNA sequences were mapped at pericentromeric region on long arms of all studied species, corresponding to the pairs 15 in *C. faber*, 21 in *L. synagris* and 14 in *R. randalli* (Figure 1A, 1B, 1C).

Discussion

The three species studied in the present work shared a karyotype composed of 24 pairs of acrocentric chromosomes, regarded as a plesiomorphic feature for Perciformes sensu Nelson 2006 (Brum 1996, Galetti et al. 2006, Molina 2007, Arai 2011) in spite of the derived position of some representatives in phylogenetic studies. Indeed, according to

the recent classification of bony fish, *C. faber* (Ephippidae) would belong to a distinct order (Ephippiformes) while Lutjanidae has been placed apart from other Perciformes families (e.g. Serranidae) within Percomorpharia (Betancur et al. 2013). Indeed, this symplesiomorphic karyotype has been commonly reported in serranids (groupers and allies) (Molina et al. 2002), just like herein described for *R. randalli*.

Likewise, lutjanids (snappers) from the Brazilian coast invariably present 2n=48 acrocentric chromosomes (Rocha and Molina 2008), as corroborated by our data in *L. synagris*. However, cytogenetic studies in Caribbean populations of *L. synagris* revealed an additional karyomorph with 2n=47 (1 metacentric and 46 acrocentric chromosomes), characterizing a polymorphic condition (Nirchio et al. 2008).

The karyotypic results in *C. faber* represent the first cytogenetic data in the order Ephippiformes (Betancur et al. 2013), which constrains inferences about chromosomal evolution in this group. Nonetheless, the karyotype macrostructure of this species follows the common trend observed in most Percomorpharia groups (e.g. Haemulidae, Scianidae, Lutjanidae and Serranidae) (Nirchio et al. 2014).

Besides the role of dispersal and formation of large populations (Molina 2007), some authors have inferred that speciation driven mainly by ecological features rather than by genetic isolation *per se* could result in a high number of species with similar karyotypes, as proposed for Haemulidae and Lutjanidae (Rocha and Molina 2008, Motta Neto et al. 2012). Moreover, intrinsic genome features could favor a conserved karyoevolution in these marine fish families. In common, most of studied species with basal karyotypes are poor in heterochromatin content and other repetitive sequences, which have been associated with the dynamics and rates of chromosomal changes (Molina 2007, Costa et al. 2013). However, detailed studies of karyotype microstructure are scarce for most marine fish species. Thus, microstructural chromosomal changes not affecting the number and morphology of chromosomes could remain undetected, misleading to the apparent chromosomal stability in Perciformes and allies (Nirchio et al. 2014). Therefore, chromosomal studies including banding methods and mapping of specific DNA sequences, as carried out in the present study, are particularly important to infer the karyotype structure of Percomorpharia and the evolutionary forces that could determine interspecific variation in spite of the conservativeness of karyotype macrostructure.

The nucleolus organizer regions (NORs) are considered a highly informative cytogenetic marker in teleosteans (Gornung 2008). The presence of single NORs at pericentromeric region is considered the plesiomorphic condition for several families in Percomorpharia, particularly those characterized by species with 2n=48a (Affonso et al. 2001, Motta Neto et al. 2011b, Molina et al. 2013), as also supported by the present results of 18S rDNA FISH and silver nitrate staining. In the case of *L. synagris*, the pattern of distribution of ribosomal genes was similar to that previously described for other populations in Brazil (Costa et al. 2016). Similarly, NOR size heteromorphism between homologous chromosomes in other fish species bearing single 18S rDNA clusters (Foresti et al. 1981) is widespread, as also detected in this report. Usually, this polymorphism is related to spontaneous duplications/deletions or unequal crossover between homologous (Affonso et al. 2002).

On the other hand, the NOR-bearing pair seems to differ according to each species in some families, like Lutjanidae (Costa et al. 2016) and Serranidae (Molina et al. 2002). Accordingly, in spite of sharing the same karyotype macrostructure and the number of 18S rDNA sites, the three species herein studied could be distinguished by the NOR-bearing pair (3 in *C. faber*, 23 in *L. synagris*, and 20 in *R. randalli*). Even though the precise establishment of pairs is hindered by the subtle size differences of acrocentric chromosomes, thereby being susceptible to some degree of subjectivity, the NOR-bearing pairs in the species clearly belong to distinctive categories according to size, ranging from large (*C. faber*) to small (*L. synagris*) pairs.

The identification of 5S rRNA genes was also informative to the karyotypic analyses of studied species. As commonly reported in marine fish, particularly Perciformes (Motta Neto et al. 2011a, Martins et al. 2011, Molina et al. 2013), the 5S rDNA clusters were non-syntenic to NORs and located close to centromeres, revealing their independent evolution in relation to 18S rDNA. Apparently, this trend is widespread in Percomorpharia once it was identified in distinct orders according to the recent phylogenetic tree of teleosteans (Betancur et al. 2013). As observed for 18S rDNA, the pairs carrying 5S rDNA clusters also differed among each species suggesting they represent species-specific markers, even though the pairs were more similar in size (15 in *C. faber*, 21 in *L. synagris*, and 14 in *R. randalli*).

In conclusion, the present results highlight the extensive karyotype macrostructure stasis in marine Percomorpha, since several cytogenetic features were shared by three species from distinct families and groups within Percomorpharia, corroborating the hypothesis of conserved karyotype macrostructure in widespread marine species. However, the evolutionary dynamics of ribosomal genes seem to play a major role in the cytotaxonomy of marine fish, as pointed out in typical marine families with basal karyotypes like Lutjanidae (Costa et al. 2016). Therefore, the mapping of distinct classes of repetitive DNA is highly recommended to provide a reliable scenario about the chromosomal evolution of groups with apparent stable karyotypes, as Perciformes and their allies.

Acknowledgments

The authors are grateful to FAPESB (PNE0019/2011, APP0064/2011) and "INCT de Ambientes Marinhos Tropicais" (SICONV 762228/2011) for the financial support, and ICMBIO/SISBIO for authorizing the collection of studied species (licenses 31360-1 and 27027-2).

References

Accioly IV, Molina WF (2008) Cytogenetic studies in Brazilian marine Sciaenidae and Sparidae fishes (Perciformes). Genetics and Molecular Research 7(2): 358–370. https://doi.org/10.4238/vol7-2gmr427

Accioly IV, Molina WF (2007) Contribuição à citogenética dos gêneros *Pomadasys* e *Anisotremus* (Haemuldae, Perciformes). PublIca III: 36–44. http://periodicos.ufrn.br/publica/article/view/109/105

Affonso PRAM, Guedes W, Pauls E, Galetti Jr. PM (2001) Cytogenetic Analysis of Coral Reef Fishes from Brazil (Families Pomacanthidae and Chaetodontidae). Cytologia 66: 379–384. http://doi.org/10.1508/cytologia.66.379

Affonso PRAM, Guedes W, Pauls E, Galetti Jr. PM (2002) Close karyotypical relationship between two species of marine angelfishes from South Atlantic: *Pomacanthus arcuatus* and *P. paru* (Perciformes, Pomacanthidae).Caryologia 55(4): 323–329. http://dx.doi.org/10.1080/00087114.2002.10797883

Arai R (2011) Fish Karyotypes: A Check List. Springer, Japan, 340 pp. https://doi.org/10.1007/978-4-431-53877-6

Blanco DR, Bertollo LAC, Lui RL, Vicari MR (2012) A new technique for obtaining mitotic chromosome spreads from fishes in the field. Journal of fish biology 81(1): 351–7. https://doi.org/10.1111/j.1095-8649.2012.03325.x

Balen RE, Noleto RB, Vicari MR, Artoni RF, Cestari MM (2013) Comparative cytogenetics among populations of *Hollandichthys multifasciatus* (Teleostei: Characidae). Zoological science 30(2): 105–9. http://dx.doi.org/10.2108/zsj.30.105

Betancur RR, Broughton RE, Wiley EO, Carpenter K, López JA, Li C (2013) The tree of life and a new Classification of bony fishes. PLOS Currents Tree of Life, 49 pp. https://doi.org/10.1371/currents.tol.53ba26640df0ccaee75bb165c8c26288

Blessing JJ, Marshall JC, Balcombe SR (2010) Humane killing of fishes for scientific research: a comparison of two methods. Journal of Fish Biology 76: 2571–2577. https://doi.org/10.1111/j.1095-8649.2010.02633.x

Brum MJI (1996) Cytogenetics studies of Brazilian marine fish. Brazilian Journal of Genetics 19(3): 421–427.

Brum MJI, Galetti Jr. PM (1997) Teleostei ground plan karyotype. Journal of Computational Biology 2: 91–102.

Cardoso AL, Sales KAH, Nagamachi CY, Pieczarka JC, Noronha RCR (2013) Comparative cytogenetics of two species of genus *Scobinancistrus* (Siluriformes, Loricariidae, Ancistrini) from the Xingu River, Brazil. Comparative cytogenetics 7(1): 43–51. https://doi.org/10.3897/CompCytogen.v7i1.4128

Cipriano RR, Fenocchio AS, Artoni RF (2008) Chromosomal Studies of Five Species of the Marine Fishes From the Paranaguá Bay and the Karyotypic Diversity in the Marine Teleostei of the Brazilian Coast. Brazilian Archives of Biology and Technology 51(April): 303–314. http://dx.doi.org/10.1590/S1516-89132008000200010

Costa GWWF, Cioffi MB, Bertollo LAC, Molina WF (2016) The Evolutionary Dynamics of Ribosomal Genes, Histone H3, and Transposable Rex Elements in the Genome of Atlantic Snappers. Journal of Heredity 107(2): 173–180. https://doi.org/10.1093/jhered/esv136

Costa GWWF, Cioffi MB, Bertollo LAC, Molina WF (2013) Transposable elements in fish chromosomes: A Study in the marine Cobia species. Cytogenetic Genome Research 1–7. https://doi.org/10.1159/000354309

Feldberg E, Porto JIR, Bertollo LAC (2003) Chromosomal changes and adaptation of cichlid fishes during evolution. In:Val AL, Kapoor BG (Eds) Fish Adaptation. IBH & Oxford, New Dehli & New York, 287–310.

Foresti F, Almeida–Toledo LF, Toledo–Filho AS (1981) Polymorphic nature of nucleolus organizer regions in fishes. Cytogenet Cell Genetics 31: 137–144. https://doi.org/10.1159/000131639

Galetti Jr. PM, Aguilar CT, Molina WF (2000) An overview of marine fish cytogenetics. Hydrobiologia 420(1): 55–62. https://doi.org/10.1023/A:1003977418900

Galetti Jr. PM, Molina WF, Affonso PRAM, Aguilar CT (2006) Assessing genetic diversity of Brazilian reef fishes by chromosomal and DNA markers. Genetica 126: 161–177. https://doi.org/10.1007/s10709-005-1446-z

Gouveia JG, Moraes VPO, Pires LB, Rosa R, Dias AL (2013) Comparative cytogenetics between two species of the family Pseudopimelodidae (Siluriformes): occurrence of natural triploidy and supernumerary chromosomes. Cytotechnology 67(2): 215–222. https://doi.org/10.1007/s10616-013-9676-x

Gornung E (2013) Twenty years of physical mapping of major ribosomal RNA genes across the teleosts: A review of research. Cytogenetic and genome research 141(2–3): 90–102. https://doi.org/10.1159/000354832

Helfman GS, Collette BB, Facey DE, Bowen BW (2009) The Diversity of Fishes. Wiley-Blackwel.

Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer region with protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. https://doi.org/10.1007/BF01953855

Lee MR, Elder FFB (1980) Yeast stimulation of bone marrow mitosis for cytogenetic investigations. Cytogenetics Cell Genetics 26: 36–40. https://doi.org/10.1159/000131419

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52: 201-220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Martins C, Cabral-de-Mello DC, Valente GT, Mazzuchelli J, Oliveira SG (2011) Cytogenetic mapping and its contribution to the knowledge of animal genomes. In: Kevin VU (Ed.) Advances in Genetics Research, Vol. 4. Nova Science Publisher, Hauppauge, NY, 1–82.

Molina WF, Martinez PA, Bertollo LAC, Bidau CJ (2014) Preferential accumulation of sex and Bs chromosomes in biarmed karyotypes by meiotic drive and rates of chromosomal changes in fishes. Anais da Academia Brasileira de Ciências 86(4): 1801–1812. doi: http://dx.doi.org/10.1590/0001-3765201420130489

Molina WF, Costa GWWF, Soares RX, Affonso PRAM (2013) Extensive chromosome conservatism in Atlantic butterflyfishes, genus *Chaetodon* Linnaeus, 1758: Implications for the high hybridization success. Zoologischer Anzeiger – A Journal of Comparative Zoology 253(2): 137–142. http://dx.doi.org/10.1016/j.jcz.2013.10.001

Molina WF, Motta Neto CC, Sena DCS, Cioffi MB, Bertollo LAC (2012) Karyoevolutionary aspects of Atlantic hogfishes (Labridae-Bodianinae), with evidence of an atypical decondensed argentophilic heterochromatin. Marine genomics 6: 25–31. http://dx.doi.org/10.1016/j.margen.2012.01.001

Molina WF (2007) Chromosome changes and stasis in marine fish groups. In: Pisano E, Ozouf-Costaz C, Foresti F, Kapoor BG (Eds) Fish Cytogenetic. CRC Press, Boca Raton (FL), 69–110.

Molina WF, Maia-Lima FA, Affonso PRAM (2002) Divergence between karyotypical pattern and speciation events in Serranidae fish (Perciformes). Caryologia 55(4): 299–305. http://dx.doi.org/10.1080/00087114.2002.10797880

Motta Neto CC,Cioffi MB, Bertollo LAC, Molina WF (2011a) Extensive chromosomal homologies and evidence of karyotypic stasis in Atlantic grunts of the genus *Haemulon* (Perciformes). Journal of Experimental Marine Biology and Ecology 401(1–2): 75–79. http://dx.doi.org/10.1016/j.jembe.2011.02.044

Motta Neto CC,Cioffi MB, Bertollo LAC, Molina WF (2011b) Molecular cytogenetic analysis of Haemulidae fish (Perciformes): Evidence of evolutionary conservation. Journal of Experimental Marine Biology and Ecology 407(1): 97–100. http://dx.doi.org/10.1016/j.jembe.2011.07.014

Motta Neto CC, Lima-Filho PA, Araújo WC (2012) Differentiated evolutionary pathways in Haemulidae (Perciformes): karyotype stasis versus morphological differentiation. Reviews in Fish Biology and Fisheries 22(2): 457–465. https://doi.org/10.1007/s11160-011-9236-4

Nelson JS (2006) Fishes of the World. John Wiley & Sons, 624 pp.

Netto MRCB, Pauls E, Affonso PRAM (2007) A standard protocol for obtaining fish chromosomes under post-mortem conditions. Micron 38: 214–217. https://doi.org/10.1016/j.micron.2006.07.019

Nirchio M, Rondón R, Oliveira C (2008) Cytogenetic studies in three species of *Lutjanus* (Perciformes: Lutjanidae: Lutjaninae) from the Isla Margarita, Venezuela. Neotropical Ichthyology 6(1): 101–108. http://dx.doi.org/10.1590/S1679-62252008000100012

Nirchio M, Rossi AR, Foresti F, Oliveira C (2014) Chromosome evolution in fishes: a new challenging proposal from Neotropical species. Neotropical Ichthyology 12(4): 761–770. http://dx.doi.org/10.1590/1982-0224-20130008

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences 83: 2934–2938.

Rocha EC, Molina WF (2008) Cytogenetic analysis in western Atlantic snappers (Perciformes, Lutjanidae). Genetics and Molecular Biology 31(2): 461–467. http://dx.doi.org/10.1590/S1415-47572008000300011

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental CellResearch 75: 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

CompCytogen 11(2): 309–324 (2017)
doi: 10.3897/CompCytogen.v11i2.12070
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Comparative analysis of chromosomes in the Palaearctic bush-crickets of tribe Pholidopterini (Orthoptera, Tettigoniinae)

Elżbieta Warchałowska-Śliwa[1], Beata Grzywacz[1], Klaus-Gerhard Heller[2], Dragan P. Chobanov[3]

1 *Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Krakow, Poland* **2** *Grillenstieg 18, 39120 Magdeburg, Germany* **3** *Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel Boul., 1000 Sofia, Bulgaria*

Corresponding author: *Elżbieta Warchałowska-Śliwa* (warchalowska@isez.pan.krakow.pl)

Academic editor: *D. Cabral-de-Mello* | Received 2 February 2017 | Accepted 28 March 2017 | Published 5 May 2017

http://zoobank.org/8ACF60EB-121C-48BF-B953-E2CF353242F4

Citation: Warchałowska-Śliwa E, Grzywacz B, Heller K-G, Chobanov DP (2017) Comparative analysis of chromosomes in the Palaearctic bush-crickets of tribe Pholidopterini (Orthoptera, Tettigoniinae). Comparative Cytogenetics 11(2): 309–324. https://doi.org/10.3897/CompCytogen.v11i2.12070

Abstract

The present study focused on the evolution of the karyotype in four genera of the tribe Pholidopterini: *Eupholidoptera* Mařan, 1953, *Parapholidoptera* Mařan, 1953, *Pholidoptera* Wesmaël, 1838, *Uvarovistia* Mařan, 1953. Chromosomes were analyzed using fluorescence *in situ* hybridization (FISH) with 18S rDNA and $(TTAGG)_n$ telomeric probes, and classical techniques, such as C-banding, silver impregnation and fluorochrome DAPI/CMA_3 staining. Most species retained the ancestral diploid chromosome number 2n = 31 (male) or 32 (female), while some of the taxa, especially a group of species within genus *Pholidoptera*, evolved a reduced chromosome number 2n = 29. All species show the same sex determination system X0/XX. In some taxa, a pericentric inversion has changed the morphology of the ancestral acrocentric X chromosome to the biarmed X. The rDNA loci coincided with active NORs and C-band/CG-rich segments. A comparison of the location of the single rDNA/NOR in the genus *Pholidoptera* suggests that reduced chromosome number results from Robertsonian translocation between two pairs of autosomes, one carrying the rDNA/NOR. The results constitute a step towards better understanding of the chromosomal reorganization and evolution within the tribe Phaneropterini and the whole subfamily Tettigoniinae.

Keywords

Orthoptera, Pholidopterini, karyotype, FISH, 18S rDNA, telomeric DNA, NOR, C-banding, fluorochrome staining

Introduction

Tettigoniinae Krauss, 1902 is amongst the largest groups of Tettigoniidae (also known as the bush-crickets or the katydids) with over 500 species occurring over five continents with the exception of the equatorial and subequatorial climatic zones (Rentz and Colless 1990, Cigliano et al. 2017). The Tettigoniinae has been considered by different authors with different systematic rank or taxonomic composition, being treated also as a family (e.g. Kevan 1982, Heller 1988). Recently, it has mostly been regarded as a subfamily including 12 tribes (Cigliano et al. 2017). However, the present tribal composition and ranking have been a matter of diverse interpretation (e.g. Gorochov 1995, Massa and Fontana 2011).

Ramme (1951) first established the group "Pholidopterini" based mostly on a comparison of the male copulatory structures including the genera *Apholidoptera* Mařan, 1953, *Parapholidoptera* Mařan, 1953, *Pholidoptera* Wesmaël, 1838, *Uvarovistia* Mařan, 1953 (the latter validated according to the ICZN by Mařan 1953). Later, the genus *Exopholidoptera* has been added by Ünal (1998).

The diploid chromosome number (2n), chromosome morphology (FN), and type of sex determination systems of Tettigoniinae have been described for more than 100 species out of 35 genera. Most of the Old World species have 31 acrocentric chromosomes, but the few New World genera diverge from this standard karyotype with one or more pairs of metacentric autosomes and often metacentric X chromosome. Hence, the karyotypes in this group range from 23 to 33 in male (reviewed in Warchałowska-Śliwa 1998).

Molecular cytogenetic methods through mapping molecular markers such as repetitive or unique sequences on chromosomes have been successfully applied for interspecific comparative karyotype structure and evolution in insects, especially coleopterans (e.g. Cabral-de Mello et al. 2011a), lepidopterans (e.g. Nguyen et al. 2010, Vershinina et al. 2015) hemipterans (Kuznetsova et al, 2015, Maryańska-Nadachowska et al. 2016), and orthopterans (grasshoppers e.g. Cabrero et al. 2009, Cabral-de-Mello 2011b, Jetybayev et al. 2012, Neto et al. 2013). Recently, a series of works using advanced, such as fluorescence *in situ* hybridization (FISH), and conventional chromosome banding techniques, showed that the number and location of rDNA (NOR) and heterochromatin, respectively, can be useful markers for the studying the karyotypes evolution in tettigoniids, and for the identification of genus/species-specific patterns, namely in the subfamilies Phaneropterinae (e.g. Warchałowska-Śliwa et al. 2011, 2013a, Grzywacz et. al. 2011, 2014a,b), Saginae (Warchałowska-Śliwa et al. 2009), Hetrodinae (Warchałowska-Śliwa et al. 2015) and Bradyporinae (Warchałowska-Śliwa et al. 2013b). However, data on NORs in Tettiginiinae genomes are scarce (Warchałowska-Śliwa et al. 2005) and no mapping location of rDNA clusters and telomeric DNA have been done so far.

Until now, the karyotypes of 16 species/subspecies of Pholidopterini, occasionally examined using C-banding and NOR staining have been studied (Warchałowska-Śliwa et al. 2005). For the first time, we performed cytogenetic analysis of the chro-

mosome number of 27 Pholidopterini taxa belonging to four genera (*Eupholidoptera* Mařan, 1953, *Parapholidoptera* Mařan, 1953, *Pholidoptera* Wesmaël, 1838, *Uvarovistia* Mařan, 1953). We examined the number and distribution of rDNA clusters in 28 taxa by FISH with 18S rDNA and telomeric $(TTAGG)_n$ probes as well as by conventional methods with the aim to estimate the role of analyzed markers of understanding genome organization in this tribe.

Material and methods

A total of 74 specimens of the genera *Eupholidoptera*, *Parapholidoptera*, *Pholidoptera* and *Uvarovistia* (Pholidopterini), belonging to 38 species/subspecies were studied cytologically. Table 1 summarizes the geographic origin and cytogenetic-molecular data of the analyzed material (including data reported earlier). Testes and ovarioles were incubated in a hypotonic solution (0.9% sodium citrate), then fixed in ethanol - acetic acid (3:1) and squashed in a drop of 45% acetic acid. The cover slip was removed using the dry ice procedure. Slides were dehydrated and air dried. Chromosome preparation for the C-banding examination was carried out according to Sumner (1972). Constitutive heterochromatin was analyzed qualitatively by CMA_3 (chromomycin A_3) and DAPI (4,6-diamidino-2-phenylindole) staining according to Schweizer (1976). Silver nitrate staining for active nucleolus organizing regions (NORs) was achieved using the protocol of Warchałowska-Śliwa and Maryańska-Nadachowska (1992).

The best chromosome preparations were used for fluorescence *in situ* hybridization (FISH) with 18S ribosomal DNA (rDNA) and telomeric DNA $(TTAGG)_n$. FISH was carried out as described earlier in Warchałowska-Śliwa et al. (2009). For detection of rDNA clusters in chromosomes, a probe containing a fragment of orthopteran 18S rDNA labeled with biotin-16-dUTP was used. In order to study the organization of telomeric repeats $(TTAGG)_n$ in chromosomes, a DNA probe was generated by non-template PCR with Tel1 [5' GGT TAG GTT AGG TTA GGT TAG G 3'] and Tel2 [5' TAA CCT AAC CTA ACC TAA 3'] primers. Visualization of hybridized DNA labeled with biotin and digoxigenin was performed with sheep avidin-FITC conjugates and anti-digoxigenin-rhodamine, respectively. The chromosomes were counterstained with DAPI solution under a cover glass. Chromosomes were studied with a Nikon Eclipse 400 microscope with a CCD DS-U1 camera and NIS-Elements BR2. For each individual (analyzed for the first time) at least five oogonial/spermatogonial mitotic metaphase and 15 meiotic divisions (in male) were examined.

Results

Table 1 shows the chromosome number (2n) and morphology of chromosomes (Fundamental Number = FN, the number of chromosome arms including of the X chromosome) of the Pholidopterini species by combining new data with previously published

Table 1. Pholidopterini taxa: collection sites, comparison of the number and morphology of chromosomes, distribution of rDNA cluster and NOR.

Species	Collection sites	Geographical coordinates	No.	2n, FN	References	rDNA-NOR
Eupholidoptera *anatolica* (Ramme, 1930)	TR: Antalya, Termessos	36°58'N, 30°30'E	2m	31,31	Warchałowska-Śliwa et al. 2005	no
Eupholidoptera annulipes (Brunner von Wattenwyl, 1882)	TR: Mersin, below Güzeloluk	36°44'N, 34°9'E	2m	31,31	Warchałowska-Śliwa et al. 2005	no
Eupholidoptera astyla (Ramme, 1927)	GR: 1) Crete, Rethimni, Mt. Ida, spring of Skaronero, above Kamares 2) Crete, Rethimini, Skaleta	1) 35°10'N, 24°48'E 2) 35°24'N, 24°37'E	1m 1m	31,31	this study	3/4i-3/4i
Eupholidoptera epirotica (Ramme, 1927)	GR: Aitolia-Akarnania (Central Greece), Mt. Karnania above Thirion	38°48'N, 20°58'E	6m, 1f	31,31	Warchałowska-Śliwa et al. 2005	no-3/4i
Eupholidoptera chabrieri garganica (La Greca, 1959)	GR: Kerkyra, near Agios Spiridon	39°48'N, 19°50'E	3m	31,31	Warchałowska-Śliwa et al. 2005	no
Eupholidoptera giuliae Massa, 1999	GR: Crete, Chania, Chora Sfakion	35°12'N, 24°8'E	3m	31,31	this study	no
Eupholidoptera icariensis Willemse, 1980	GR: Samos, Ikaria	37°36'N, 26°9'E	1m	31,31	Warchałowska-Śliwa et al. 2005	no
Eupholidoptera karabagi Salman, 1983	GR: Antalya, Termessos	36°58'N, 30°30'E	3m	31,31	Warchałowska-Śliwa et al. 2005	no-3/4i
Eupholidoptera krueperi (Ramme, 1930)	TR: Antalya, Kozdere Köprü	36°36'N, 30°31'E	1m	31,31	this study	3/4i-3/4i
Eupholidoptera latens Willemse & Kruseman, 1976)	GR: Crete, Chania, Rodopos	35°33'N, 23°45'E	2m	31,31	this study	no
Eupholidoptera megastyla (Ramme, 1939)	GR: 1) Myrsini, Pd Peloponez 2) Arta, Tsoumerka Mt.	39°25'N, 21°10'E	4 m 2m	31,31	this study Warchałowska-Śliwa et al. 2005	3/4i-3/4i no
Eupholidoptera mersinensis Salman, 1983	TR: 1) Mersin, Fýndýkpýnarý 2) Mersin, below Güzeloluk (near Köserlý)	1) 36°50'N, 34°20'E 2) 36°45'N, 34°7'E	1m 1m	31,31	Warchałowska-Śliwa et al. 2005	no 3/4i
Eupholidoptera prasina (Brunner von Wattenwyl, 1882)	TR: Mersin, Kasyayla (above Anamur)	36°15'N, 32°54'E	5m	31,31	Warchałowska-Śliwa et al. 2005	no
Eupholidoptera schmidti (Fieber, 1861)	MK: 1) Gorna Belica 2) Korab Mt.	1) 41°13'N, 20°33'E 2) 41°41'N, 20°39'E	2m 1m	31,31	this study	3/4i-3/4i
Eupholidoptera smyrnensis (Brunner von Wattenwyl, 1882)	1) TR: N Sindrigi 2) BG: Melnik	1) 39°18'N, 28°12'E 2) 41°31'N, 23°23'E	1m 2m	31,31	this study	3/4i-3/4i

Species	Collection sites	Geographical coordinates	No.	2n, FN	References	rDNA-NOR
Eupholidoptera tauricola (Ramme, 1930)	TR: Mersin, below Güzeloluk	36°44'N, 34°9'E	2m	31,31	Warchałowska-Śliwa et al. 2005	no
Eupholidoptera tahtalica (Uvarov, 1949)	TR: Aspendos close Antalya	36°56'N, 31°10'E	1m	31,31	this study	3/4i-3/4i
Parapholidoptera *castaneoviridis* (Brunner von Wattenwyl, 1882)	BG: 1) Stara Planina Mts, Karandila site 2) Strandzha Mts, Kovach place	1) 42°43'N, 26°22'E 2) 42°05'N, 27°25'E	2m 1m	31,32	this study	3/4i-3/4i
Parapholidoptera cf. *belen* Ünal, 2006	TR: Yiglica	40°58'N, 31°34'E	1m	31,31	this study	3/4i-3/4i
Parapholidoptera distincta (Uvarov, 1921)	GE: E Nailevi	41°38'N, 42°35'E	2m	31,31 +B	this study	3/4i-3/4i
Parapholidoptera noxia (Ramme, 1930)	GE: 1) Gombori range 2) SW of Gora vill.	1) 41°52'N, 45°18'E 2) 41°14'N, 44°17'E	1m 2m	31,31	this study	3/4i-3/4i
Parapholidoptera grandis (Karabag, 1952)	TR: E Ibradi	37°03'N, 31°44'E	4m	31,32	this study	3/4i-3/4i*
Parapholidoptera signata (Brunner von Wattenwyl, 1861)	TR: Mersin, below Güzeloluk	36°44'N, 34°9'E	1m	31,31	Warchałowska-Śliwa et al. 2005	no
Parapholidoptera cf. *signata*	TR: above Zara	39°37'N, 37°56'E	1m	31,31	this study	no
Parapholidoptera aff. *syriaca*	TR: Demre	36°27'N, 30°26'E	3m	29,31	this study	3/4i-3/4i*
Pholidoptera *dalmatica maritima/ebneri*	MO: Cetinje, Skader lake	42°23'N, 55°45'E	2m	31,31	this study	3/4i-3/4i
Pholidoptera fallax (Fischer, 1853)	1) BG: Veliko Tarnovo 2) MO: Cetinje, Skader lake	1) 43°05'N, 25°39'E 2) 42°23'N, 55°45'E	2m 2m	31,31	this study	3/4i-3/4i
Pholidoptera frivaldszkyi (Herman, 1871)	1) BG: Batak 2) BG: Iskar 3) GR: Drama, ca. 5 km north of Elatia	1) 43°05'N, 25°39'E 2) 41°57'N, 24°12'E 3) 41°30'N, 24°18'E	3m 1m 1m	31,31	this study Warchałowska-Śliwa et al. 2005	3/4i- 3/4i no
Pholidoptera griseoaptera Mařan, 1953	1) CR: Chetyr Dag 2) PL: Ojców National Park, Sąspowska valley	1) 44°44'N, 34°19'E 2) 50°15'N, 19°50'E	1m 6m	31,31	this study Warchałowska-Śliwa et al. 2005	3/4i-3/4i no-3/4i
Pholidoptera littoralis (Fieber, 1853)	BG: Belogradchik	43°38'N, 22°41'E	1m, 1f	31,31	this study	3/4i 3/4i
Pholidoptera pustulipes (Motschulsky, 1846)	CR: 1) Chetyr Dag 2) Karadag Reserve	1) 44°44'N, 34°19'E 2) 44°55'N, 35°12'E	1m 1m, 1f	31,31	this study	3/4i-3/4i*
Pholidoptera aptera aptera (Fabricius, 1793)	PL: Pieniny Mts, polana Walusiówka	49°25'N, 20°28'E	4m	29,31	Warchałowska-Śliwa et al. 2005	no-1p

Species	Collection sites	Geographical coordinates	No.	2n, FN	References	rDNA-NOR
Pholidoptera aptera bulgarica Mařan, 1953	BG: E Rhodopi Mts, Shturets vill	41°37'N, 25°32'E	1m	29,31	this study	1p – 1p
Pholidoptera cf. *aptera bulgarica*	MK: Strumica, Cham Chiflik	41°25'N, 22°38'E	1f	30,32	this study	1p-1p
Pholidoptera aptera karnyi Ebner, 1908	BG: 1) Stara Planina Mts, Uzana place	1) 42°46'N, 25°14'E	2m 1m	29,31	this study	1p-1p
	2) Lyulin Mt.	2) 42°39'N, 23°12'E			Warchałowska-Śliwa 1988	no
Pholidoptera brevipes Ramme, 1939	BG: 1) Sakar Mt., Matochina vill;	1) 41°51'N, 26°33'E	1m	29,32	this study	3/4i-3/4i
	2) Gorska Polyana vill.	2) 42°06'N, 26°58'E	2m			
Pholidoptera aff. *brevipes*	TR: Coroglu	40°53'N, 32°57'E	1m	29,32	this study	3/4i-3/4i
Pholidoptera macedonica Ramme, 1928	1) AL: Galichica Mt., Pilcina	1) 40°54'N, 20°52'E	2m		this study	1p-1p
	2) MK: Nidzhe Mt.	2) 41°00'N, 21°41'E	2f	29,31		
	3) MK: Gorna Belica	3) 41°13'N, 20°33'E	1m			
	4) GR: Drama, near Elatia	4) 41°30'N, 24°18'E	2m		Warchałowska-Śliwa et al. 2005	no
Pholidoptera rhodopensis Maran, 1953	BG: 1) near Goce Delcev	1) 41°47'N, 23°33'E	2m	29,31	this study	1p-1p
	2) Pirin Mt. above Bansko	2) 41°46'N, 23°27'E	1m			
Pholidoptera stankoi Karaman, 1960	MK: Ribnicka Riv	41°42'N, 20°39'E	3m	29,31	this study	1p-1p
Uvarovistia *satunini* (Uvarov, 1934)	TR: 1) Tunceli-Mazgirt	1) 39°04'N, 39°34'E	2m	31,31	this study	4/5i 4/5i
	2) Yanıkcay	2) 38°15'N, 42°54'E	1m			

Note: AL = Albania, BG= Bulgaria, CR = Crimea, GE = Georgia, GR = Greece, MK = Macedonia, MO = Montenegro, TR = Turkey, PL = Poland. No = numbers of individuals studied, m = male and f = female; FN = fundamental number of chromosome arms in male (except female of *Pholidoptera* cf. *aptera bulgarica*); B = B chromosome; a slash between two numbers indicates imprecise identification of the pair of bivalents; *high or low signal of 18S rDNA probes between homologous chromosomes.

information (Warchałowska-Śliwa 1988, Warchałowska-Śliwa et al. 2005). All analyzed species show the same sex determination system X0 (male) and XX (female). All species of the genera *Eupholidoptera*, (Fig. 1a–c), most *Parapholidoptera* (Fig. 1d–l) (except *P.* aff. *syriaca*), six out of the 16 taxa of *Pholidoptera* (Fig. 2a-c) and one *Uvarovistia* species have 2n = 31 in male and 32 in female (Fig. 2h, i). Fifteen pairs of acrocentric autosomes can be subdivided into the three size groups: one long, five medium and nine short. On the other hand, in *Parapholidoptera* aff. *syriaca* (Fig. 1j–l) and in 10 out of 16 taxa of *Pholidoptera* (Fig. 2d–g), the complement is reduced to 2n = 29 (male) and 30 (female) as a result of one Robertsonian fusion (Warchałowska-Śliwa 1998). In this case, the bivalents may be classified according to size as one long submetacentric pair, four medium and nine short acrocentric pairs. Often, minor length differences in chromosome pairs cause problem with precise identification. In most Pholidopterini taxa, the X chromosome was acrocentric, except for the bi-armed X chromosomes in *Parapholidoptera grandis* (Karabag, 1952) (Fig. 1g–i) and *P. castaneoviridis* (Brunner von Wattenwyl, 1882) (not shown). B chromosome, a supernumerary element to the standard karyotype, was found in one *Parapholidoptera distincta* (Uvarov, 1921) male, being acrocentric and meiotically stable and similar in size to the short autosomes (Fig. 1f).

Cytogenetic maps of 18S rDNA were obtained for six *Eupholidoptera*, six *Parapholidoptera*, 15 *Pholidoptera* taxa and one *Uvarovistia* species. In each of the species, FISH revealed a single cluster of rDNA (per haploid genome), located on one autosome pair (Table 1). Its location was observed at mitotic metaphases or one bivalent from diakinesis to metaphase I. In all taxa with 31 (male) and 32 (female) chromosomes, 18S rDNA loci with different intensity signals were located in the interstitial area of the chromosomes – closer to their paracentromeric region (Figs 1a, b; 2a,c) or in the middle of probably the 3rd or 4th bivalent (Fig. 1g, j) or of 4/5th bivalent in *Uvarovistia satunini* (Uvarov, 1934) (Fig. 2h). On the other hand, in most taxa with 29/30 chromosomes, a high-intensity signal located in the paracentromeric region was found on the bi-armed first pair of autosomes (Fig. 2d, e); only in *Pholidoptera brevipes* Ramme, 1939 it appeared in the interstitial region in 3rd/4th bivalent (Fig. 2g).

Two species of *Parapholidoptera* and one *Pholidoptera* exhibited heteromorphism in the rDNA-FISH signal and NOR in terms of the size and presence/absence between homologous chromosomes (indicated with an asterisk in Table 1). In both *Parapholidoptera grandis* (Fig. 1g) and *Pholidoptera pustulipes* (Motschulsky, 1846) (Fig. 2b, c), one of the males analyzed showed size variation for the 18S rDNA clusters, whereas presence/absence heteromorphism was observed in *Parapholidoptera* prope *syriaca* (Fig. 1j, k). The signals produced by $(TTAGG)_n$ FISH occurred at the distal ends of each chromosome in most the taxa analyzed (invisible in some species, probably due to a technical error or to a low number of copies of telomeric repeats) (Figs 1g, j; 2h). These signals were not observed in the centromere region of bi-armed chromosomes.

Generally, regardless of the number of chromosomes in karyotype, after both C-band staining and fluorochrome double-staining, chromosome regions showed a similar pattern in analyzed species in terms of constitutive heterochromatin. Most species had heterochromatin blocks in the paracentromeric region with thin C-bands, very

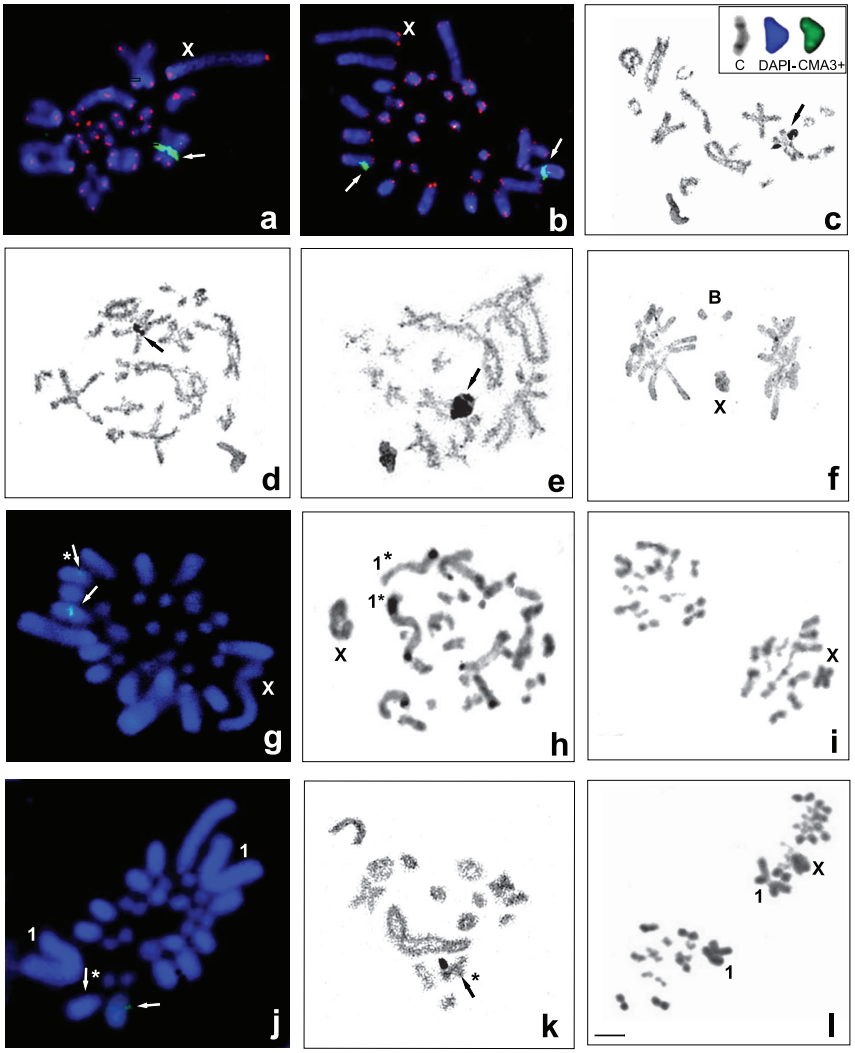


Figure 1. Examples of chromosome banding in different species of the tribes *Eupholidoptera* (**a–c**) and *Parapholidoptera* with 2n = 31 (**d–i**) and 2n = 29 (**j–l**) studied using different techniques. 18S rDNA FISH revealed a single interstitial cluster (per haploid genome) located in the 3/4 or 4/5 bivalent (**a, b, g, j**) and co-localized with the active NOR visualized by $AgNO_3$ staining (**c–e, k**). **a** *E. astyla,* diakinesis and **b** *E. megastyla*, spermatogonial metaphase with 18S rDNA loci (green, arrows) located close to the paracentromeric region of bivalent 3/4 and telomeric DNA probes (red) **c** *E. schmidti*, diakinesis with NOR (arrow) and selected C+, DAPI- and CMA_3+ bands located interstitially on 3/4 bivalent (in the right corner) **d** *P.* cf. *belen* and **e** *P. distincta*, diplotene, arrows indicate NOR located in the middle of bivalent 3/4 **f** *P. distincta*, anaphase I with B chromosomes **g, h, i** *P. grandis* **g** spermatogonial metaphase with the rDNA cluster with different size between homologous chromosomes (arrows) **h** heterochromatin heteromorphism in respect to the pattern of C-bands in the first autosome pair (1, asterisks) **i** two metaphase II with bi-armed X chromosome **j, k, l** *P.* aff. *syriaca* **j** spermatogonial metaphase, rDNA-FISH signal present/absent in homologous chromosomes (arrows and an asterisk) correspond to **k** NOR **l** two metaphase II with bi-armed first pair of autosomes (1) and acrocentric X. Bar = 10 μm.

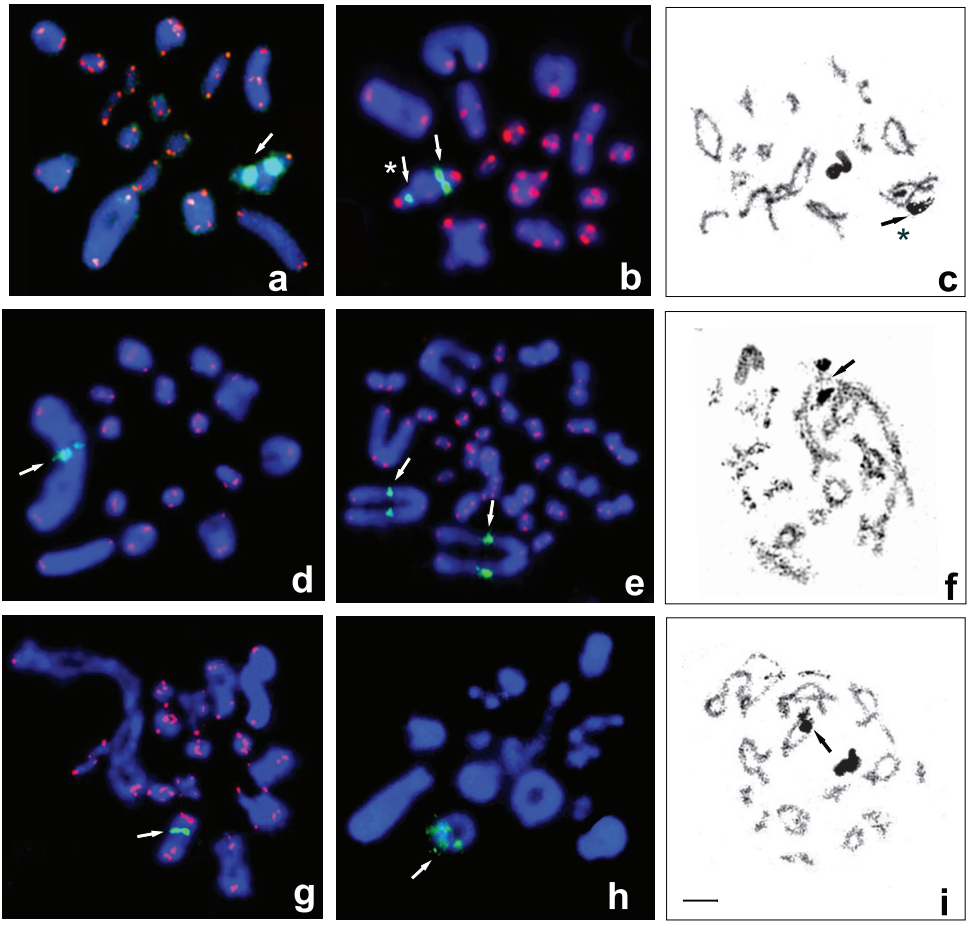


Figure 2. FISH using 18S rDNA (green) and telomeric TTAGG (red) probes on male (**a, b, d, g, h**) and female (**e**) karyotypes and silver staining (**c, f, i**). Diplotene of *Pholidoptera* (**a–g**) and *Uvarovistia* (**h, i**) species. White arrows point to rDNA clusters on the medium acrocentric pair or on the bi-armed first pair of autosomes. Black arrows indicate the active NOR co-localized with rDNA. **a** *P. fallax* and **b, c** *P. pustulipes* (2n = 31). Asterisks point to differences in size/strength of rDNA/NOR between homologous chromosomes **d–f** *P. macedonica* (2n = 29). Arrows indicate high-intensity rDNA signal and NOR located in the paracentromeric region of the bi-armed first pair of autosomes **g** *P. brevipes* (2n = 29). Arrows point 18S rDNA cluster on medium-sized bivalent **h, i** *U. satunini* (2n = 31). Bar = 10 μm.

weakly staining with DAPI and CMA_3 (CMA_3-) negative (not shown). Only in the first chromosome pair of *Parapholidoptera grandis*, thick C-bands in paracentromeric and distal regions were visualized with both DAPI-positive (AT-rich) and CMA_3-positive (GC-rich) signals (not shown). In this case, a heterochromatin heteromorphism in respect to the pattern of C-bands (Fig. 1h) and fluorochrome bands (not shown) was observed between individuals. However, in all analyzed Pholidopterini, thin or thick C-band, DAPI- and CMA_3+ band were located interstitially on the 3rd/4th (Fig. 1c)

or 4/5th bivalent in species with 2n = 31, 32 and in *Pholidoptera brevipes* with 2n = 29, as well as in the paracentromeric region on the long bi-armed autosome pair in species with 2n =29, 30 (not shown).

Discussion

Up to now, cytotaxonomic studies of the Palaearctic Tettigoniinae in more than 60 species out of 22 genera, including 30 species of 11 genera from Europe showed that the most of species had 31 acrocentric chromosomes. However, in *Pholidoptera macedonica* and *Ph. aptera*, the chromosome number is reduced to 29 with one submetacentric long pair (Warchałowska-Śliwa 1988, 1998, Warchałowska-Śliwa et al. 2005).

Representatives of four Pholidopterini genera examined in this study have two different male karyotypes including 31 or 29 chromosomes, respectively, and the same sex determination system. The diploid number 2n = 31 (male) of all species belonging to *Eupholidoptera* and *Uvarovistia* (only one species was analyzed) as well as to *Parapholidoptera* (excluding *Parapholidoptera* prope *syriaca*) corroborates previous studies, which revealed that most species of the Palaearctic Tettigoniinae were characterized by such basic/ancestral karyotype (e.g. for review see Warchałowska-Śliwa 1998). Additionally, a pericentric inversion modifying the position of the centromere has changed the morphology of the ancestral acrocentric X chromosome to the biarmed X in two *Parapholidoptera* and two *Pholidoptera* species. A similar type of translocation was reported in some other species of Tettigoniinae (Warchałowska-Śliwa 1998), Phaneropterinae (Warchałowska-Śliwa et al. 2011) and Bradyporinae (e.g. Warchałowska-Śliwa et al. 2013a). Further analysis is required to confirm this assumption of polymorphism of such chromosome changes.

The ancestral chromosome number is reduced to 2n = 29 in almost half of the analyzed *Pholidoptera* species and only one *Parapholidoptera* species (*P.* prope *syriaca*) as a result of one Robertsonian translocation (the first autosome pair in the set becomes submetacentric). This reduction appears to be more frequent in the chromosomal evolutionary history of the subfamilies Tettigoniinae and Bradyporinae (e.g. White 1973, Hewitt 1979, Warchałowska-Śliwa 1988, 1998, Grzywacz et al. 2017 in press).

B chromosomes are frequent in orthopterans, especially in superfamilies Pyrgomorphoidea, Grylloidea, Acridoidea, Tetrigoidea (e.g. Palestis et al. 2010) while rare and often unstable in Tettigonioidea, (e.g. Warchałowska-Śliwa et al. 2005, 2008, Hemp et al. 2010a, 2015b). The origin of B chromosome in *Parapholidoptera distincta* is currently not clearly known and needs a comparison of the DNA sequences shared by both autosomes and Bs.

In the Pholidopterini chromosomes described in this paper, one 18S rDNA FISH locus (per haploid genome) coincides with a single active NOR detected by Ag-NO_3 staining and with a C-band/CG-rich segment, independently from the number of chromosomes in the set. However, in analyzed species/subspecies two different patterns of the location of rDNA/NOR were observed. A single bivalent carrying 18S

rDNA clusters in the interstitial region on a medium-sized chromosome (3rd/4th pair) seems to be typical feature of the representatives of *Eupholidoptera*, some *Pholidoptera* and *Parapholidoptera* taxa with 31 chromosomes as well as *Parapholidoptera* prope *syriaca* with chromosome number reduced to 29 in male. The 18S rDNA location on the 4/5th pair of *Uvarovistia satunini* might represent a derived aspect. Additionally, some structural rearrangements, e.g. a small inversion, may have been involved in changes of the rDNA location in two *Parapholidoptera* species. By contrast, in seven species/subspecies with 29 chromosomes, one paracentromeric rDNA site, exhibited a unique rDNA distribution pattern in the long bi-armed autosome. The distribution of rDNA loci/active NORs show that a Robertsonian translocation between the first pair and medium sized pair of chromosome-bearing rDNA cluster (probably 3rd/4th) has reduced the chromosome number from 2n = 31 (FN=31, 32) to 29 (FN=31, 32).

The presence of interstitial rDNA loci on a single bivalent (acrocentric or bi-armed) has been observed in the same subfamily in the tribe Platycleidini (Grzywacz et al. 2017 in press) and in some Bradyporinae karyotypes where a reduction of the chromosome number as the result of tandem fusion or Robertsonian translocation was suggested (Warchałowska-Śliwa et al. 2013a). These observations show that frequently rDNA/NOR-bearing chromosomes take part in karyotype rearrangements. The rDNA of terminal NORs is considered to be involved in Robertsonian translocations and in this case either very small centric or acentric fragments may be eliminated during subsequent mitotic or meiotic divisions or be undetectable by microscopic visualization techniques (Lysák and Schubert 2013). The lack of interstitial $(TTAGG)_n$ sequences (tDNA-FISH signal) in the centromeric region of bi-armed chromosomes, observed in some Pholidopterini species, could probably be due to a loss of telomeric repeats during karyotype evolution.

The presence of paracentromeric 18S rDNA cluster on a single bivalent was previously observed in different size chromosomes of other tettigoniids: in European (e.g. Warchałowska-Śliwa et al. 2013b) and African (Hemp et al. 2010b, 2013, 2015a) Phaneropterinae likewise in European Saginae (Warchałowska-Śliwa et al. 2009) and Bradyporinae (Warchałowska-Śliwa et al. 2013a), as well as in some grasshoppers (e.g. Cabral-de-Mello et al. 2011b). Probably, 18S rDNA cluster in Pholidopterini (present results) and Platycleidini (Grzywacz et al. 2017 in press) is syntenic in the interstitial position on chromosome pair 3rd/4th in karyotype with 31 chromosomes. This localization suggests occurrence of chromosome reorganization that probably occurred in their common ancestor by inversion causing displacement of the 18S rDNA cluster from paracentromeric to an interstitial position.

Generally, the Pholidopterini are characterized by chromosomes with a small amount of constitutive heterochromatin in the paracentromeric region and interstitially located C-bands in a medium sized autosome. In most of species and genera belonging to European Tettigoniinae, thin paracentromeric C-bands were uniformly present in chromosomes, but the C-banding patterns and distribution of interstitial and telomeric heterochromatin are usually found to vary among genera and sometimes between species of one genus (e.g. Warchałowska-Śliwa et al. 2005). In most of analyzed spe-

cies only the interstitial region of the 3rd/4th autosome pair showed thin C-bands and bright CMA_3 (DAPI-negative) CG-rich segments (except the first chromosome pair of *Parapholidoptera grandis* with thick C-bands/DAPI+/CMA_3+ signals). Our results demonstrate a coincidence between the location of rDNA loci and active NOR and C/GC-rich heterochromatin regions, which indicates the presence of multiple repetitive DNA sequences. In a few Pholidopterini species, the size of the rDNA hybridization signals on homologous pairs of autosomes (Table 1; indicated by an asterisk) and the pattern of heterochromatin distribution have revealed size heteromorphism in the NOR and C-bands. Present results indicate different intensities of hybridization signals on the autosomes (3rd/4th) of *Parapholidoptera grandis* and *Pholidoptera pustulipes*, reaching an extreme case in *Parapholidoptera* prope *syriaca* (only one chromosome showed a hybridization signal). These differences, detected by e.g. Ag-NOR banding technique, suggested the occurrence of a polymorphism in the number of rDNA sequence copies. Similar heteromorphism has been observed in other tettigoniids (e.g. Warchałowska-Śliwa et al. 2013a,b, Grzywacz et al. 2014a,b) as a result of different mechanisms, i.e. homologous translocation, unequal crossing-over, homologous translocation or specific rearrangements of repetitive DNA families (e.g. Bressa et al. 2008, Cabrel-de-Mello et al. 2011b).

In conclusion, the results described in this paper demonstrate the usefulness of molecular techniques as tools for better understanding of chromosomal organization and evolutionary history in the tribe Pholidopterini. This study show that the karyotypes of the species analyzed have undergone evolution including changes in chromosome number and morphology by one Robertsonian translocation and sporadically inversion in the X chromosome. The location of the single rDNA/NOR coinciding with C-band/CG-rich segment in the genus *Pholidoptera* suggests that reduced chromosome number from 31 to 29 in male resulted from a Robertsonian translocation between two pairs of autosomes, one carrying the rDNA/NOR. Thus, FISH for identifying the location of 18S rDNA has proved to be a good marker for distinguishing species in this genus. On the other hand, the tendency of interstitial distribution of repeated DNA sequences may represent a cytogenetic marker for distinguishing some genera in Tettigoniinae. In contrast, interspecific autosomal differentiation has involved minor differences concerning the heterochromatin composition and distribution obtained by C-banding and fluorochrome staining. Furthermore, the additionally detected taxon-specific karyotypic differences in Pholidopterini need to be compared with phylogenetic data to get a clearer idea of their importance both for understanding the karyotype evolution and speciation within this group.

Acknowledgements

This work was supported by grant 2011/01/B/NZ8/01467 from the National Science Centre, Poland (B. Grzywacz) and project between the Bulgarian Academy of Sciences and the Polish Academy of Sciences (E. Warchałowska-Śliwa and D.P. Chobanov).

References

Bressa MJ, Franco MJ, Toscani MA, Papeschi AG (2008) Heterochromatin polymorphism in *Holhymenia rubiginosa* (Heteroptera: Coreidae). European Journal of Entomology 105: 65–72. https://doi.org/10.14411/eje.2008.009

Cabral-de-Mello DC, Martins C, Souza MJ, Moura RC (2011b) Cytogenetic mapping of 5S and 18S rDNA and H3 histone genes in 4 ancient Proscopiidae grasshopper species: contribution to understanding the evolutionary dynamics of multigene families. Cytogenetic and Genome Research 132: 89–93. https://doi.org/10.1159/000317476

Cabral-de-Mello DC, Oliveira SB, Moura RC, Martins C (2011a) Chromosomal organization of the 18S and 5S rRNAs and histone H3 genes in Scarabaeinae coleopterans: insights into the evolutionary dynamics of multigene families and heterochromatin. BMC Genetics 12: 88. https://doi.org/10.1186/1471-2156-12-88

Cabrero J, López-León MD, Teruel M, Camacho JPM (2009) Chromosome mapping of H3 and H4 histone gene clusters in 35 species of acridid grasshoppers. Chromosome Research 17: 397–404. https://doi.org/10.1007/s10577-009-9030-5

Cigliano MM, Braun H, Eades DC, Otte D (2017) Orthoptera Species File. Version 5.0/5.0. http://Orthoptera.SpeciesFile.org [accessed 14.01.2017]

Gorochov AV (1995) System and evolution of the suborder Ensifera (Orthoptera). Part I & II. Proceedings of the Zoological Institute, Russian Academy of Sciences 260: 1–224.

Grzywacz B, Chobanov DP, Maryańska-Nadachowska A, Karamysheva TV, Heller K-G, Warchałowska-Śliwa E (2014a) A comparative study of genome organization and inferences for the systematics of two large bushcricket genera of the tribe Barbitistini (Orthoptera: Tettigoniidae: Phaneropterinae). BMC Evolutionary Biology 14: 48. http://www.biomedcentral.com/1471-2148/14/48

Grzywacz B, Heller K-G, Chobanov DP, Warchałowska-Śliwa E (2017) Conventional and molecular chromosome study in the European genus *Parnassiana* Zeuner, 1941 (Orthoptera, Tettigoniinae, Platycleidini). Folia Biologica (Kraków) 65(1): 1–8. https://doi.org/10.3409/fb65_1.01.

Grzywacz B, Heller K-G, Lehmann AW, Warchałowska-Śliwa E, Lehmann GUC (2014b) Chromosomal diversification in the flightless Western Mediterranean bushcricket genus *Odontura* (Orthoptera: Tettigoniidae: Phaneropterinae) inferred from molecular data. Journal of Zoological Systematics and Evolutionary Research 52(2): 109–118. https://doi.org/10.1111/jzs.12046

Grzywacz B, Maryańska-Nadachowska A, Chobanov DP, Karamysheva TV, Warchałowska-Śliwa E (2011) Comparative analysis of the location of rDNA in the Palaearctic bushcricket genus *Isophya* (Orthoptera: Tettigoniidae: Phaneropterinae). European Journal of Entomology 108(4): 509–517. https://doi.org/10.14411/eje.2011.066

Heller K-G (1988) Bioakustik der Europäischen Laubheuschrecken. Ökologie in Forschung und Anwendung 1. Josef Margraf.

Hemp C, Heller K-G, Warchałowska-Śliwa E, Grzywacz B, Hemp A (2013) Biogeography, ecology, acoustics and chromosomes of East African *Eurycorypha* Stål species (Orthoptera,

Phaneropterinae) with the description of new species. Organisms Diversity and Evolution 13(3): 373–395. https://doi.org/10.1007/s13127-012-0123-1

Hemp C, Heller K-G, Warchałowska-Śliwa E, Grzywacz B, Hemp A (2015a) Review of the *Plangia graminea* (Serville) complex and the description of new *Plangia* species from East Africa (Orthoptera: Phaneropteridae, Phaneropterinae) with data on habitat, bioacoustics, and chromosomes. Organisms Diversity and Evolution 15(3): 471–488. https://doi.org/10.1007/s13127-015-0216-8

Hemp C, Heller K-G, Warchałowska-Śliwa E, Hemp A (2010b) A new genus and species of African Phaneropterinae (Orthoptera: Tettigoniidae), with data on its ecology, bioacoustics and chromosomes. Organisms Diversity and Evolution 10: 215–226. https://doi.org/10.1007/s13127-010-0013-3

Hemp C, Heller K-G, Warchałowska-Śliwa E, Hemp A (2015b) A new species of *Philoscirtus* (Orthoptera: Phaneropterinae: Mecopodinae) from the West Usambara Montains of Tanzania and its conservation status. Zootaxa 3905(2): 273–282. doi: http://dx.doi.org/10.11646/zootaxa.3905.2.8

Hemp C, Voje KL, Heller K-G, Warchałowska-Śliwa E, Hemp A (2010a) A new genus in African Acrometopini (Tettigoniidae: Phaneropterinae) based on morphology, chromosomes, acoustics, distribution, and molecular data, and the description of a new species. Zoological Journal of the Linnean Sociaty 158: 66–82. https://doi.org/10.1111/j.1096-3642.2009.00542.x

Hewitt GM (1979) Grasshoppers and Crickets. Animal Cytogenetics,3. Insecta I. Orthoptera. Borntraeger, Berlin, Stuttgart, 170 pp.

Jetybayev IE, Bugrov AG, Karamysheva TV, Camacho JPM, Rubtsov NB (2012) Chromosomal localization of ribosomal and telomeric DNA provides new insights on the evolution of Gomphocerinae grasshoppers. Cytogenetics & Genome Research 138: 36–45. https://doi.org/10.1159/000341571

Kevan DKM (1982) Orthoptera. In: Parker SP (Ed.) Synopsis and Classification of Living Organisms. Vol. 2. McGraw-Hill Book Company, New York.

Kuznetsova VG, Maryańska-Nadachowska A, Karmysheva TV (2015) Chromosome analysis and FISH mapping of 18S rDNA and $(TTAGG)_n$ telomere sequences in the spittlebug genus *Philaenus* Stål (Hemiptera, Auchenorrhyncha, Aphrophoridae), Charper 10. In: Sharakhov IV (Ed.) Cytogenetic Mapping of Arthropod Genomes. CRC Press, Taylor & Francis Group, Boca Raton, 351–380.

Lysák MA, Schubert I (2013) Mechanisms of chromosome rearrangements. In: Leicht IJ et al. (Eds) Plant genome diversity. Springer-Verlag, Wien, 137–147. https://doi.org/10.1007/978-3-7091-1160-4_9

Mařan J (1953) Contribution to the knowledge of the genus *Pholidoptera* Wesm. Acta entomologica Musei Nationalis Pragae 28 (1952): 209–221.

Maryańska-Nadachowska A, Anokhin BA, Gnezdilov VM, Kuznetsova VG (2016) Karyotype syability in the family Issidae (Hemiptera, Auchenorrhyncha) revealed by chromosome techniques and FISH with $(TTAGG)_n$ and 18S rDNA probes. Comparative Cytogenetics 10(3): 347–369. https://doi.org/10.3897/CompCytogen.v10i3.9672

Massa B, Fontana P (2011) Supraspecific taxonomy of Palaearctic Platycleidini with unarmed prosternum: a morphological approach (Orthoptera: Tettigoniidae, Tettigoniinae). Zootaxa 2837: 1–47.

Neto MSR, de Souza MJ, Loreto V (2013) Chromosomal evolution of rDNA and H3 histone genes in representative Romaleidae grasshoppers from northern Brazil. Molecular Cytogenetics 6: 41. https://doi.org/10.1186/1755-8166-6-41

Nguyen P, Sahara K, Yoshido A, Marec F (2010) Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). Genetica 138: 343–354. https://doi.org/10.1007/s10709-009-9424-5

Palestis BG, Cabrero J, Trivers R, Yamacho JPM (2010) Prevalence of B chromosomem in Orthoptera is associated with shape and number of A chromosomes. Genetica 138: 1181–1189. https://doi.org/10.10007/s10709-010-9509-1

Ramme W (1951) Zur Systematik, Faunistik und Biologie der Orthopteren von Südost-Europa und Vorderasien. Mitteilungen aus dem Zoologischen Museum in Berlin 27: 1–432.

Rentz DCF, Colless DH (1990) A classification of the shield-backed katydids (Tettigoniinae) of the World. In: Bailey WJ, Rentz DCF (Eds) The Tettigoniidae: biology, systematics and evolution. Crawford House Press, Bathurst.

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58(4): 307–324. https://doi.org/10.1007/bf00292840

Sumner SG (1972) A simple technique for demonstrating centromere heterochromatin. Experimental Cell Research 75: 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

Ünal M (1998) A new genus and new species of Pholidopterini from Turkey (Orthoptera: Tettigoniidae). Center of Entomological Studies, Miscellaneous Papers 51: 1–5.

Vershinina AO, Anokhin BA, Lukhtanov VA (2015) Ribosomal DNA cluster and telomeric $(TTAGG)_n$ repeates in blue butterflies (Lepidoptera, Lycaenida) with low and high chromosome numbers. Comparative Cytogenetics 9(2): 161–171. https://doi.org/10.3897/CompCytogen.v9i2.4715

Warchałowska-Śliwa E (1988) Karyotype of *Pholidoptera aptera karnyi* Ebner. from Bulgaria and its comparison with that of *Pholidoptera aptera aptera* (Fabr.) from Poland (Orthoptera, Decticidae). Caryologia 41(2): 161–168. http://dx.doi.org/10.1080/00087114.1988.10797857

Warchałowska-Śliwa E (1998) Karyotype characteristics of katydid orthopterans (Ensifera, Tettigoniidae) and remarks on their evolution at different taxonomic levels. Folia Biologica (Kraków) 46: 143–176.

Warchałowska-Śliwa E, Maryańska-Nadachowska A (1992) Karyotypes, C-bands, NORs location in spermatogenesis of *Isophya brevipennis* Brunner (Orthoptera: Phaneropteridae). Caryologia 45(1): 83–89. http://dx.doi.org/10.1080/00087114.1992.10797213

Warchałowska-Śliwa E, Chobanov DP, Grzywacz B, Maryańska-Nadachowska A (2008) Taxonomy of the genus *Isophya* (Orthoptera, Phaneropteridae, Barbitistinae): Comparison of karyological and morphological data. Folia Biologica (Kraków) 56: 227–241. http://dx.doi.org/10.3409/fb.56_3-4.227-241

Warchałowska-Śliwa E, Heller K-G, Maryańska-Nadachowska A (2005) Cytogenetic variability of European Tettigoniinae (Orthoptera, Tettigoniidae): Karyotypes, C-

and Ag-NOR-banding. Folia Biologica (Kraków) 53(3–4): 161–171. https://doi.org/10.3409/173491605775142800

Warchałowska-Śliwa E, Grzywacz B, Maryańska-Nadachowska A, Hemp A, Hemp C (2015) Different steps in the evolution of neo-sex chromosomes in two East African *Spalacomimus* species (Orthoptera: Tettigoniidae: Hetrodinae). European Journal of Entomology 112(1): 1–10. https://doi.org/10.14411/eje.2015.024

Warchałowska-Śliwa E, Grzywacz B, Maryańska-Nadachowska A, Karamysheva T, Chobanov DP, Heller K-G (2013a) Cytogenetic variability among Bradyporinae species (Orthoptera: Tettigoniidae). European Journal of Entomology 110(1): 1–12. https://doi.org/10.14411/eje.2013.001

Warchałowska-Śliwa E, Grzywacz B, Maryańska-Nadachowska A, Karamysheva TV, Rubtsov NB, Chobanov DP (2009) Chromosomal differentiation among bisexual European species of *Saga* Charp. (Orthoptera, Tettigoniidae, Saginae) detected by both classical and molecular methods. European Journal of Entomology 106(1): 1–9. https://doi.org/10.14411/eje.2009.001

Warchałowska-Śliwa E, Maryańska-Nadachowska A, Grzywacz B, Karamysheva T, Lehmann AW, Lehmann GUC, Heller K-G (2011) Changes in the numbers of chromosomes and sex determination system in bushcrickets of the genus *Odontura* (Orthoptera, Tettigoniidae, Phaneropterinae). European Journal of Entomology 108(2): 183–195. https://doi.org/10.14411/eje.2011.025

Warchałowska-Śliwa E, Grzywacz B, Maryańska-Nadachowska A, Karamysheva TV, Heller K-G, Lehmann AW, Lehmann GUC, Chobanov DP (2013b) Molecular and classical chromosomal techniques reveal diversity in bushcricket genera of Barbitistini (Orthoptera). Genome 56(11): 667–676. dx.doi.org/10.1139/gen-2013-0119

White MJD (1973) Animal Cytology and Evolution (3rd edn). Cambridge University Press, London.

CompCytogen 11(2): 325–357 (2017)
doi: 10.3897/CompCytogen.v11i2.12370
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

A new species of *Melitaea* from Israel, with notes on taxonomy, cytogenetics, phylogeography and interspecific hybridization in the *Melitaea persea* complex (Lepidoptera, Nymphalidae)

Vladimir A. Lukhtanov[1,2,3,4]

1 *Department of Karyosystematics, Zoological Institute of the Russian Academy of Sciences, Universitetskaya nab. 1, St. Petersburg 199034, Russia* **2** *Department of Entomology, St. Petersburg State University, Universitetskaya nab 7/9, St. Petersburg 199034, Russia* **3** *McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, University of Florida, 3215 Hull Rd., UF Cultural Plaza, PO Box 112710, Gainesville, Florida, 32611-2710 USA* **4** *Museum of Comparative Zoology, Harvard University, 26 Oxford Street, Cambridge, Massachusetts, 02138 USA*

Corresponding author: *Vladimir A. Lukhtanov* (lukhtanov@mail.ru)

Academic editor: *V. Kuznetsova* | Received 22 February 2017 | Accepted 31 March 2017 | Published 5 May 2017

http://zoobank.org/DF575903-DA4B-4586-A149-9D9470D29260

Citation: Lukhtanov VA (2017) A new species of *Melitaea* from Israel, with notes on taxonomy, cytogenetics, phylogeography and interspecific hybridization in the *Melitaea persea* complex (Lepidoptera, Nymphalidae). Comparative Cytogenetics 11(2): 325–357. https://doi.org/10.3897/CompCytogen.v11i2.12370

Abstract

Specimens with intermediate morphology are often considered to be the result of ongoing interspecific hybridization; however, this conclusion is difficult to prove without analysis of chromosomal and/or molecular markers. In the butterfly genus *Melitaea*, such an intermediacy can be detected in male genitalia, and is more or less regularly observed in localities where two closely related, presumably parental species are found in sympatry. Here I analyze a high altitude *Melitaea* population from Mt. Hermon in north Israel and show that its male genitalia are clearly differentiated from those found in phenotypically similar *M. persea* and *M. didyma*, but in some aspects intermediate between them. This hybrid-like population is unique because, although *M. didyma* is present on Mt. Hermon, the true, low-altitude *M. persea* has never been reported from Israel. Cytogenetic analysis revealed no apomorphic chromosomal characters to distinguish the Mt. Hermon population from other known taxa of the *M. persea* and *M. didyma* species groups. At the same time, DNA barcode-based phylogeographic study showed that this population is ancient. It was estimated to originate 1–1.6 million years ago in the Levantine refugium from a common ancestor with *M. persea*. Generally, the data obtained are incompatible with interpretation of the studied population as a taxon conspecific with *M. persea* or *M. didyma*, or a swarm of recent hybrids between *M. persea* and *M. didyma*,

although the possibility of ancient homoploid hybrid speciation cannot be ruled out. I also argue that the name *Melitaea montium* assigned to butterflies from north Lebanon cannot be applied to the studied taxon from Mt. Hermon. Here I describe this morphologically and ecologically distinct entity as a new species *Melitaea acentria* **sp. n.**, and compare it with other taxa of the *M. persea* complex.

Keywords

Chromosomes, *COI*, DNA barcoding, genitalia, homoploid hybrid speciation, interspecific hybridization, Middle East, *Melitaea casta*, *Melitaea eberti*, *Melitaea higginsi*, *Melitaea deserticola*, *Melitaea trivia*, morphology, nomenclature, taxonomy

Introduction

Butterflies of the genus *Melitaea* Fabricius, 1807 are distributed throughout the warm and temperate part of the Palaearctic region and occupy a wide range of habitat types, including meadows, grasslands, steppe, alpine biotopes, arid mountains and deserts (Tuzov and Churkin 2000). This group was revised by Higgins (1941, 1955) and more recently by Oorschot and Coutsis (2014) who used analysis of male genitalia as a main tool to document taxonomic structure of the genus. Despite these revisions, a large number of unresolved taxonomic questions persist among *Melitaea*, where species-level boundaries remain poorly defined. For example, DNA-barcode analysis revealed multiple deeply diverged lineages with properties of phylogenetic and partially biological species within *Melitaea didyma* (Esper, 1779) sensu lato, a widely distributed and common *Melitaea* species (Pazhenkova et al. 2015, Pazhenkova and Lukhtanov 2016).

Recent progress in improving our knowledge of relationships in *Melitaea* was made by using chromosomal (de Lesse 1960, Larsen 1975, Lukhtanov and Kuznetsova 1989, Hesselbarth et al. 1995) and molecular markers (Zimmermann et al. 1999, Long et al. 2014). In particular, molecular studies have helped to resolve some of the issues related to the composition of species groups within *Melitaea* (Wahlberg and Zimmermann 2000, Leneveu et al. 2009). However, with few exceptions (Kuznetsov et al. 2014, Toth et al. 2014, Pazhenkova et al. 2015, Pazhenkova and Lukhtanov 2016), molecular markers have not been used for analysis of taxonomic structure of *Melitaea* on level of closely related species or on intraspecific level.

One of the most serious problems of the *Melitaea* taxonomy is the presence of so called "intermediates" (Oorschot and Coutsis 2014). The closely related sympatric species of the genus *Melitaea* can be distinguished by male genitalia structure; however, specimens with intermediate genitalia can be more or less regularly found in nature. Most likely, these intermediates represent results of recent interspecific hybridization (Oorschot and Coutsis 2014), but such a conclusion is difficult to prove without analysis of genetic markers. The majority of these intermediates are concentrated in south-west Asia where the widely distributed species *M. persea* Kollar, 1849 contacts with *M. didyma* (in Turkey and Armenia), *M. interrupta* Kolenati, 1848 (in the Russian Caucasus, Azerbaijan, Armenia, east Turkey, Iran and Turkmenistan), *M. gina* Higgins, 1941 (in Iran) and *M. mixta* Evans, 1912 (in Afghanistan and Pakistan) (Oorschot and Coutsis 2014).

While analyzing specimens of the genus *Melitaea* collected in 2013-2016 in Israel as a part of the Israeli butterflies DNA barcoding survey project, I encountered a series of distinctive samples, collected in June 2013 at high altitude of Mt. Hermon by Asya Novikova (the Hebrew University of Jerusalem). These specimens were preliminarily identified as *M. persea montium* Belter, 1934, a name described from north Lebanon (Belter 1934) and recently established to be a synonym of *M. didyma* (Oorschot and Coutsis 2014, pages 17–18). Analysis of their male genitalia revealed them to be clearly different from phenotypically most similar *M. persea* and *M. didyma*, but in some aspects intermediate between them. A subsequent search and collecting in 2013, 2014 and 2016 resulted in a number of additional specimens from Mt. Hermon and demonstrated that this population was sympatric and partially syntopic with phenotypically similar *M. didyma liliputana* Oberthür, 1909, *M. deserticola* Oberthür, 1909 and *M. trivia syriaca* Rebel, 1905 as well as with phenotypically differentiated *M. cinxia* Linnaeus, 1758, *M. telona* Fruhstorfer, 1908 and *M. collina* Lederer, 1861.

In an effort to analyze the origin of these unusual Israeli specimens and to determine their taxonomic status, their karyotype and morphology were studied and compared to those of *M. persea* and *M. didyma*. In addition, legs were sampled from all species and major populations in the *M. didyma* and *M. persea* groups (except for the extremely rare and local *M. eberti* Koçak, 1980 from N. Iran), and sequence data from the DNA barcode region of *COI* were obtained. The results of the *M. didyma* DNA barcode survey have already been published (Pazhenkova et al. 2015, Pazhenkova and Lukhtanov 2016). Herein I present the results of the *M. persea* DNA barcode analysis, and describe the distinctive Israeli *Melitaea* as a new species, *Melitaea acentria* sp. n.

Material and methods

Samples

Specimens examined are deposited in the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia and in the McGuire Center for Lepidoptera and Biodiversity (MGCL), Florida Museum of Natural History, University of Florida, Gainesville, Florida, USA. Photographs of all specimens used in the analysis, as well as collecting data, are available on the Barcode of Life Data System (BOLD) at http://www.boldsystems.org/. Localities where specimens of the *M. persea* group were collected are shown in Figure 1.

Morphological analysis

For genitalia preparation, abdomens removed from adults were soaked in hot (90°C) 10% KOH for 3–10 min. Then they were transferred to water, the genitalia were carefully extracted and examined under a stereo-microscope using a pair of prepara-

Figure 1. Localities from where specimens of the *M. persea* group were collected for molecular studies. **1** *M. acentria*, haplogroup A **2** *M. acentria*, haplogroup P2 **3** *M. persea*, haplogroup P2 **4** *M. persea persea*, haplogroup P1 **5** *M. persea paphlagonia*, haplogroup P3 **6** *M. higginsi*, haplogroup H.

tion needles or a needle and a watchmaker's tweezer. Once cleansed of all unwanted elements they were transferred and stored in tubes with glycerine. Cleansed genitalia armatures were handled, studied and photographed while immersed in glycerine, free from pressure due to mounting, and therefore free from the ensuing distortion. Genitalia photographs were taken with a Leica M205C binocular microscope equipped with a Leica DFC495 digital camera, and processed using the Leica Application Suite, version 4.5.0 software.

The terminology of genitalia structures follows Oorschot and Coutsis (2014).

Butterfly photographs were taken with a Nikon D810 digital camera equipped with Nikon AF-S Micro Nikkor 105 mm lens.

Molecular methods and DNA barcode-based phylogeographic study

Standard *COI* barcodes (658-bp 5' segment of mitochondrial *cytochrome oxidase subunit I*) were studied. *COI* sequences were obtained from 92 specimens representing *M. acentria* sp. n. (25 samples), *M. persea persea* (18 samples), *M. persea* ssp. from Lebanon (2 samples), *M. persea paphlagonia* Fruhstorfer, 1917 (4 samples), *M. higginsi* Sakai, 1978 (2 samples), *M. casta* Kollar, 1849 (11 samples), *M. didyma liliputana* (7 samples), *M. deserticola* (14 samples) and *M. trivia syriaca* (9 samples) (Appendix 1: Table 1).

88 samples were processed at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using protocols described in

deWaard et al. (2008), Ivanova et al. (2006) and Hajibabaei et al. (2005). Photographs of these specimens are available in the Barcode of Life Data System (BOLD) at http://www.boldsystems.org/. Legs of two samples (castaM8 and castaM9) were processed by Elena Pazhenkova at the Department of Karyosystematics of the Zoological Institute of the Russian Academy of Sciences as described earlier (Pazhenkova and Lukhtanov 2016). Two sequences, NW43-10 (http://www.nymphalidae.net/story.php?code=NW43-10) and NW43-9 (http://www.nymphalidae.net/story.php?code=NW43-9), were provided by Niklas Wahlberg. Four sequences were downloaded from GenBank: AF187796 (*M. persea*), FJ462273 (*M. persea*), FJ462238 (*M. casta*) and FJ462288 (*M. casta wiltshirei* Higgins, 1941) (Wahlberg and Zimmermann 2000, Wahlberg et al. 2005, Leneveu et al. 2009).

The barcode analysis involved 96 *COI* sequences (Appendix 1: Table 1) including 53 samples of the species close to *M. persea* (*M. persea*, *M. acentria* sp. n. and *M. higginsi*) and 13 samples of *M. casta* that was previously recovered as a sister group to *M. persea* (Leneveu et al. 2009). It also involved samples of the phenotypically similar species *M. didyma liliputana* (7 samples), *M. deserticola* (14 samples) and *M. trivia syriaca* (9 samples) collected in Israel, Jordan and Syria. Nine *M. trivia syriaca* samples were selected as an outgroup.

Sequences were aligned using the BioEdit software (Hall 1999) and edited manually. Phylogenetic hypotheses were inferred using Bayesian inference as described previously (Vershinina and Lukhtanov 2010, Lukhtanov et al. 2016a, b). Briefly, the Bayesian analysis was performed using the program MrBayes 3.2 (Ronquist et al. 2012) with default settings as suggested by Mesquite (Maddison and Maddison 2015): burnin=0.25, nst=6 (GTR + I + G). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. The consensus of the obtained trees was visualised using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

I used two criteria to evaluate the level of DNA barcode divergence between taxa and haplogroups. First, I calculated the number of fixed DNA substitutions, i.e. the number of invariable differences in the studied *COI* fragment. Second, I calculated the minimal uncorrected *COI p*-distance between taxa and haplogroups. For this calculation, two genetically closest samples from each taxon pair were selected, and the distance between them was calculated using both fixed and non-fixed substitutions.

Chromosomal analysis

Karyotypes were obtained from fresh adult males and processed as previously described (Lukhtanov et al. 2014, 2015a, Vishnevskaya et al. 2016). Briefly, gonads were removed from the abdomen and placed into freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing the butterfly in the field. Testes were stored in the fixative for 1 month at +4°C. Then the gonads were stained in 2% acetic orcein for 7–10 days at +18-20°C. Haploid chromosome numbers (n) were counted in meiotic prometaphase, metaphase I (MI) and metaphase II (MII).

Results

New species description

Melitaea acentria **Lukhtanov, sp. n.**
http://zoobank.org/A2179B2C-0B7C-4CA5-8A41-EDF93BD27D92

Holotype (Fig. 2a, b), male, BOLD process ID BPAL2191-13, field # CCDB-17949_A06, GenBank accession number # KY777529; Israel, Mt. Hermon, 33°18'45.6"N; 35°47'11.9"E, 2050 m, 01 June 2013, A. Novikova leg., deposited in the Zoological Institute of the Russian Academy of Science (St. Petersburg).

COI barcode sequence of the holotype (BOLD process ID BPAL2191-13; GenBank accession number # KY777529): ACTTTATATTTTATCTTTGGAATTTGAGCAGGTATATTGGGAACTTCTTTAAGACTTTTAATTCGAACTGAATTAGGAAATCCAGGATCTTTAATTGGTGATGATCAAATTTATAATACTATTGTTACAGCTCATGCTTTTATTATAATTTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATGTTAGGAGCCCCTGATATAGCATTCCCACGAATAAATAATATAAGATTTTGATTGCTCCCCCCCTCATTAATCTTATTAATTTCTAGAAGAATTGTAGAAAATGGTGCAGGTACAGGATGAACAGTTTACCCCCCACTTTCATCCAATATTGCTCATAGAGGATCATCTGTTGATTTAGCAATTTTTTTCTCTTCATTTAGCTGGAATTTCTTCAATTTTAGGGGCTATTAATTTTATTACCACTATTATTAACATACGCATTAATAATATATCATTCGATCAAATACCTTTATTTGTTTGAGCTGTAGGTATTACAGCTCTTTTATTATTATTATCTTTACCAGTTTTAGCAGGAGCAATTACAATACTTCTTACTGATCGAAATATTAATACTTCATTTTTTGACCCTGCTGGAGGAGGAGATCCTATTTTATACCAACATTTA

Paratypes. 26 males and 10 females collected on Mt. Hermon, Israel.

Four males with codes CCDB-17949_E01, BPAL2234-13; KT874736, BPAL2236-13, CCDB-17949_E03; CCDB-25452_C10, BPAL3359-16 and BPAL3360-16, CCDB-25452_C11. Two females with codes BPAL3361-16, CCDB-25452_C12; CCDB-17949_E02, KT874697, BPAL2235-13. Two females without codes. Israel, Mt. Hermon, 33°18'45.6"N; 35°47'11.9"E, 2040 m, 22 June 2013, V.A. Lukhtanov & A. Novikova leg.

Four males with codes CCDB-25453_E10, BPAL3193-16; CCDB-25453_E08, BPAL3191-16; CCDB-25453_E09, BPAL3192-16; CCDB-25454_C03, BPAL3257-16; CCDB-25453_E11, BPAL3194-16. Six males and one female without codes. Israel, Mt. Hermon, 33°18'20"N; 35°47'09"E, 2030 m, 17 May 2014, A. Novikova leg.

One male with codes CCDB-17969_A04, BPAL2759-15. Israel, Mt. Hermon, 33°18'45.6"N; 35°47'11.9"E, 2040 m, 03 July 2014, V.A. Lukhtanov & A. Novikova leg.

Ten males with codes CCDB-25458_C06, BPALB125-16; CCDB-25458_C07, BPALB126-16; CCDB-25458_C08, BPALB127-16; CCDB-25458_C09, BPALB128-16; CCDB-25458_C10, BPALB129-16; CCDB-25458_C11, BPALB130-16; CCDB-

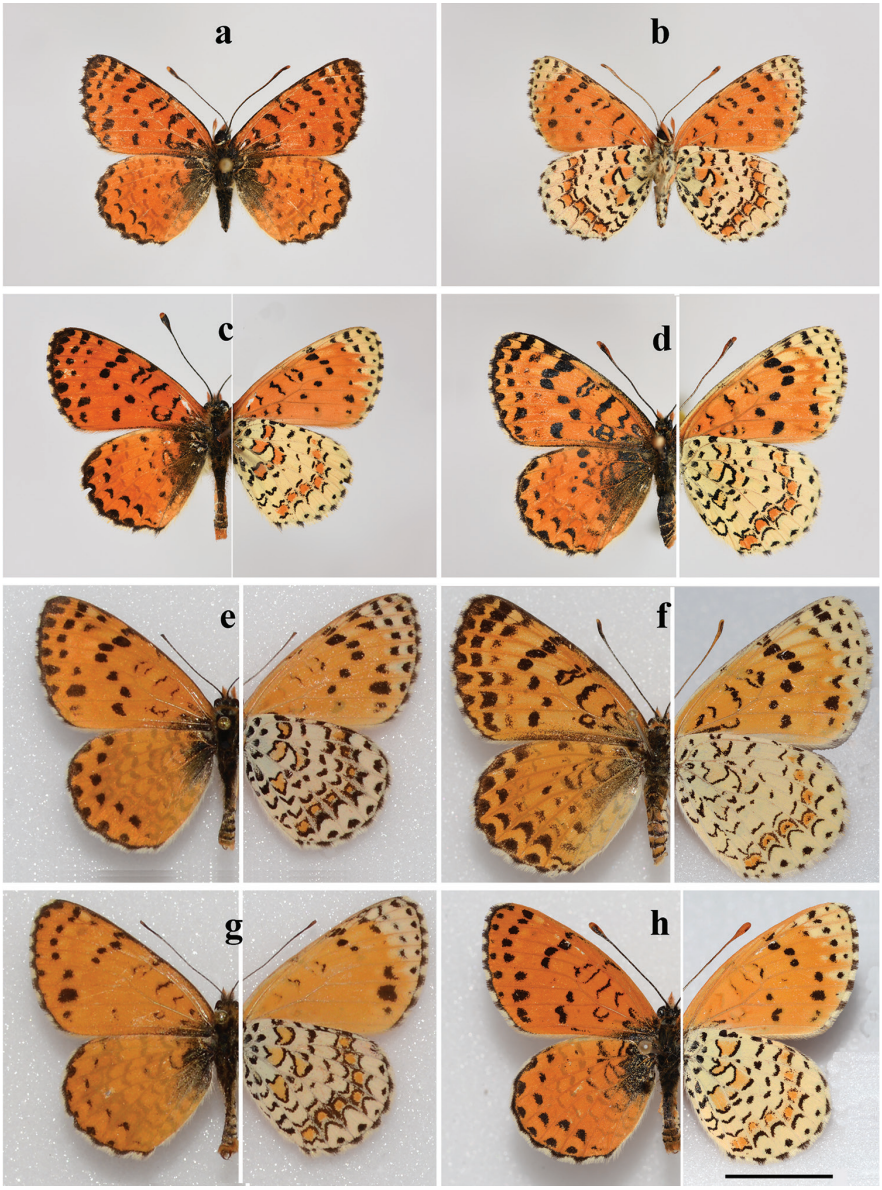


Figure 2. *Melitaea acentria* sp. n. and *M. persea persea*. Photos by V. Lukhtanov **a** *M. acentria* sp. n., holotype, male, sample 17949_A06, Israel, Mt. Hermon; upperside **b** *M. acentria* sp. n., holotype, male, sample 17949_A06, Israel, Mt. Hermon; underside **c** *M. acentria* sp. n., paratype, male, sample 25453_E09, Israel, Mt. Hermon **d** *M. acentria* sp. n., paratype, female, sample 25453_E11, Israel, Mt. Hermon **e** *M. persea persea*, male, 17966_A10, Iran, Fars prov., Fasa area, 20 km W Estahban, 2200 m, 9-11 May 2007, B. Denno coll., MGCL accession # 2010-20 **f** *M. persea persea*, female, 17951_B01, Iran, Fars prov., 20 km N Darab, 2100-2300 m, 24.05.1999, leg. P. Hofmann, MGCL **g** *M. persea persea*, male, 17966_A11, Iran, Fars prov., Fasa area, 20 km W Estahban, 2200 m, 9–11.05.2007, MGCL accession # 2008-43 **h** *M. persea persea*, male, 17951_B02, Iran, Char Mahall-o-Bahtiyari, Umg. Shahr-e-Kord, 2000 m, 28 May 2002, leg. P. Hofmann, MGCL. Scale bar corresponds to 10 mm in all figures.

25458_C12, BPALB131-16; CCDB-25458_D01, BPALB132-16; CCDB-25458_D02, BPALB133-16; CCDB-25458_D07, BPALB138-16. One male and three females without codes. Israel, Mt. Hermon, 33°18'41"N; 35°46'49"E, 1750-1900 m, 03 May 2016, V.A. Lukhtanov & E. Pazhenkova leg.

Two females with codes 25458 E06, BPALB149-16; 25458_E08 BPALB151-16. Israel, Mt. Hermon, 33°18'51"N; 35°46'31"E, 1800 m, 07 May 2016, V.A. Lukhtanov, A. Novikova & E. Pazhenkova leg.

All paratypes are deposited in the Zoological Institute of the Russian Academy of Science (St. Petersburg).

Males (Fig. 2a–c). Forewing length 16–19 mm. Forewing is roundish.

Upperside: ground color orange-red; the wing markings are small and delicate when compared to those in *M. didyma* and *M. persea*. Forewings with very narrow black marginal border fused with internervural marginal black spots. Submargimal series formed by black triangular spots on the forewings and by fine lunules on the hindwing. Forewing postdiscal series formed by small 1-3 black spots. Forewing discal series is complete or nearly complete, formed by black spots of variable size, the first four spots near costa are often enlarged. Hindwing discal series reduced or absent. Basal marking of the fore- and hindwings is delicate. Black basal suffusion is developed only near the base of hindwings. Fringe is white, checkered by black dots.

Underside: forewing ground color orange-red except for the apical part which is yellowish. Black markings delicate, reduced as compared with those of the upperside of the wing. Hindwing ground colour yellowish-white with two orange-red fascias. The red-orange submarginal fascia shows segmentation as the yellowish-white ground color spreads along the nervures. The orange-red macules of this fascia are bordered by back lunules from the outer side. From the inner side these macules are edged by black scales and additionally bordered by black lunules, giving the appearance that the proximal border of the submarginal fascia is doubly edged. Fringe white, checkered by black dots.

Females (Figs 2d, 3). Forewing length 17–20 mm. Forewing is roundish. Ground color of the upperside is slightly lighter and black markings heavier than in males. Costal area of the wing apex yellow-orange. Underside of the forewings as in males but black markings are heavier and there are additional yellowish maculae between discal and postdiscal spots. Underside of the hindwings as in males. Fringe white, checkered by black dots.

Karyotype. The genus *Melitaea* is known for its relatively low interspecific chromosome number variation (Pazhenkova and Lukhtanov 2016). However, in certain cases, the chromosome numbers are key to distinguish between closely related *Melitaea* species. For example, karyotype differences in combination with information about parapatric distribution were the main argument for the non-conspecificity of *M. didyma* and *M. latonigena* Eversmann, 1847 (Lukhtanov and Kuznetsova 1989). Therefore, chromosomal analysis is highly desirable in any taxonomic study of *Melitaea*. Here I conducted the chromosomal analysis of the high altitude population from Mt. Hermon (*M. acentria* sp. n.). The haploid chromosome number n=27 was found

Figure 3. *Melitaea acentria* in nature. Female. Israel, Mt. Hermon, 1800 m, 07 May 2016. Photo by V. Lukhtanov.

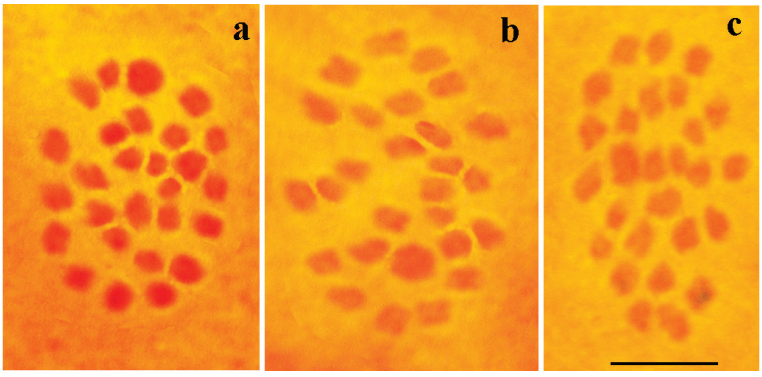


Figure 4. Karyotype in male meiosis of *Melitaea acentria* sp. n. from Israel. **a, b, c** sample CCDB-25458_D01, MI, n = 27. Scale bar corresponds to 5μ in all figures.

in prometaphase I, MI and MII cells of three studied individuals (2016-006, CCDB-25458_C11; 2016-008, CCDB-25458_D01; 2016-009, CCDB-25458_D02) (Fig. 4). The MI karyotype contained one chromosome bivalent that was significantly larger than the rest of the bivalents.

The same chromosome number (n=27) was previously reported for *M. persea* from Iran (de Lesse 1960). A karyotype characterized by n=27 including one large chromosome element was also found in *M. didyma neera* Fischer de Waldheim, 1840 from the North Caucasus (Russia), although in some other studied populations of *M. didyma*

n=28 was found (de Lesse 1960, Lukhtanov and Kuznetsova 1989). The chromosome number n=27 was also mentioned for "*M. didyma libanotica*" from Lebanon (Larsen 1975), but the vouchers for this chromosomal analysis were larvae, and in my opinion their identification was not certain. They could represent *M. didyma liliputana*, but also *M. acentria* as well as *M. persea* (but certainly not *M. deserticola* in which n=29 was found and not *M. trivia* in which n=31 was found, Larsen 1975). Finally, n=27 was reported for "*M. montium*" from Lebanon (de Lesse 1960), but in the last case the identity of the studied samples was also not clear because the identification was not supported by genitalia analysis.

Thus, no fixed karyotype difference is known to exist between *M. acentria* and *M. persea* as well as between *M. acentria* and *M. didyma*. Therefore we cannot use the available chromosomal data for delimitation between these species.

Male genitalia structure. *M. didyma* from Israel (Mt. Hermon) and *M. persea* from Iran and Azerbaijan were analyzed and were found to possess typical characters described previously (Higgins 1941, Oorschot and Coutsis 2014).

In *M. persea* all the main structures (ring-wall, tegumen, saccus, valvae) are elongated (Fig. 5a, b), longest in the genus *Melitaea* (Oorschot and Coutsis 2014). The valva is elongated from lateral view (Fig. 6a) and the valval distal process is massive (Fig. 6b). The dorsum of the valval distal process lies nearly in line with the remainder of the valval dorsum (Fig. 6a). The ventrum of the valval distal process possesses a keel bearing strong teeth (Fig. 6b). The saccus is bifurcate, with long, distally pointed branches (Fig. 5a, b). The aedeagus is curved, with a pronounced dorso-lateral ridge (Fig. 7a). The lateral sclerotized element of the tegumen is massive and its distal half is shaped like a smoker's pipe (Fig. 7b).

In *M. didyma liliputana* from Mt. Hermon all the main structures (ring-wall, tegumen, saccus, valvae) are significantly shorter than in *M. persea* (Fig. 5e, f). The valva is trapezoidal from lateral view (Fig. 6e). The valval distal process is delicate (Fig. 6f) and the dorsum of the valval distal process forms a clear angle with the remainder of the valval dorsum (Fig. 6e). The ventrum of the valval distal process is smooth, without a keel and/or teeth (Fig. 6f). The saccus is bifurcate, with short, distally rounded branches (Fig. 5e, f). The aedeagus is curved, without a pronounced dorso-lateral ridge (Fig. 7e). The lateral sclerotized element of the tegumen is delicate and its distal half is T- or Γ-shaped (Fig. 7f).

In *M. acentria* genitalia are clearly different from both *M. persea* and *M. didyma*, but at the same time are intermediate in some aspects. All the main structures (ring-wall, tegumen, saccus, valvae) are similar to those in *M. persea* but shorter (however, longer than in *M. didyma*) (Fig. 5c,d). The valva is cylindrical from lateral view (Fig. 6c). The valval distal process is intermediate in its shape between *M. persea* and *M. didyma* (Fig. 6c, d). Its dorsal and ventral borders are roughly parallel from lateral view (Fig. 6c). The dorsum of the valval distal process forms a clear angle with the remainder of the valval dorsum (similarly to *M. didyma*) (Fig. 6c). At the same time, the ventrum of the valval distal process possesses a keel bearing teeth (similarly to *M. persea*) (Fig. 6d). However, this keel and teeth are smaller and more delicate than

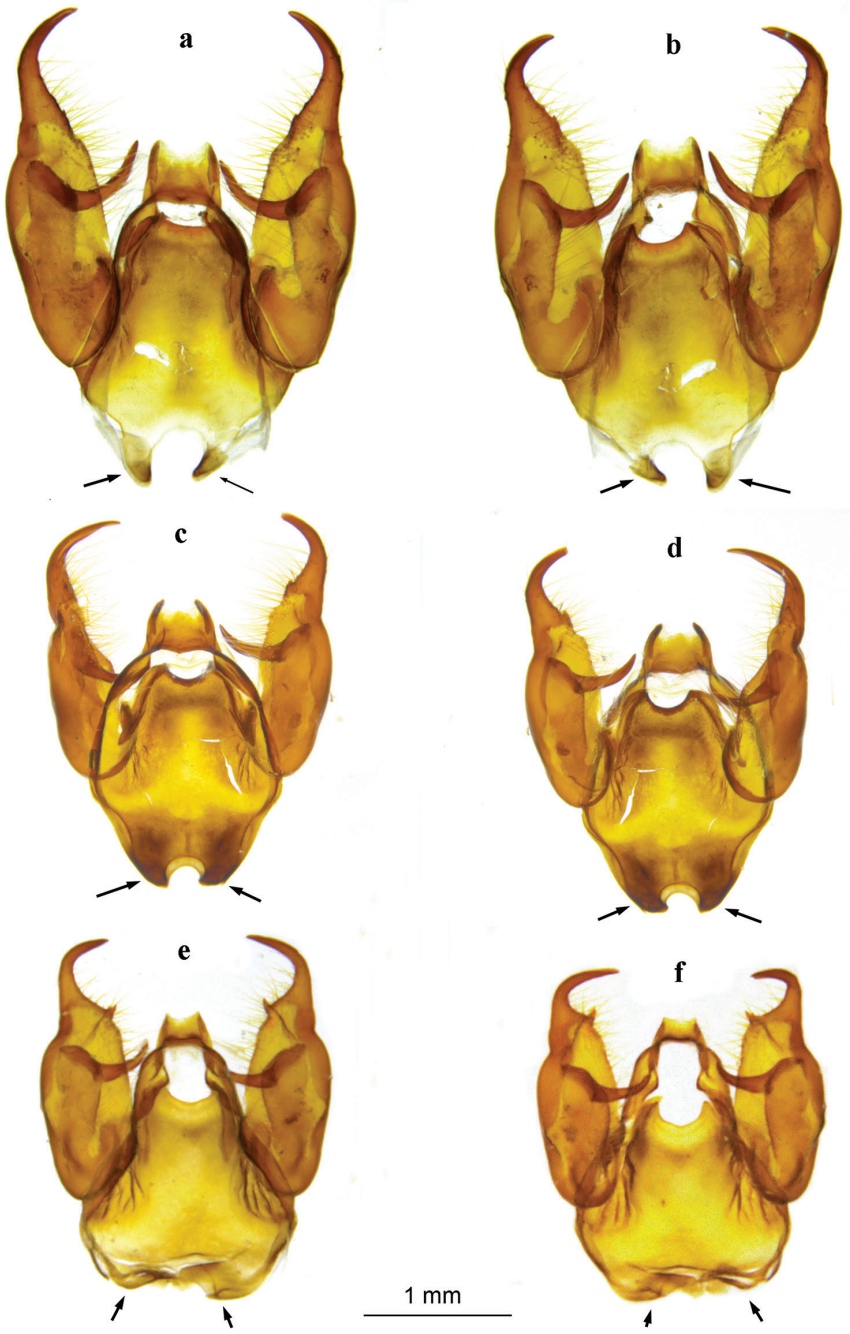


Figure 5. Male genitalia of *Melitaea persea persea* (sample 25450_C06, Azerbaijan, Talysh), *M. acentria* sp. n. (holotype, sample 17949_A06, Israel, Mt. Hermon) and *M. didyma liliputana* (sample 17698_E10 Israel, Mt. Hermon) (aedeagus is not shown; branches of saccus are indicated by arrows). **a** *M. persea persea*, dorsal view **b** *M. persea persea*, ventral view **c** *M. acentria* sp. n., dorsal view **d** *M. acentria* sp. n., ventral view **e** *M. didyma liliputana*, dorsal view **f** *M. didyma liliputana*, ventral view.

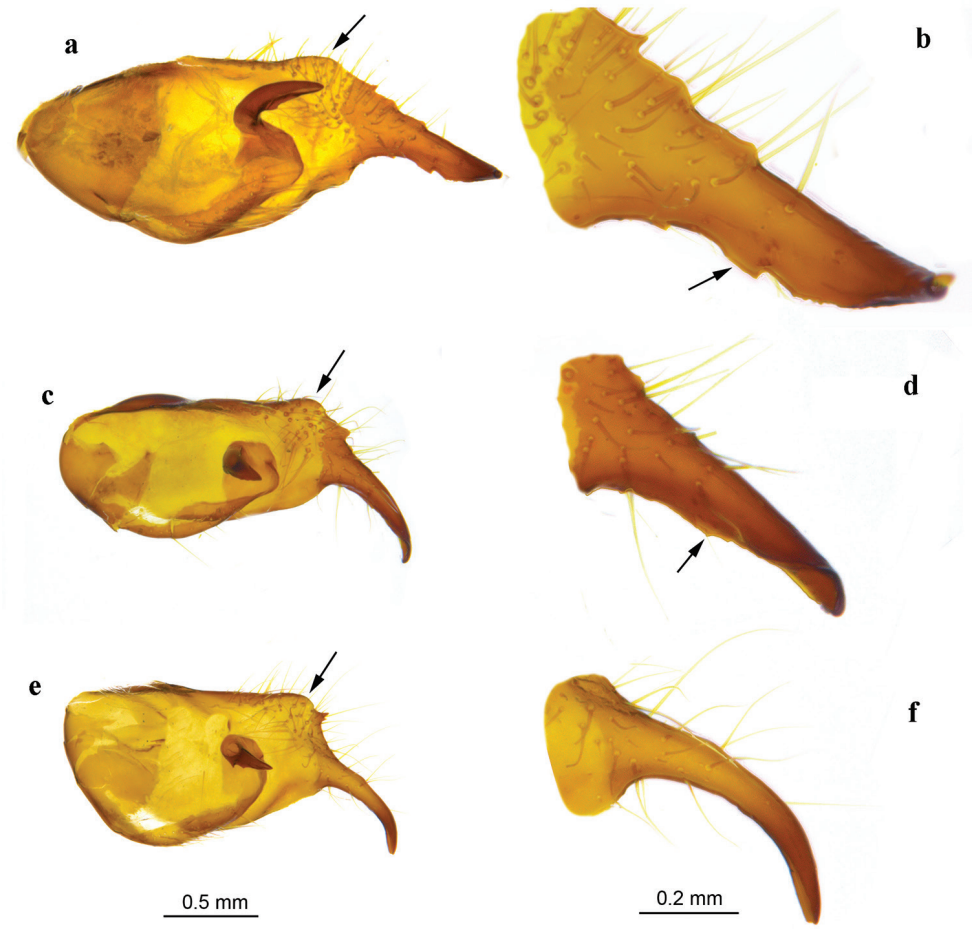


Figure 6. Lateral view of the inner face of right valva (left panel) and distal process (right panel). The angle between the valval dorsum and the distal process (left panel), and the keel of the distal process (right panel), are indicated by arrows. **a, b** *Melitaea persea persea* (sample 25450_C06, Azerbaijan, Talysh) **c, d** *M. acentria* sp. n. (holotype, sample 17949_A06, Israel, Mt. Hermon) **e, f** *M. didyma liliputana* (sample 17698_E10 Israel, Mt. Hermon).

in *M. persea* (Fig. 6d). The saccus is bifurcate, with relatively long, distally pointed branches; however, these branches are shorter than in *M. persea*, but longer than in *M. didyma liliputana*, where they are almost absent (Fig. 5c, d). The aedeagus is curved, with a dorso-lateral ridge (Fig. 7c); thus the aedeagus of *M. acentria* is not intermediate between *M. persea* and *M. didyma*, but similar to *M. persea*. The lateral sclerotized element of the tegumen is massive and its distal half is shaped like a smoker's pipe (Fig. 7d). This type of male genitalia was found in all seven studied samples including two samples (25453_E08 and 25458_C09) that were characterized by the mitochondrial haplogroup P2 (Figs 8 and 9).

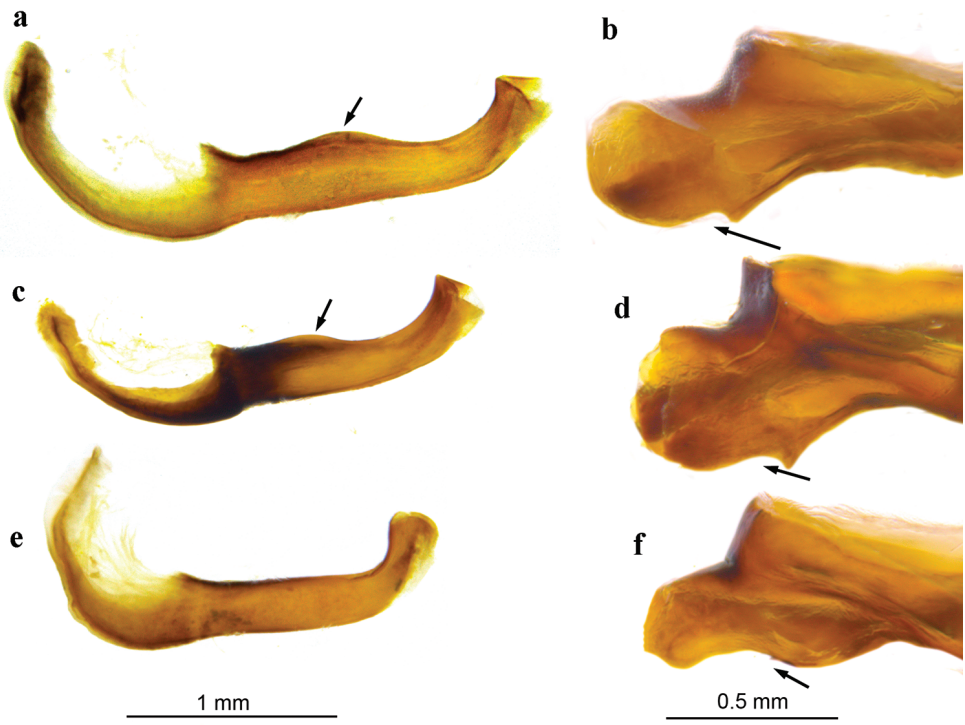


Figure 7. Lateral view of the left side of aedeagus and (left panel), and lateral view of the right side of tegumen (right panel). The post-zonal dorso-lateral ridge (left panel) and lateral sclerotized element (right panel) are indicated by arrows. **a, b** *Melitaea persea persea* (sample 25450_C06, Azerbaijan, Talysh) **c, d** *M. acentria* sp. n. (holotype, sample 17949_A06, Israel, Mt. Hermon) **e, f** *M. didyma liliputana* (sample sample 17698_E10 Israel, Mt. Hermon).

COI barcode analysis. The *COI* barcode analysis revealed five major clusters represented by (1) *M. trivia syriaca*, (2) *M. deserticola*, (3) *M. didyma liliputana*, (4) *M. casta* and (5) taxa of the *M. persea* group (haplogroups A, H, P1, P2 and P3) (Fig. 8). Interestingly, this analysis showed that the phenotypically similar species *M. trivia syriaca*, *M. deserticola* and *M. didyma liliputana*, can be easily separated by their DNA barcodes.

The analysis recovered the *M. persea* group (*M. acentria* + *M. persea* +*M. higginsi*) as a strongly supported monophyletic clade sister to *M. casta* (Fig. 9). This clade was divided into five lineages.

The first lineage (haplogroup P1) includes a huge range of *M. persea* populations from Daghestan (Russia) in the north to Shiraz province (Iran) in the south, including samples from Shiraz in SW Iran, which represents the type locality of *M. persea*. Across this range, *M. persea* shows various degrees of localized morphological diversification, and from this territory several taxa, currently attributed to *M. persea*, were described: *M. didyma caucasica* Staudinger, 1861; *M. didyma kaschtschenkoi* Christoph, 1889; *M. didyma araratica* Verity, 1929; *M. didyma magnacasta* Verity, 1929; *Melitaea tauricus*

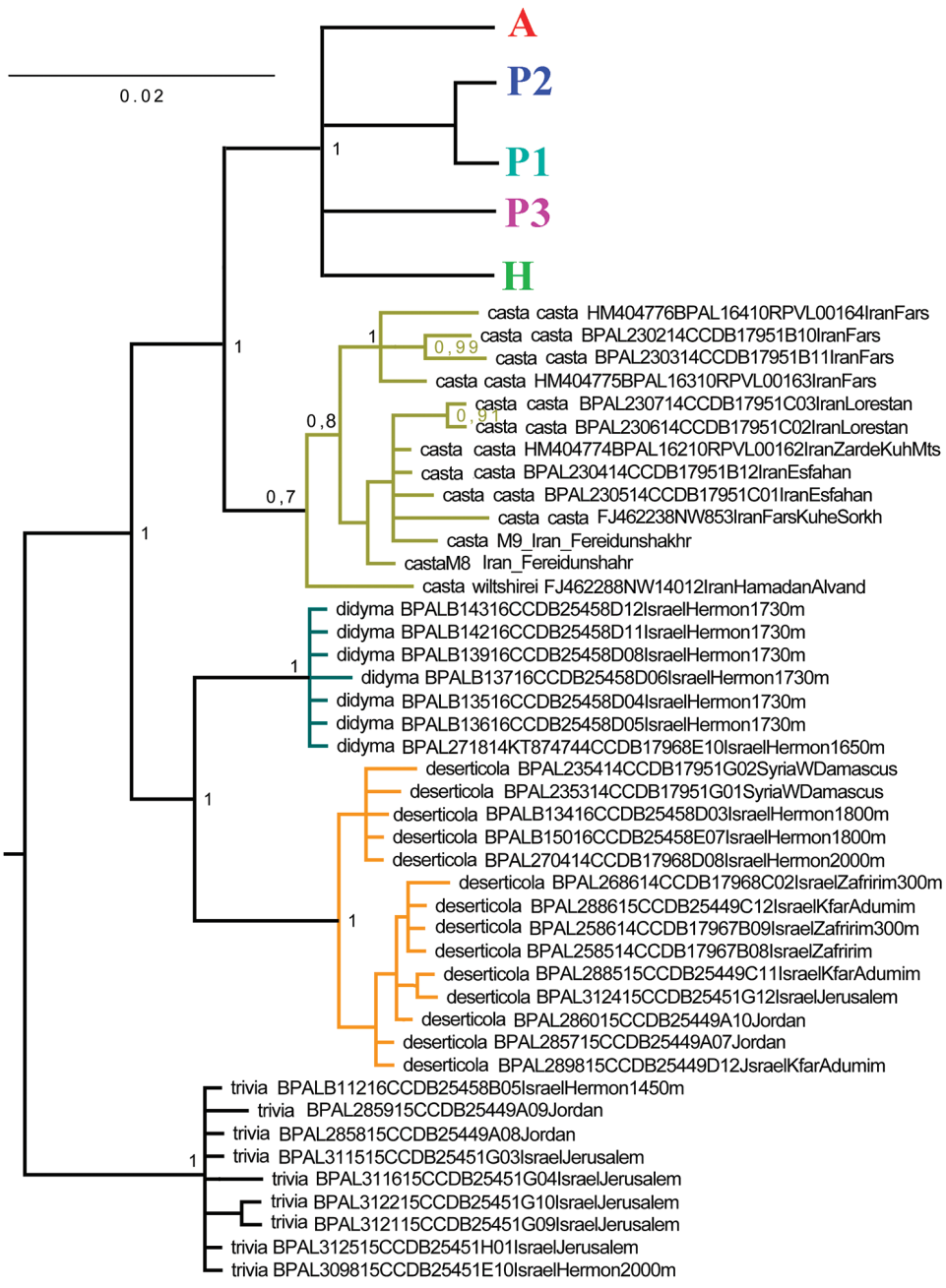


Figure 8. The Bayesian tree of studied *Melitaea* samples based on analysis of *the cytochrome oxidase subunit I* (*COI*) gene. Numbers at nodes indicate Bayesian posterior probability. A, H, P1, P2 and P3 are recovered haplogroups of the *M. persea* species complex (see Fig. 9 for more details).

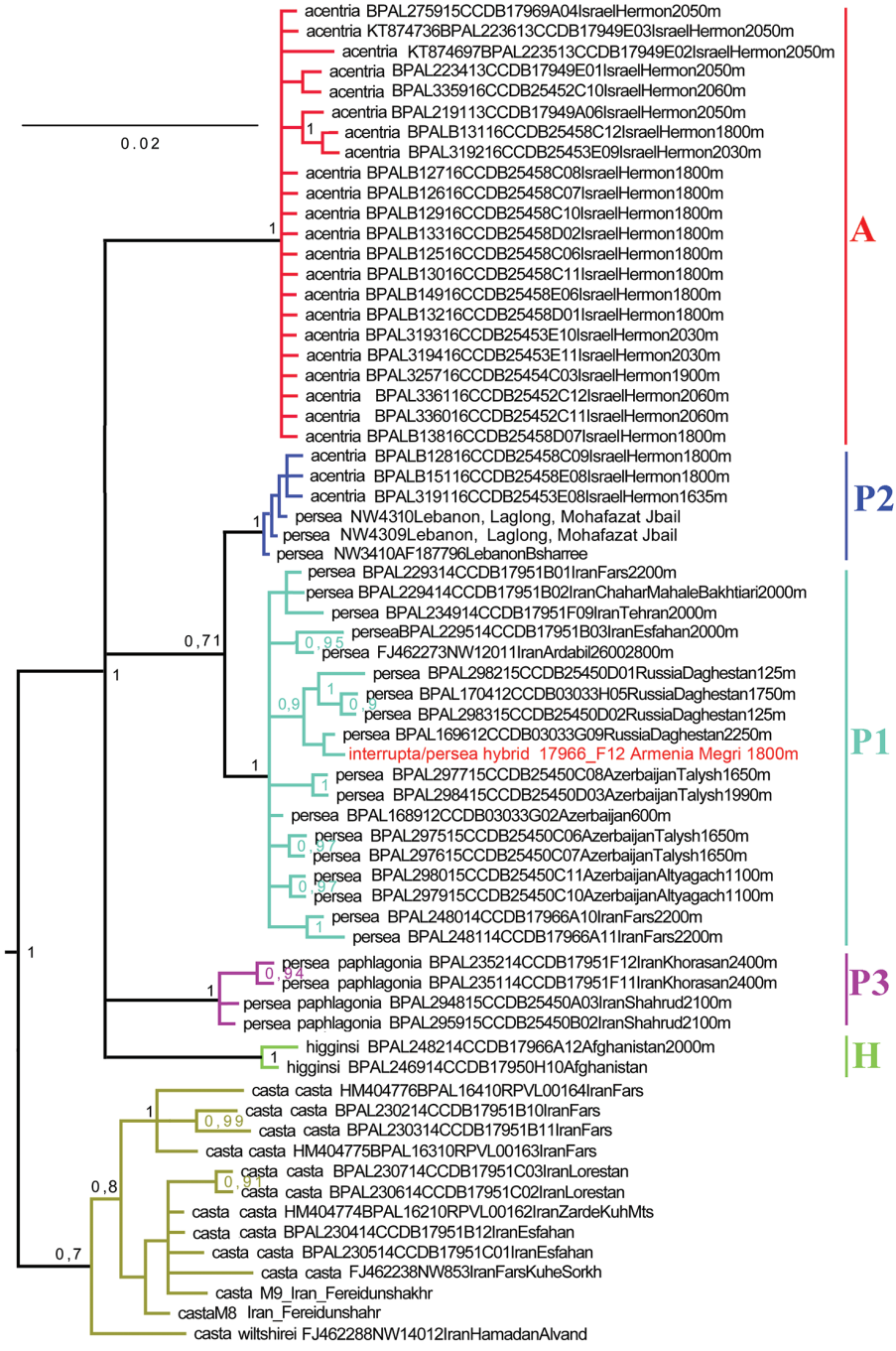


Figure 9. Fragment of the Bayesian tree of the studied *Melitaea* samples based on analysis of the *cytochrome oxidase subunit I* (*COI*) gene. *M. casta* and species of the *M. persea* species complex are shown. Numbers at nodes indicate Bayesian posterior probability. A, H, P1, P2 and P3 are recovered haplogroups of the *M. persea* species complex.

Belter, 1934; *M. pesea hafiz* Higgins, 1941; *M. hafiz darius* Gross & Ebert, 1975 and *M. jitka* D.Weiss & Major 2000. The taxonomy of these taxa was studied in more detail by Oorschot and Coutsis (2014), who found that they are closely related and should be considered no more than synonyms of *M. persea persea*. My DNA barcode results are consistent with this conclusion (Fig. 9).

The haplogroup P1 includes also a female sample 17966_F12 possessing intermediate morphological characters between *M. interrupta* and *M. persea* (Fig. 10b). In this specimen wing upperside is similar to that in *M. interrupta*, whereas the wing underside is without black scales along the veins which are typical for *M. interrupta* (Fig. 10h), but with orange-red submarginal spots edged by black scales typical for *M. persea* (Fig. 2e–h, 10a). This sample was collected at the same place with three typical *M. interrupta* males (samples17966_F09, 17966_F10 and 17966_F11) possessing typical *M. interrupta* phenotype (Fig. 10h) and *COI* haplotypes (GenBank # KT874702, KT874740 and KT874741), which were very different from those of *M. persea* (see Fig. 4 in Pazhenkova and Lukhtanov 2016). It is thus probable that the female 17966_F12 is a result of a more or less recent hybridization between *M. interrupta* and *M. persea*. Thus, it likely represents a first molecular evidence for sporadic interspecific hybridization in *Melitaea*.

The second lineage (haplogroup P2) is represented by three specimens from north Lebanon originally identified as *M. persea* (Wahlberg et al. 2005) and by three samples of *M. acentria* from Mt. Hermon: two males (25453_E08 and 25458_C09) that were indistinguishable in their genitalia from *M. acentria* of the haplogroup A and a single female (25458_E08). This lineage was found to be closest to P1 (*Melitaea persea persea*). It differed from P1 by 7 fixed DNA substitutions in the studied 658 bp fragment of the mitochondrial *COI* gene. The minimal uncorrected *COI p*-distance between the representatives of these two haplogroups was calculated using both fixed and non-fixed substitutions and was found to be 2.0 %.

The third lineage (haplogroup P3) includes samples from NE Iran (*M. persea paphlagonia*). It differed from P1 (*M. persea persea*) by 10 fixed DNA substitutions in the studied 658 bp fragment of the mitochondrial *COI* gene. The minimal uncorrected *COI p*-distance between these two haplogroups was found to be 2.3 %. They were also distinct in wing pattern: on the upper surface all the markings were well developed and the first four spots of the discal series were nearly fused to form a prominent costal bar (Fig. 10c, d). The male genitalia of *M. persea paphlagonia* were similar to those found in *M. persea persea* (Higgins 1941). This lineage was not recognized as a taxon by Oorschot and Coutsis (2014). However, it was recognized as a distinct subspecies by Higgins (1941), and my DNA barcode results corroborate this conclusion. The level of *COI* differentiation between M. *persea paphlagonia* and *M. persea persea* (10 fixed DNA substitutions) was found to be equal to that found between *M. persea persea* and *M. higginsi* (10 fixed DNA substitutions).

The forth lineage (haplogroup A), one of the most diverged lineages, is represented by samples from Mt. Hermon (*M. acentria*). It differed from P1 (*M. persea persea*) by 11 fixed nucleotide substitutions in the studied 658 bp fragment of the mitochondrial

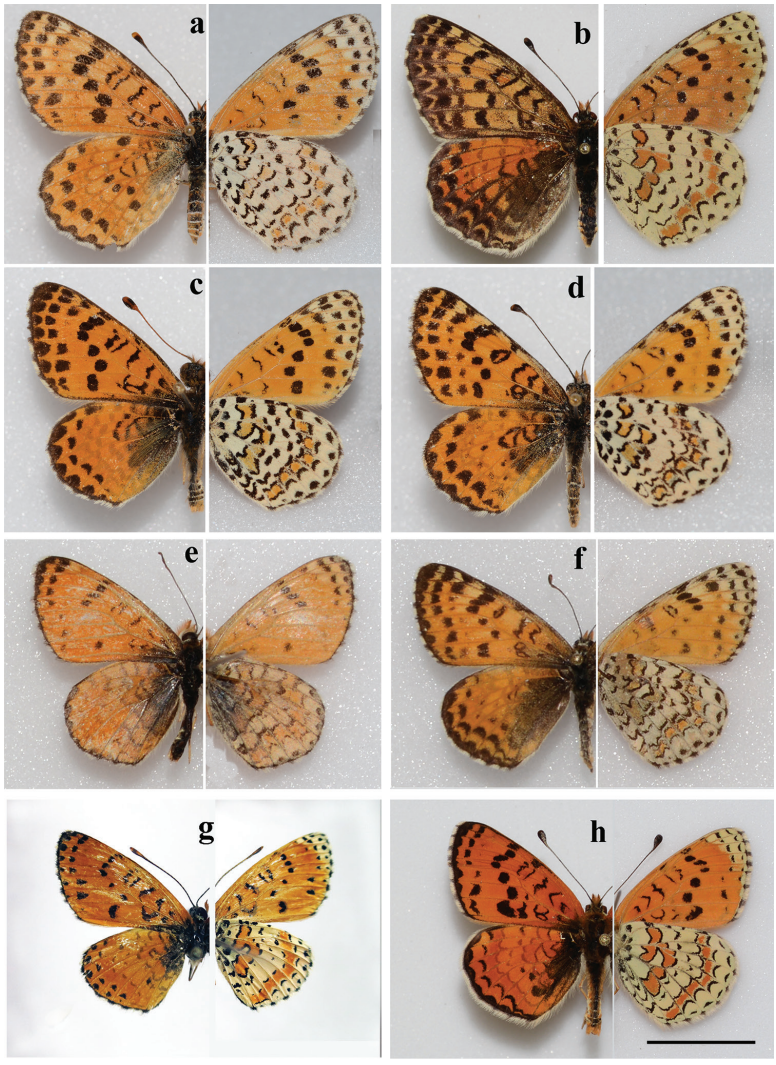


Figure 10. *Melitaea persea persea*, presumptive hybrid between *M. interrupta* and *M. persea*, *M. persea paphlagonia, M. higginsi*, *M. didyma liliputana* and *M. interrupta*. Photos by V. Lukhtanov **a** *Melitaea persea persea*, female, 17951_B03, Iran, Esfahan, Kuh-e-Marsenan, near Zefre, 2000 m, 26 May 2002, leg. Hofmann, MGCL **b** presumptive hybrid female between *M. interrupta* and *M. persea*, 17966_F12, Armenia, Zhangezur Range, Megri district, Litchk, 1800 m, 23 July 1999, A. Dantchenko leg., MGCL **c** *M. persea paphlagonia*, male, 17951_F11, Iran, Khorasan, Kuh-e-Binalut, 15 km SW Zoshk, 2300–2500 m, 7 June 1999, leg. P. Hofmann, MGCL **d** *M. persea paphlagonia*, male, 17951_F11, Iran, Khorasan, Kuh-e-Binalut, 15 km SW Zoshk, 2300-2500 m, 7 June 1999, leg. P. Hofmann, MGCL **e** *M. higginsi*, male, 17966_A12, Afghanistan, Hindukush, Panchir Valley, 20 June 2004, M.J.Simon collection, MGCL **f** *M. higginsi*, female, 17950_H10, Afghanistan, Badakhshan, Mt. Yamak N of Anjuman Pass, 3500-4000 m, 1-25 July 2004 M.J.Simon collection, MGCL **g** *M. didyma liliputana*, male, 17968_E10, Israel, Mt. Hermon **h** *M. interrupta*, male, 17966_F11, Armenia Armenia, Zhangezur Range, Kadjaran, 2500 m, 21–22 July 1999, leg. A. Chuvilin, MGCL; the wing underside is with black scales along the veins. Scale bar corresponds to 10 mm in all figures.

COI gene. The minimal uncorrected *COI p*-distance between these two haplogroups was found to be 2.4 %.

The fifth lineage (haplogroup H) includes samples of *M. higginsi* (Fig. 10e, f). This taxon is very rare in collections, and I have been lucky to find two specimens in the McGuire Center. It differed from P1 (*M. persea persea*) by 10 fixed DNA substitutions in the studied 658 bp fragment of the mitochondrial *COI* gene. The minimal uncorrected *COI p*-distance between these two haplogroups was found to be 2.4 %. This taxon is similar to *M. persea* with respect to male genitalia structure (Oorschot and Coutsis 2014), but quite different in wing pattern. Particularly, in males the hindwing uppersurface is without black spots which are always present in *M. persea*, and in both sexes hindwing underside veins are scaled with black, similar to *M. interrupta* and different from *M. persea*. My DNA barcode results confirm the distinctness of this high altitude very local Afghani taxon. They also confirm that this taxon is a member of the *M. persea* species group as suggested by Oorschot and Coutsis (2014), and not related to the Mongolian *M. didymina* Staudinger, 1895 as was supposed by Sakai (1978), as well as not related to *M. didyma* as was supposed by Kolesnichenko and Churkin (2004).

Diagnosis. Butterfly wing pattern and male genitalia morphology, as well as DNA barcodes certaintly indicate that *Melitaea acentria* belongs to the *M. persea* species complex. After Oorschot and Coutsis (2014) this complex includes three closely related species: *M. persea*, *M. eberti* and *M. higginsi*. Male genitalia of these three species were analysed by Oorschot and Coutsis (2014) and were found to be virtually indistinguishable. *Melitaea acentria* differs from these most closely related species by several characters in male genitalia. In *M. acentria* main genitalia structures (ring-wall, tegumen, saccus, valvae) are significantly shorter. The valva is cylindrical from lateral view, not elongated (Fig. 6c). The valval distal process is intermediate in its form between *M. persea* and *M. didyma* (Fig. 6c, d). The dorsum of the valval distal process forms a clear angle with the remainder of the valval dorsum (in similar way as in *M. didyma*) (Fig. 6c), but very different from *M. persea*. The keel and teeth of the valval distal process are smaller and more delicate than in *M. persea* (Fig. 6d). On average, in *M. acentria* the ground color of the wing upperside is more orange-red (Fig. 2a–d). In other species of the *M. persea* complex it is yellowish-orange (Fig. 2e-g, 10a, c–f). However, this character is not constant (e.g. see *M. persea* with orange-red wing color on Fig. 2h). The great majority of *M. acentria* samples significantly differ from all other taxa by their DNA barcodes; however, probably due to mitochondrial introgression, a minor part of the samples cluster with the haplogroup P2 of *M. persea*.

Melitaea acentria significantly differs from the distantly related but phenotypically similar species *M. didyma*, *M. deserticola* and *M. trivia* by DNA barcodes and male genitalia structures. Particularly, it differs from *M. didyma* by the ventrum of the valval distal process possessing a keel bearing teeth and by the elongated shape of the ring-wall, tegumen, saccus and valvae. *Melitaea acentria* mostly differs from *M. didyma* by the hindwing underside with submarginal macules that are edged by black scales and then bordered by black lunules, giving the impression that the proximal border of the

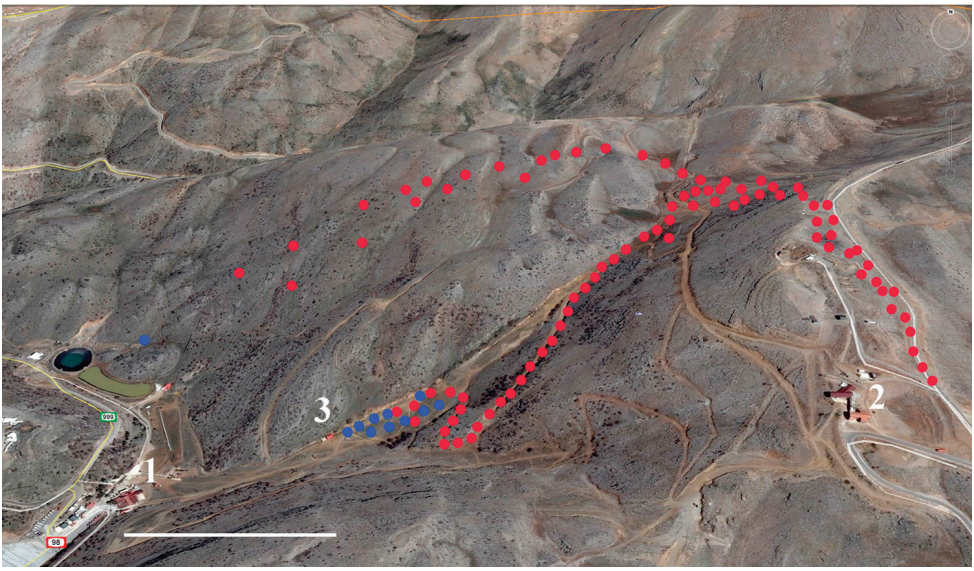


Figure 11. Points where *M. acentria* (red spots) and *M. didyma* (blue spots) were sampled or observed on Mt. Hermon, Israel. **1** lower station of the Hermon ski lift **2** upper station of the Hermon ski lift **3** winter café. Scale bar = 400 m.

submarginal fascia is doubly edged; *M. acentria* shares this character with *M. persea*. In *M. didyma* submarginal macules of the hindwing underside are usually not edged by black scales and simply bordered by black strokes (Fig. 10g). However, elements of the black scaling of the submarginal macules can be found in few *M. didyma* samples, and sometimes this black scaling is strongly reduced in species of the *M. persea* complex.

Distribution. *Melitaea acentria* is known to occur at high altitudes (1730–2060 m above the sea level) of Mt. Hermon (Fig. 11). Within these altitudes it is sympatric and syntopic with *M. trivia syriaca*, *M. deserticola* and *M. cinxia*. At the altitudes 1730–1780 m there is an essential overlapping of the *M. acentria* and *M. didyma liliputana* ranges where both species were found to fly together in early May 2016. Two other *Melitaea* species known from Mt. Hermon, *M. collina* and *M. telona*, were found to fly mostly at lower altitudes 1000–1600 m.

Habitat and phenology. Three main vegetation belts have been described from Mt. Hermon: (i) evergreen Mediterranean maquis (300–1250 m); (ii) xero-montane open forest (1250–1850 m) and (iii) subalpine mountain steppe, or "Tragacanthic belt" (1850–2814 m) (Kent et al. 2013). Adults of *M. acentria* were found to fly in open grassy (Fig. 12) and stony (Fig. 13) areas of the upper part of the xero-montane open forest belt (1750–1850 m) (Fig. 14) and of the subalpine mountain steppe belt (1850–2060 m) (Fig. 15). Butterflies were observed from 3 May to 3 July. On the 3rd of May 2016 they were abundant at altitudes from 1780 to 1900 m, therefore I conclude that they can start to fly at the end of April and continue to fly at least until mid-July.

Figure 12. Habitat of *M. acentria*. Israel, Mt. Hermon, 1920 m, 7 May 2016. Photo by A. Novikova.

Figure 13. *Melitaea acentria* and its habitat. Israel, Mt. Hermon, 2040 m, 22 June 2013. Photo by V. Lukhtanov.

Figure 14. Habitat of *M. acentria* and *M. didyma liliputana.* Israel, Mt. Hermon, 1750 m, 3 May 2016. The building with red roof is the winter café shown as 3 on Figure 11. Photo by V. Lukhtanov.

Figure 15. Habitat of *M. acentria*. Israel, Mt. Hermon, 2040 m, 22 June 2013. Photo by V. Lukhtanov

Etymology. The name *acentria* is a noun of the feminine gender. This name originates from the Greek prefix "a" that means "not" and from the Latin word "centrum" (centre) derived from the Greek "κέντρον" (kentron, a sharp point). Acentria is the Internet nickname of Asya Novikova who collected the samples initiated this research. This name indicates also the peripheral position of the new species within the distribution range of the *M. persea* species complex.

Discussion

Hypothesized evolutionary history of *Melitaea acentria*

Melitaea acentria was recovered as a diphyletic group with respect to *COI* barcodes being represented by two haplogroups A and P2. The major haplogroup A (22 samples of 25 studied) represents one of the most differentiated and thus most ancient mitochondrial lineages within the *M. persea* complex. The minor haplogroup P2 (3 samples of 25 studied) is also differentiated, but is more similar to the haplogroup P1 found in the core part of the *M. persea* species range.

To estimate the age of the haplogroup A (and the Israeli lineage as a whole) I used two calibration points: a lower rate of 1.5% uncorrected pairwise distance per million years estimated using a variety of invertebrates (Quek et al. 2004) for *COI*, and a faster rate of 2.3% uncorrected pairwise distance per million years for the entire mitochondrial genome of various arthropod taxa (Brower 1994). Using these points and the value 2.4% as the minimal uncorrected *COI p*-distance between the haplogroups A and P1, the haplogroup A can be estimated to originate approximately 1-1.6 million years ago from a common ancestor with the haplogroup P1 of *M. persea*, a species currently distributed throughout the whole Middle East. The Israeli lineage represented by haplogroup A evolved in isolation in the Levantine refugium and, most likely, relatively recently experienced episodes of hybridization with *M. persea* (haplogroup P2) resulting in mitochondrial introgression observed in the samples 25453_E08, 25458_C09 and 25458_E08. Thus, the haplogroup P2 of *M. acentria* seems to be a footprint of this introgression. Despite this supposed sporadic hybridization, the population from Mt. Hermon preserves clear diagnostic characters in male genitalia.

Melitaea acentria possesses male genitalia which are different from those found in both *M. persea* and *M. didyma*, but in some aspects intermediate between them. Such an intermediacy can theoretically be interpreted as a consequence of (i) an ancient hybridization resulting in homoploid hybrid speciation or (ii) a more recent hybridization resulting in the formation of a swarm of recently obtained hybrids (Lukhtanov et al. 2015b). In the first case (homoploid hybrid speciation), a new ***reproductively isolated***, sexually reproducing ***species*** arises through hybridization and combination of parts of the parental genomes, but without an increase in ploidy (Rieseberg et al. 1995, Rieseberg 1997, Coyne and Orr 2004, Lai et al. 2005). In the second case (formation of hybrid swarms), interspecific hybridization results in a number of individuals which

are ***not reproductively isolated*** from their parents and, thus, do not represent a new species (Lukhtanov et al. 2015b). The second scenario caused by occasional hybridization probably occurs in *Melitaea* as demonstated by the sample 17966_F12 with an intermediate morphology and, most likely, introgressed mitochondria.

Oorschot and Coutsis (2014) treated the Lebanese samples with mixed *M. persea* - *M. didyma* genitalia type as intermediates (i.e. hybrids) between *M. persea* and *M. didyma*. Such an interpretation seems to be logical for Lebanon where *M. persea* in its more or less typical form has been reported (Higgins 1941, Larsen 1974, Racheli 1980). However, this interpretation is inappropriate for the Israeli population (i.e. for *M. acentria*). First, typical *M. persea* has never been reported from Israel, which is one of the best studied territories in the world with respect to the butterfly fauna. Second and most importantly, the *M. acentria* samples posses very divergent *COI* haplotypes which can be attributed neither to *M. persea* and nor to *M. didyma*. Thus, *M. acentria* is not a swarm of recently obtained hybrids, but an old, well-established, morphologically and ecologically differentiated lineage with clear properties of phylogenetic and biological species.

At the same time, the hypothesis that *M. acentria* is a result of ancient homoploid hybrid speciation can not be ruled out. This highly speculative hypothesis should be tested in future through full genome molecular and chromosomal studies. While such a mode of speciation is widely accepted in plants (e.g., Soltis 2013), it has only relatively recently been thoroughly investigated in animals, including butterflies (Gompert et al. 2006, Mavárez et al. 2006, Mallet 2007, Kunte et al. 2011, Dupuis and Sperling 2015, Lukhtanov et al. 2015b).

Preimaginal stages and larval hostplant

Preimaginal stages of *Melitaea acentria* (originally identified as "*Melitaea persea montium"*) were recently described from Mt. Hermon with a comparison to the metamorphosis of *M. cinxia* (Benyamini 2016). These two species were shown to share the same larval hostplant *Plantago atrata* Hoppe, 1799 (Plantaginaceae) (Benyamini 2016).

Why *Melitaea montium* Belter, 1934 cannot be used as a valid name?

The identity of the taxon described under the name *Melitaea montium* Belter, 1934 has never been clear. Belter (1934) reported a difference between *M. montium* and *M. didyma* in the shape of the male genitalia valva. Although this supposed difference looks very distinct in Belter's drawing (Fig. 13 in Belter 1934) in fact it can hardly be traced. The real difference between these taxa is in the form of the tegumen, the distal process and, especially, of the saccus; however these structures were not shown on the very schematic drawings from Belter's paper. Thus, the genitalia description and figures provide little information on the identity of the taxon described as *M. montium* (see a more detailed discussion on this topic in the monograph by Oorshot and Coutsis (2014)).

The same can be said about wing pattern. In fact, Belter was the first author who described two types of hindwing underside in *Melitaea*: (i) with the proximal double (see Fig. 2b–h; 10a, c, d, f) and (ii) with the proximal single black border of the submarginal fascia (see Fig. 10b, g, h), and mentioned that both types existed in *M. montium*. These two types were later referred to as types "a" and "b" (Hesselbarth et al. 1995). None of these two types is species-specific, although the type (i) is much more common in the *M. persea* complex, and the type (ii) is more common in the *M. didyma* complex (Hesselbarth et al. 1995, Larsen 1974).

After Belter, the name *Melitaea montium* was used in literature for the Middle Eastern (Higgins 1941, Hesselbarth et al. 1995, Tshikolovets 2011), Lebanese (Larsen 1974, Gross and Ebert 1975, Racheli 1980) and Israeli (Benyamini 2002) butterflies close or supposedly identical to *M. persea*. It was also used as a synonym of *M. didyma* (Oorshot and Coutsis 2014).

I should note that the identity of butterflies in these publications has never been clear, except for the monograph by Oorshot and Coutsis (2014) since at least three different groups of populations close to *M. persea* are recorded from the Middle East: 1) the populations close (but probably not identical) to true *M. persea* (Higgins 1941, Larsen 1974, Racheli 1980, Oorshot and Coutsis 2014), 2) *M. acentria* from Israel (this study), and 3) intermediates (hybrids) between *M. persea* and *M. didyma* from north Lebanon (Oorshot and Coutsis 2014). These three groups could be identified on basis of male genitalia characters; however, until the work of Oorshot and Coutsis (2014), the genitalia of these butterflies were not carefully studied. Higgins (1941) provided only schematic genitalia drawings that were good enough to exclude *M. didyma* from consideration, but not detailed enough to distinguish between *M. persea* and *M. persea-M. didyma* intermediates, and the consequent authors did not provide genitalia drawings at all (see the monograph of Oorshot and Coutsis (2014) for a more detailed analysis of the previous taxonomic interpretations).

The Gordian knot of this taxonomic and nomenclatural uncertainty was cut by Oorshot and Coutsis (2014) through a careful analysis of genitalia morphology, checking of all taxonomically important publications, studies of type material and subsequent designation of the lectotype of *Melitaea montium* (male, sample HO0937 in Zoologische Staatssammlung, München, collected in Lebanon, Bcharré, genitalia preparation no. 4822, figured in Oorshot and Coutsis (2014), page 188, pl. 51, figs 14, 35 and 38).

The lectotype of *Melitaea montium* was found to have typical *M. didyma* genitalia, having nothing in common with those of *M. persea*, and devoid of any intermediate characters between *M. persea* and *M. didyma* (Oorshot and Coutsis 2014, page 18). Thus, the name *Melitaea montium* represents a nominal taxon conspecific with *M. didyma*, and therefore can be synonymized with *M. didyma liliputana*, the oldest available name representing the distinct phylogenetic lineage (subspecies) distributed from north Israel, through Lebanon, Syria and east Turkey to Armenia (Pazhenkova et al. 2015, Pazhenkova and Lukhtanov 2016): *Melitaea didyma* race *liliputana* Oberthür, 1909 (Études de lépidoptérologie compare 3: 244, TL "Akbès" [SE Turkey, prov. Hatay, Akvez]) = *Melitaea montium* Belter, 1934, **syn. n.**

I should also note that, despite the valid lectotype designation resulting in this synonymy, the name *M. montium* could theoretically be preserved for a valid taxon under the plenary power of the International Commission on Zoological Nomenclature through a neotype designation. Such a possibility exists for the cases in which the existing name-bearing type of a nominal species-group taxon is not in taxonomic accord with the prevailing usage of names and stability or universality is threatened thereby (Article 75.6, http://iczn.org/iczn/index.jsp). However, in case of *M. montium* the article 75.6 can hardly be applied because de facto the prevailing usage cannot be calculated. After Belter's publication there were few cases when this name was used, and in each case the identity of the butterflies called *M. montium* was unclear.

In this situation I see no other way than following the latest comprehensive revision (Oorshot and Coutsis 2014) that established the synonymy: *M. didyma* = *M. montium* based on lectotype designation and analysis.

Conclusion

The *Melitaea persea* species complex consists of the following taxa:

M. persea Kollar, 1849
M. persea persea Kollar, 1849 (East Turkey, Armenia, Azerbaijan, Daghestan in Russian Caucasus, western, central and nothern parts of Iran)
M. persea paphlagonia Fruhstorfer, 1917 (NE Iran, probably also S. Turkmenistan)
M. eberti Koçak, 1980 (N. Iran)
M. higginsi Sakai, 1978 (Afghanistan)
M. acentria Lukhtanov sp. n. (Mt. Hermon in Israel, definitely also the neighboring territories of Syria and Lebanon)

The identity and taxonomic status of the *M. persea*-similar samples from north Lebanon, Jordan, Iraq, Pakistan, and Afghanistan remain still unclear. The populations from Lebanon characterized by the mitochondrial haplogroup P2 (Fig. 9) could actually represent (i) a distinct subspecies of *M. persea*, (ii) an undescribed subspecies of *M. acentria*, or even (iii) an undescribed species. Further morphological, molecular and chromosomal studies are required to select between these hypotheses.

Acknowledgements

I thank Dubi Benyamini, Vlad Dincă, Oleg Kosterin and Niklas Wahlberg who provided valuable comments on the paper. Asya Novikova (the Hebrew University of Jerusalem) and Elena Pazhenkova (Zoological Institute, St. Petersburg) helped with field studies in Israel. Elena Pazhenkova and Niklas Wahlberg donated their unpublished sequences: castaM8 and castaM9 (EP) and NW43-09 and NW43-10 (NW).

The financial support for all molecular, chromosomal and morphological studies was provided by the grant N 14-14-00541 from the Russian Science Foundation to the Zoological Institute of the Russian Academy of Sciences. The travels to Israel and field studies of V. Lukhtanov were supported by RFBR grants 15-04-01581 and 15-29-02533. The work was partially performed using equipment of the 'Chromas' Core Facility, the Centre for Molecular and Cell Technologies and the Department of Entomology of St. Petersburg State University. A part of this equipment was purchased with support of the St. Petersburg University grant 1.40.490.2017.

References

Belter G (1934) Neues aus der *Melitaea didyma* Esp.-Gruppe mit Berücksichtigung des Materials des Deutschen Entomologischen Instituts. Arbeiten über morphologische und taxonomische Entomologie aus Berlin-Dahlem 1(2): 105–115.

Benyamini D (2002) A Field Guide to the Butterflies of Israel. Keter Publishing House, Jerusalem, Israel, 248 pp. [In Hebrew]

Benyamini D (2016) The biology of the Persian Fritillary (*Melitaea persea*) and Glanville Fritillary (*Melitaea cinxia*) sharing the same hostplant on Mt. Hermon. Bulletin of the Israeli Lepidopterists Society 33(2): 22–25. [In Hebrew]

Brower AVZ (1994) Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proceedings of the National Academy of Sciences of the United States of America 91: 6491–6495. https://doi.org/10.1073/pnas.91.14.6491

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, Ma, USA, 545 pp.

de Lesse H (1960) Speciation et variation chromosomique chez les Lépidoptères Rhopalocères. Annales des Sciences Naturelles Zoologie et Biologie Animale, 12e série. 2: 1–223

deWaard JR, Ivanova NV, Hajibabaei M, Hebert PDN (2008) Assembling DNA barcodes: analytical protocols. In: Martin CC (Ed.) Environmental Genomics, Methods in Molecular Biology. Humana Press, Totowa, New Jersey, 410: 275–283. https://doi.org/10.1007/978-1-59745-548-0_15

Dupuis JR, Sperling FAH (2015) Repeated reticulate evolution in North American *Papilio machaon* group swallowtail butterflies. PLoS ONE 10(10): e0141882. https://doi.org/10.1371/journal.pone.0141882

Gompert Z, Fordyce JA, Forister MA, Shapiro AM, Nice CC (2006) Homoploid hybrid speciation in an extreme habitat. Science 314: 1923–1925. https://doi.org/10.1126/science.1135875

Gross FJ, Ebert G (1975) 2. Beitrag. Neue Taxa der Pieridae, Satyridae und Nymphalidae. Journal of Entomological Society of Iran. Supplementum 1: 8–45.

Hajibabaei M, deWaard JR, Ivanova NV, Ratnasingham S, Dooph RT, Kirk SL, Mackie PM, Hebert PDN (2005) Critical factors for assembling a high volume of DNA barcodes. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 360: 1959–1967. https://doi.org/10.1098/rstb.2005.1727

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Hesselbarth G, Oorschot H, Wagener S (1995) Die Tagfalter der Türkei unter Berücksichtigung der angrenzenden Länder. Selbstverlag Siegbert Wagener, Bocholt, Vol. 1–3, 1354 pp.

Higgins LG (1941) An illustrated catalogue of the Palearctic *Melitaea* (Lep. Rhopalocera). Transactions of the Royal Entomological Society of London 91: 175–365. https://doi.org/10.1111/j.1365-2311.1941.tb01045.x

Higgins LG (1955) A descriptive catalogue of the genus *Mellicta* Billberg (Lepidoptera: Nymphalidae) and its species, with supplementary notes on the genera *Melitaea* and *Euphydryas*. Transactions of the Royal Entomological Society of London 106: 1–131. https://doi.org/10.1111/j.1365-2311.1955.tb01262.x

Ivanova NV, deWaard JR, Hebert PDN (2006) An inexpensive, automation-friendly protocol for recovering high quality DNA. Molecular Ecology Resources 6: 998–1002. https://doi.org/10.1111/j.1471-8286.2006.01428.x

Kent R, Levanoni O, Banker E, Pe'er G, Kark S (2013) Comparing the response of birds and butterflies to vegetation-based mountain ecotones using boundary detection approaches. PLoS ONE 8(3): e58229. https://doi.org/10.1371/journal.pone.0058229

Kolesnichenko KA, Churkin SV (2004) Taxonomic notes on *Melitaea didymina* Staudinger, 1895 (Lepidoptera, Nymphalidae). Helios. Collection of lepidopterological articles 5: 91–100.

Kunte K, Shea C, Aardema ML, Scriber JM, Juenger TE, Gilbert LE, Kronfrost MR (2011) Sex chromosome mosaicism and hybrid speciation among tiger swallowtail butterflies. PLoS Genet 7(9): e1002274. https://doi.org/10.1371/journal.pgen.1002274

Kuznetsov GV, Stradomsky BV, Fomina ES (2014) Two forms of *Melitaea trivia* ([Denis et Schiffermuller], 1775) (Lepidoptera: Nymphalidae) from Volgograd Region (Russia): the systematic position on the basis of molecular genetic studies. Kavkazskii Entomologicheskii Byulleten 10(1): 119-120.

Lai Z, Nakazato T, Salmaso M, Burke JM, Tang S, Knapp SJ, Rieseberg LH (2005) Extensive chromosomal repatterning and the evolution of sterility barriers in hybrid sunflower species. Genetics 171: 291–303. https://doi.org/10.1534/genetics.105.042242

Larsen TB (1974) Butterflies of Lebanon. Classey Publications, Beirut, Lebanon, 256 pp.

Larsen TB (1975) Chromosome numbers and notes on testicular morphology of some Lebanese Rhopalocera (Insecta Lepidoptera). Entomologica Scandinavica 6(3/4): 253–260. https://doi.org/10.1163/187631275X00091

Leneveu J, Chichvarkhin A, Wahlberg N (2009) Varying rates of diversification in the genus *Melitaea* (Lepidoptera: Nymphalidae) during the past 20 million years. Biological Journal of the Linnean Society 97: 346–361. https://doi.org/10.1111/j.1095-8312.2009.01208.x

Long EC, Thomson RC, Shapiro AM (2014) A time-calibrated phylogeny of the butterfly tribe Melitaeini. Molecular Phylogenetics and Evolution 79: 69–81. https://doi.org/10.1016/j.ympev.2014.06.010

Lukhtanov VA, Kuznetsova VG (1989) The analysis of the karyotype variability in the butterflies of the *Melitaea didyma* group along with the evidence of the species distinctness of *M. latonigena* (Lepidoptera, Nymphalidae). Zoologichesky Zhurnal 68 (12): 38–46.

Lukhtanov VA, Pazhenkova EA, Novikova AV (2016a) Mitochondrial chromosome as a marker of animal migratory routes: DNA barcoding revealed Asian (non-African) origin of a tropical migrant butterfly *Junonia orithya* in south Israel. Comparative Cytogenetics 10(4): 671–677. https://doi.org/10.3897/CompCytogen.v10i4.11085

Lukhtanov VA, Shapoval NA, Dantchenko AV (2014) Taxonomic position of several enigmatic *Polyommatus* (*Agrodiaetus*) species (Lepidoptera, Lycaenidae) from Central and Eastern Iran: insights from molecular and chromosomal data. Comparative Cytogenetics 8(4): 313–322. https://doi.org/10.3897/CompCytogen.v8i4.8939

Lukhtanov VA, Sourakov A, Zakharov EV (2016b) DNA barcodes as a tool in biodiversity research: testing pre-existing taxonomic hypotheses in Delphic Apollo butterflies (Lepidoptera, Papilionidae). Systematics and Biodiversity 14(6): 599–613. https://doi.org/10.1080/14772000.2016.1203371

Lukhtanov VA, Dantchenko AV, Vishnevskaya MS, Saifitdinova AF (2015a) Detecting cryptic species in sympatry and allopatry: analysis of hidden diversity in *Polyommatus* (*Agrodiaetus*) butterflies (Lepidoptera: Lycaenidae). Biological Journal of the Linnean Society 116(2): 468–485. https://doi.org/10.1111/bij.12596

Lukhtanov VA, Shapoval NA, Anokhin BA, Saifitdinova AF, Kuznetsova VG (2015b) Homoploid hybrid speciation and genome evolution via chromosome sorting. Proceedings of the Royal Society B: Biological Sciences 282(1807): 20150157. https://doi.org/10.1098/rspb.2015.0157

Maddison WP, Maddison DR (2015) Mesquite: a modular system for evolutionary analysis. Version 3.04. http://mesquiteproject.org

Mallet J (2007) Hybrid speciation. Nature 446: 279–283. https://doi.org/10.1038/nature05706

Mavárez J, Salazar CA, Bermingham E, Salcedo C, Jiggins CD, Linares M (2006) Speciation by hybridization in *Heliconius* butterflies. Nature 441: 868–871. https://doi.org/10.1038/nature04738

Oorschot H van, Coutsis J (2014) The Genus *Melitaea* Fabricius, 1807 (Lepidoptera: Nymphalidae, Nymphalinae). Taxonomy and systematics with special reference to the male genitalia. Tshikolovets Publications, Pardubice, Czech Republic, 360 pp.

Pazhenkova EA, Lukhtanov VA (2016) Chromosomal and mitochondrial diversity in *Melitaea didyma* complex (Lepidoptera, Nymphalidae): eleven deeply diverged DNA barcode groups in one non-monophyletic species? Comparative Cytogenetics 10(4): 697–717. https://doi.org/10.3897/CompCytogen.v10i4.11069

Pazhenkova EA, Zakharov EV, Lukhtanov VA (2015) DNA barcoding reveals twelve lineages with properties of phylogenetic and biological species within *Melitaea didyma* sensu lato (Lepidoptera, Nymphalidae). ZooKeys 538: 35–46. https://doi.org/10.3897/zookeys.538.6605

Quek SP, Stuart JD, Itino T, Pierce NE (2004) Codiversification in an ant-plant mutualism: stem texture and the evolution of host use in *Crematogaster* (Formicidae: Myrmicinae) inhabitants of Macaranga (Euphorbiaceae). Evolution 58: 554–570. https://doi.org/10.1111/j.0014-3820.2004.tb01678.x

Racheli T (1980) Papilionoidea and Hesperioidea (Lepidoptera) collected during 1976 in Fars, south Iran. Nota Lepidopterologica 3(1-2): 73–89.

Rieseberg LH, Van Fossen C, Desrochers A (1995) Hybrid speciation accompanied by genomic reorganization in wild sunflowers. Nature 375: 313–316. https://doi.org/10.1038/375313a0

Rieseberg LH (1997) Hybrid origins of plant species. Annual Review of Ecology and Systematics 28: 359–389. https://doi.org/10.1146/ annurev.ecolsys.28.1.359

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Sakai S (1981) Butterflies of Afghanistan. Koudansha, Tokyo, 272 pp. [In Japanese]

Soltis PS (2013) Hybridization, speciation and novelty. Journal of Evolutionary Biology 26: 291–293. https://doi.org/10.1111/jeb.12095

Toth JP, Bereczki J, Varga Z, Rota J, Sramko G, Wahlberg N (2014) Relationships within the *Melitaea phoebe* species group (Lepidoptera: Nymphalidae): new insights from molecular and morphometric information. Systematic Entomology 39: 749–757. https://doi.org/10.1111/syen.12083

Tshikolovets VV (2011) Butterflies of Europe and the Mediterranean Area. Tshikolovets Publications, Pardubice, Czech Republic, [i–v]+544 pp.

Tuzov VK, Churkin S (2000) Genus *Melitaea*. In: Tuzov VK (Ed.) Guide to the Butterflies of Russia and Adjacent Territories (Lepidoptera, Rhopalocera). Volume 2, Pensoft Publishers, Sofia-Moscow, 59–76.

Vershinina AO, Lukhtanov VA (2010) Geographical distribution of the cryptic species *Agrodiaetus alcestis alcestis*, *A. alcestis karacetinae* and *A. demavendi* (Lepidoptera, Lycaenidae) revealed by cytogenetic analysis. Comparative Cytogenetics 4(1): 1–11. https://doi.org/10.3897/compcytogen.v4i1.21

Vishnevskaya MS, Saifitdinova AF, Lukhtanov VA (2016) Karyosystematics and molecular taxonomy of the anomalous blue butterflies (Lepidoptera, Lycaenidae) from the Balkan Peninsula. Comparative Cytogenetics 10(5): 1–85. https://doi.org/10.3897/CompCytogen.v10i5.10944

Wahlberg N, Zimmermann M (2000) Pattern of phylogenetic relationships among members of the tribe Melitaeini (Lepidoptera: Nymphalidae) inferred from mitochondrial DNA sequences. Cladistics 16: 347–363. https://doi.org/10.1006/clad.2000.0136

Wahlberg N, Brower AVZ, Nylin S (2005) Phylogenetic relationships and historical biogeography of tribes and genera in the subfamily Nymphalinae (Lepidoptera: Nymphalidae). Biological Journal of the Linnean Society 86(2): 227–251. https://doi.org/10.1111/j.1095-8312.2005.00531.x

Zimmermann M, Aubert J, Descimon H (1999) Molecular systematics of the Melitaeinae. Comptes Rendus de l'Acadmie des Sciences - Series III - Sciences de la Vie. 322(5): 429–439. https://doi.org/10.1016/S0764-4469(99)80079-1

Appendix

Table 1. List of *Melitaea* samples used in this study.

Taxon	BOLD ID	Field ID	GenBank	Locality	Reference
M. acentria	BPAL2759-15	CCDB-17969 A04	KY777527	Israel Hermon	This study
M. acentria	BPAL2236-13	CCDB-17949 E03	KT874736	Israel Hermon	Pazhenkova et al. 2015
M. acentria	BPAL2235-13	CCDB-17949 E02	KT874697	Israel Hermon	Pazhenkova et al. 2015
M. acentria	BPAL2234-13	CCDB-17949 E01	KY777528	Israel Hermon	This study
***M. acentria* Holotype**	**BPAL2191-13**	**CCDB-17949 A06**	**KY777529**	**Israel Hermon**	**This study**
M. acentria	BPALB127-16	CCDB-25458 C08	KY777530	Israel Hermon	This study
M. acentria	BPALB126-16	CCDB-25458 C07	KY777531	Israel Hermon	This study
M. acentria	BPALB129-16	CCDB-25458 C10	KY777532	Israel Hermon	This study
M. acentria	BPALB133-16	CCDB-25458 D02	KY777533	Israel Hermon	This study
M. acentria	BPALB131-16	CCDB-25458 C12	KY777534	Israel Hermon	This study
M. acentria	BPALB125-16	CCDB-25458 C06	KY777535	Israel Hermon	This study
M. acentria	BPALB130-16	CCDB-25458 C11	KY777536	Israel Hermon	This study
M. acentria	BPALB149-16	CCDB-25458 E06	KY777537	Israel Hermon	This study
M. acentria	BPALB132-16	CCDB-25458 D01	KY777538	Israel Hermon	This study
M. acentria	BPAL3193-16	CCDB-25453 E10	KY777539	Israel Hermon	This study
M. acentria	BPAL3194-16	CCDB-25453 E11	KY777540	Israel Hermon	This study
M. acentria	BPAL3257-16	CCDB-25454 C03	KY777541	Israel Hermon	This study
M. acentria	BPAL3361-16	CCDB-25452 C12	KY777542	Israel Hermon	This study
M. acentria	BPAL3360-16	CCDB-25452 C11	KY777543	Israel Hermon	This study
M. acentria	BPAL3359-16	CCDB-25452 C10	KY777544	Israel Hermon	This study
M. acentria	BPALB138-16	CCDB-25458 D07	KY777545	Israel Hermon	This study
M. acentria	BPAL3192-16	CCDB-25453 E09	KY777546	Israel Hermon	This study
M. acentria	BPALB128-16	CCDB-25458 C09	KY777574	Israel Hermon	This study
M. acentria	BPALB151-16	CCDB-25458	KY777575	Israel Hermon	This study
M. acentria	BPAL3191-16	CCDB-25453 E08	KY777576	Israel Hermon	This study
M. casta casta	BPAL164-10	RPVL-00164	HM404776	Iran Fars	This study
M. casta casta	BPAL2302-14	CCDB-17951 B10	KY777549	Iran Fars	This study
M. casta casta	BPAL2303-14	CCDB-17951 B11	KY777550	Iran Fars	This study
M. casta casta	BPAL163-10	RPVL-00163	HM404775	Iran Fars	This study
M. casta casta	BPAL2307-14	CCDB-17951 C03	KY777551	Iran Lorestan	This study
M. casta casta	BPAL2306-14	CCDB-17951 C02	KY777552	Iran Lorestan	This study
M. casta casta	BPAL162-10	RPVL-00162	HM404774	Iran Zarde-Kuh Mts	This study
M. casta casta	BPAL2304-14	CCDB-17951 B12	KY777553	Iran Esfahan	This study
M. casta casta	BPAL2305-14	CCDB-17951 C01	KY777554	Iran Esfahan	This study
M. casta casta	NW85-3		FJ462238	Iran Fars Kuh-e Sorkh	This study
M. casta casta	M8		KY867399	Iran, Fereidunshahr	This study
M. casta casta	M9		KY867400	Iran, Fereidunshahr	This study
M. casta wiltshirei	NW140-12		FJ462288	Iran Hamadan Alvand	Leneveu et al. 2009
M. deserticola	BPAL2354-14	CCDB-17951 G02	KY086108	Syria W Damascus	Pazhenkova and Lukhtanov 2016

Taxon	BOLD ID	Field ID	GenBank	Locality	Reference
M. deserticola	BPAL2353-14	CCDB-17951 G01	KY086107	Syria W Damascus	Pazhenkova and Lukhtanov 2016
M. deserticola	BPALB134-16	CCDB-25458 D03	KY086186	Israel Hermon 1800m	Pazhenkova and Lukhtanov 2016
M. deserticola	BPALB150-16	CCDB-25458 E07	KY086193	Israel Hermon 1800m	Pazhenkova and Lukhtanov 2016
M. deserticola	BPAL2704-14	CCDB-17968 D08	KY777564	Israel Hermon 2000m	This study
M. deserticola	BPAL2686-14	CCDB-17968 C02	KY777565	Israel Zafririm 300m	This study
M. deserticola	BPAL2857-15	CCDB-25449 A07	KY777566	Jordan	This study
M. deserticola	BPAL2886-15	CCDB-25449 C12	KY777567	Israel Kfar-Adumim	This study
M. deserticola	BPAL2885-15	CCDB-25449 C11	KY777568	Israel Kfar-Adumim	This study
M. deserticola	BPAL2860-15	CCDB-25449 A10	KY777569	Jordan	This study
M. deserticola	BPAL2898-15	CCDB-25449 D12	KY777570	Jsrael Kfar-Adumim	This study
M. deserticola	BPAL3124-15	CCDB-25451 G12	KY086157	Israel Jerusalem	Pazhenkova and Lukhtanov 2016
M. deserticola	BPAL2586-14	CCDB-17967 B09	KY777571	Israel Zafririm 300m	This study
M. deserticola	BPAL2585-14	CCDB-17967 B08	KY777572	Israel Zafririm	This study
M. didyma liliputana	BPALB143-16	CCDB-25458 D12	KY086192	Israel Hermon 1730m	Pazhenkova and Lukhtanov 2016
M. didyma liliputana	BPALB142-16	CCDB-25458 D11	KY086191	Israel Hermon 1730m	Pazhenkova and Lukhtanov 2016
M. didyma liliputana	BPALB139-16	CCDB-25458 D08	KY086190	Israel Hermon 1730m	Pazhenkova and Lukhtanov 2016
M. didyma liliputana	BPALB137-16	CCDB-25458 D06	KY086189	Israel Hermon 1730m	Pazhenkova and Lukhtanov 2016
M. didyma liliputana	BPALB135-16	CCDB-25458 D04	KY086187	Israel Hermon 1730m	Pazhenkova and Lukhtanov 2016
M. didyma liliputana	BPALB136-16	CCDB-25458 D05	KY086188	Hermon 1730m	Pazhenkova and Lukhtanov 2016
M. didyma liliputana	BPAL2718-14	CCDB-17968 E10	KT874743	Israel Hermon 1650m	Pazhenkova et al. 2015
M. higginsi	BPAL2482-14	CCDB-17966 A12	KY777547	Afghanistan	This study
M. higginsi	BPAL2469-14	CCDB-17950 H10	KY777548	Afghanistan	This study
M. persea (?)	NW34-10		AF187796	Lebanon Bsharree	Wahlberg and Zimmermann 2000
M. persea (?)	NW43-09		KY867398	Lebanon Laglong Mohafazat Jbail	This study
M. persea (?)	NW43-10		KY867397	Lebanon Laglong Mohafazat Jbail	This study
M. persea paphlagonia	BPAL2352-14	CCDB-17951 F12	KY777523	Iran Khorasan 2400m	This study
M. persea paphlagonia	BPAL2351-14	CCDB-17951 F11	KY777524	Iran Khorasan 2400m	This study
M. persea paphlagonia	BPAL2948-15	CCDB-25450 A03	KY777525	Iran Shahrud 2100m	This study
M. persea paphlagonia	BPAL2959-15	CCDB-25450 B02	KY777526	Iran Shahrud 2100m	This study

Taxon	BOLD ID	Field ID	GenBank	Locality	Reference
M. persea persea	BPAL2480-14	CCDB-17966 A10	KY777505	Iran Fars 2200m	This study
M. persea persea	BPAL2481-14	CCDB-17966 A11	KY777506	Iran Fars 2200m	This study
M. persea persea	BPAL2293-14	CCDB-17951 B01	KY777507	Iran Fars 2200m	This study
M. persea persea	BPAL2295-14	CCDB-17951 B03	KY777508	Iran Esfahan 2000m	This study
M. persea persea	BPAL2294-14	CCDB-17951 B02	KY777509	Iran Chahar Mahal-e Bakhtiari 2000m	This study
M. persea persea	BPAL2982-15	CCDB-25450 D01	KY777510	Russia Daghestan 125m	This study
M. persea persea	BPAL1704-12	CCDB-03033 H05	KY777511	Russia Daghestan 1750m	This study
M. persea persea	BPAL2983-15	CCDB-25450 D02	KY777512	Russia Daghestan 125m	This study
M. persea persea	BPAL1696-12	CCDB-03033 G09	KY777513	Russia Daghestan 2250m	This study
M. persea persea	BPAL2977-15	CCDB-25450 C08	KY777515	Azerbaijan Talysh 1650m	This study
M. persea persea	BPAL2984-15	CCDB-25450 D03	KY777516	Azerbaijan Talysh 1990m	This study
M. persea persea	BPAL1689-12	CCDB-03033 G02	KY777517	Azerbaijan 600m	This study
M. persea persea	BPAL2975-15	CCDB-25450 C06	KY777518	Azerbaijan Talysh 1650m	This study
M. persea persea	BPAL2976-15	CCDB-25450 C07	KY777519	Azerbaijan Talysh 1650m	This study
M. persea persea	BPAL2980-15	CCDB-25450 C11	KY777520	Azerbaijan Altyagach 1100m	This study
M. persea persea	BPAL2979-15	CCDB-25450 C10	KY777521	Azerbaijan Altyagach 1100m	This study
M. persea persea	BPAL2349-14	CCDB-17951 F09	KY777522	Iran Tehran 2000m	This study
M. persea persea	NW120-11		FJ462273	Iran Ardabil 2600–2800 m	Leneveu et al. 2009
M. persea/M. interrupta hybrid (?)	BPAL2542-14	CCDB-17966 F12	KY777514	Armenia Megri 1800m	This study
M. trivia syriaca	BPAL3098-15	CCDB-25451 E10	KY777555	Israel Hermon 2000m	This study
M. trivia syriaca	BPAL3125-15	CCDB-25451 H01	KY777556	Israel Jerusalem	This study
M. trivia syriaca	BPAL3122-15	CCDB-25451 G10	KY777557	Israel Jerusalem	This study
M. trivia syriaca	BPAL3121-15	CCDB-25451 G09	KY777558	Israel Jerusalem	This study
M. trivia syriaca	BPAL3116-15	CCDB-25451 G04	KY777559	Israel Jerusalem	This study

Taxon	BOLD ID	Field ID	GenBank	Locality	Reference
M. trivia syriaca	BPAL3115-15	CCDB-25451 G03	KY777560	Israel Jerusalem	This study
M. trivia syriaca	BPAL2858-15	CCDB-25449 A08	KY777561	Jordan	This study
M. trivia syriaca	BPAL2859-15	CCDB-25449 A09	KY777562	Jordan	This study
M. trivia syriaca	BPALB112-16	CCDB-25458 B05	KY777563	Israel Hermon 1450m	This study

CompCytogen 11(2): 359–373 (2017)
doi: 10.3897/CompCytogen.11i2.11142
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotypic differentiation of populations of the common shrew *Sorex araneus* L. (Mammalia) in Belarus

Yury M. Borisov[1], Iryna A. Kryshchuk[2], Helen S. Gaiduchenko[2], Elena V. Cherepanova[1], Svetlana V. Zadyra[3], Elena S. Levenkova[1], Dmitriy V. Lukashov[3], Victor N. Orlov[1]

1 *Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskij Prosp. 33, 119071 Moscow, Russia* **2** *Scientific and Practical Center for Bioresources, National Academy of Sciences of Belarus, Akademicheskaya St. 27, 220072 Minsk, Republic of Belarus* **3** *Shevchenko Kiev National University, Educational–Scientific Center Institute of Biology, Kiev, 03187 Ukraine*

Corresponding author: *Yury M. Borisov* (borisov2693@gmail.com)

Academic editor: *V. Lukhtanov* | Received 10 October 2016 | Accepted 8 April 2017 | Published 11 May 2017

http://zoobank.org/73C9364A-A3EF-4AF4-82BA-A5EB7A6D8925

Citation: Borisov YM, Kryshchuk IA, Gaiduchenko HS, Cherepanova EV, Zadyra SV, Levenkova ES, Lukashov DV, Orlov VN (2017) Karyotypic differentiation of populations of the common shrew *Sorex araneus* L. (Mammalia) in Belarus. Comparative Cytogenetics 11(2): 359–373. https://doi.org/10.3897/CompCytogen.11i2.11142

Abstract

The common shrews, *Sorex araneus* Linnaeus, 1758, inhabiting the territory of Belarus, are characterized by a significant variation in the frequency of Robertsonian (Rb) translocations. The frequency clines for translocations specific of three chromosome races: the West Dvina (*gm*, *hk*, *ip*, *no, qr*), Kiev (*g/m*, *hi*, *k/o*, *n*, *p*, *q*, *r*), and Białowieża (*g/r*, *hn*, *ik*, *m/p*, *o*, *q*) have already been studied in this territory. In this communication we report new data on polymorphic populations with Rb metacentrics specific of the Neroosa race (*go*, *hi*, *kr*, *mn*, *p/q*) in south-eastern Belarus, analyse the distribution of karyotypes in southern and central Belarus and draw particular attention to the fixation of the acrocentric variants of chromosomes in this area. The results show that certain Rb metacentrics specific of the Neroosa, West Dvina, Kiev, and Białowieża races (namely, *go* and *pq*; *ip*; *ko*; *hn* and *ik*, respectively) are absent in many polymorphic populations. Thus, the karyotypic differentiation of *S. araneus* in the studied area is determined by unequal spread of different Rb translocations and by fixation of acrocentric variants of specific chromosomes.

Keywords

Chromosome races, Robertsonian translocations, chromosomal differentiation, *Sorex araneus*

Introduction

The common shrew, *Sorex araneus* Linnaeus, 1758, a species inhabiting Eurasia, is a model object for population genetic studies due to its exclusive chromosomal polymorphism (for review, see: Wójcik et al. 2002, Shchipanov and Pavlova 2016). Four metacentric autosomes (*af*, *bc*, *jl*, and *tu*) and sex chromosomes (XX in females and XY1Y2 in males) are characteristic of species *S. araneus* (acrocentric morphs of chromosome arms *j* and *l* sporadically occur in populations through the species area), while ten autosomal arms (*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, and *r*) can be presented as acrocentrics or be fused as metacenetrics (Searle et al. 1991). The designations of chromosome arms are constant irrespective of their condition either as separate acrocentrics (*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*) or as arms of metacentrics (*gm*, *hk*, *ip*, *no*, *qr*).

A convenient methodic approach to describe the chromosomal polymorphism of *S. araneus* is a subdivision of populations into chromosome races. "A chromosome race of *Sorex araneus* is defined as a group of geographically contiguous or recently separated populations which share the same set of metacentrics and acrocentrics by descent" (Hausser et al. 1994). Chromosome races of *S. araneus* differ in the composition and numbers (one–five pairs) of metacentrics, which were formed by Robertsonian (Rb) translocations involving 10 pairs of acrocentric chromosomes, *g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, and *r* (Searle et al. 1991, Hausser et al. 1994). The karyotype with ten pairs of acrocentric autosomes *g–i*, *k*, *m–r* (number of autosomes in diploid set, 2NA, in this karyotype equals 28) is considered as initial in chromosomal evolution of *S. araneus* (Wójcik et al. 2002). Metacentrics formed from Rb translocations are referred to as "race-specific fused chromosomes" (or "race-specific metacentrics") (Zima et al. 1996). Monomorphic karyotypes are characteristic of some races (all race-specific Rb translocations are fixed), while polymorphism for 2–5 translocations was revealed in majority of chromosome races (see list of Shchipanov and Pavlova 2016).

The fixation of Rb translocations may have occurred in isolated small-size populations, for example, in glacial refugia. The data on mtDNA polymorphism in some European species of small mammals, including species of the genus *Sorex*, testify to the existence of multiple glacial refugia in Mediterranean and central Europe (Bilton et al. 1998, Deffontaine et al. 2005).

In the postglacial period, the previously isolated populations which migrated from refugia came into contact and hybridized with each other. The width of hybrid zones depends on the degree of chromosomal differences between contacting races. When races that differ in the combination of chromosome arms (metacentrics with monobrachial homology) make contact, narrow hybrid zones (0.5–5 km) are formed (Banaszek 1994, Narain and Fredga 1996, Szałaj et al. 1996, Bulatova et al. 2011, Orlov et al. 2012). If races which come into contact have no metacentrics with monobrachial homologies, wide hybrid zones are formed, and the clinal variation in Rb metacentric frequency can stretch for 50–100 km (Lukáčová et al. 1994, Brünner et al. 2002, Zima et al. 2003).

Six chromosome races: Kiev, Bobruysk, Białowieża, Turov, West Dvina, and Borisov, are known in the territory of Belarus by present time (Mishta et al 2000, Borisov et al 2010, 2014). Three of them, the Kiev, Białowieża, and West Dvina races, have continuous distribution ranges beyond the territory of Belarus. The Kiev race (*g/m*, *hi*, *k/o*, *n*, *p*, *q*, *r*) (Mishta 1994) inhabits the western territory of the Ukraine, and this race was recently discovered in southern Belarus (Borisov et al. 2014, 2016). The distribution area of the Białowieża race (*g/r*, *hn*, *ik*, *m/p*, *o*, *r*; Fredga and Nawrin 1977) stretches from eastern Poland to western Belarus (Mishta et al., 2000; Borisov et al. 2016). The West Dvina race (*gm*, *hk*, *ip*, *no*, *qr*; Bulatova et al. 2002) occupies a vast territory from the Valdai Hills to the Smolensk Upland, its southern boundary passes through Vitebsk region of Belarus (Bulatova et al. 2002, Borisov et al. 2008, Orlov and Borisov 2009). The Borisov race (*g/m*, *h/k*, *i*, *n/o*, *q/r*, *p*; Orlov and Borisov 2009), which is a derivative of race West Dvina, is distributed in the middle Berezina basin (Borisov et al. 2010). The Bobruysk (*g*, *h/i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*) and Turov (*g*, *h/k*, *i*, *m*, *n*, *o*, *p*, *q*, *r*) races were described in the vicinities of Bobruisk and Turov towns, respectively (Mishta et al. 2000).

The clinal variation in the frequencies of Rb metacentrics, similar to the clinal variation in wide hybrid zones, was observed in the polymorphic populations of the Kiev, Białowieża, West Dvina, and Borisov races in Belarus. Karyotypes with ten pairs of acrocentric chromosomes (*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*) were found in some of these polymorphic populations (Borisov et al. 2010, 2014). Such a pattern of karyotype distribution may be associated either with the selection against heterozygotes in the interracial hybrid zone ("acrocentric peak") (term by J. Searle 1986) or with the spread of the Rb translocations of the metacentric races in populations characterized by ten pairs of acrocentric chromosomes (Borisov et al. 2014, 2016).

In this communication, we report new data on the distribution of Rb metacentrics specific of the Neroosa race (*go*, *hi*, *kr*, *mn*, *p/q*; Bulatova et al. 2000) in southeastern Belarus, analyse the distribution of the Białowieża, and Kiev races in southern and central Belarus and draw particular attention to the fixation of the acrocentric chromosomes in this area.

Materials and methods

Animals were captured at seven sites within the low Pripyat and Dnieper River basins (Gomel' and Mogilev regions) in July–September, 2014 and in September, 2015 (Table 1).

The new material includes 75 *S. araneus* individuals (37 males and 38 females). Our karyological data on 290 shrews trapped in 2009–2013 (Borisov et al., 2010, 2014, 2016) and data on 252 shrews presented by other authors were used to determine the distribution range of race-specific metacentrics. In total, 603 individuals from 43 sites (an area from 51°22' to 54°55'N; from 23°10' to 34°10'E) have been analysed in this work (Suppl. material 1, Fig. 1). The study area is a mosaic of forest and meadow biotopes with occurrences of man-made landscapes.

Table 1. Collection sites, chromosome races and karyotypes of common shrews in the territory of Belarus. The numbers indicate localities in Fig. 1. Polymorphism for Rb translocation is indicated by slash (/). Ne, Neroosa; Ki, Kiev; Bi, Białowieża; Wd, West Dvina; Bs, Borisov. S.s., sample size; ?, attribution to any race is unclear.

No.	Locality	Latitude; Longitude	S.s.	Race	2NA	Karyotypes
4	Dobrush	52°24'59"N; 31°17'12"E	**7**			
			1	Ne	23	*g, hi, k/r, mn, o, p, q*
			3	Ne	24	*g, hi, k/r, m/n, o, p, q*
			1	Ne	24	*g, hi, k, mn, o, p, q, r*
			2	Ne	26	*g, hi, k, m, n, o, p, q, r*
5	Gomel'	52°25'29"N; 30°52'31"E	**4**			
			1	Ne	23	*g, hi, k/r, mn, o, p, q*
			2	Ne	24	*g, hi, k/r, m/n, o, p, q*
	*		1	Ne	24	*g, hi, k, mn, o, p, q, r*
6	settl. Chernoye (Rechitsa distr.)	52°26'47"N; 30°22'50"E	**23**			
			4	Ne	24	*g, hi, k/r, m/n, o, p, q*
			9	Ne	25	*g, hi, k, m/n, o, p, q, r*
			9	?	26	*g, hi, k, m, n, o, p, q, r*
			1	Ki	25	*g, hi, k/o, m, n, p, q, r*
7	settl. Krasnoye (Bragin distr.)	51°33'50"N; 30°29'55"E	**14**			
			5	Ne		*g, hi, k/r, m/n, o, p, q*
			3	Ne	25	*g, hi, k/r, m, n, o, p, q*
			4	Ne	26	*g, hi, k, m, n, o, p, q, r*
			2	Ki	25	*g, hi, k/o, m, n, p, q, r*
18.3	Bobruisk	53°4'12"N; 29°14'28"E	**8**			
			2	Ki	25	*g, hi, k/o, m, n, p, q, r*
			2	Ki	26	*g, h/i, k/o, m, n, p, q, r*
			3	Ki	26	*g, hi, k, m, n, o, p, q, r*
			1	Ki	27	*g, h, i, k/o, m, n, p, q, r*
31	settl. Elizovo (Bobruisk distr.)	53°24'20"N; 29°0'30"E	**4**			
			1	Bi	25	*g, hn, i/k, m, o, p, q, r*
			1	Bi	25	*g, h/n, ik, m, o, p, q, r*
			2	Bi	26	*g, h/n, i/k, m, o, p, q, r*
32	settl. Lyubonichi (Bobruisk Distr.)	53°15'19"N; 29°10'21"E	**14**			
			2	Bi	25	*g, hn, i/k, m, o, p, q, r*
			2	Bi	25	*g, h/n, ik, m, o, p, q, r*
			5	Bi	26	*g, h/n, i/k, m, o, p, q, r*
			1	Bi	27	*g, h/n, i, k, m, o, p, q, r*
			2	Bi	27	*g, h, i/k, m, n, o, p, q, r*
			2	Bi	28	*g, h, i, k, m, n, o, p, q, r*

Chromosome preparations were obtained from bone marrow and spleen cells after a routine technique with colchicine treatment (Ford and Hamerton 1956). Chromosome identification was carried out by G-banding method with trypsin (Seabright 1971) in accordance with the international common shrew chromosome nomenclature (Searle et al. 1991).

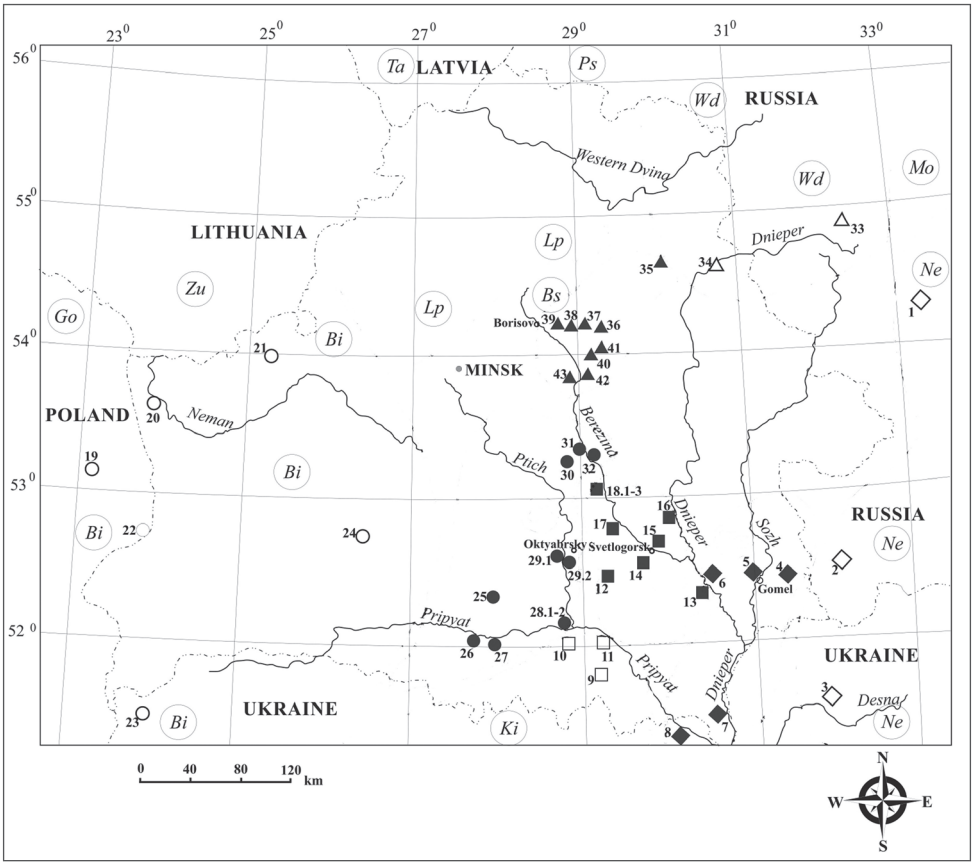


Figure 1. The distribution of the chromosome races of the common shrew in Belarus and neighbouring territories: Ne, Neroosa (diamonds); Ki, Kiev (squares); Bi, Białowieża (circles); Wd, West Dvina (light triangles); Bs, Borisov (black triangles); Go, Goldap; Zu, Zuvintas; Ta, Tallin; Ps, Pskov; Lp, Lepel; Mo, Moscow. See Suppl. material 1 for numbers of the collection sites. Dotted line indicates state borders.

Results

Karyotyping of *S. araneus* individuals captured in the southeastern territory of Belarus (at three sites to the east and one site to the west of the Dnieper River) helped to identify three Rb metacentrics, *hi*, *kr* and *mn* (Table 1, Fig. 1, nos. 4–7). These metacentrics are specific of the Neroosa race (*go*, *hi*, *kr*, *mn*, *p/q*); however, other two metacentrics of this race, *go* and *pq*, have not been found at the sites studied. The Rb translocation *hi* is fixed in all the examined samples, and chromosome arms *k*, *r* and *m*, *n* occur both as metacentric and acrocentric morphs (Table 1). The *kr* translocation was only found in heterozygous condition. Karyotypes with metacentric *ko* which is characteristic of the Kiev race (*g/m*, *hi*, *k/o*) were found in two samples from Rechitsa and Bragin districts (nos. 6 and 7).

In karyotypes of eight shrews captured near Bobruisk town (the west bank of the Berezina River), two Rb metacentrics of the Kiev race (*g/m*, *hi*, *k/o*), *hi* and *ko*, were observed (Table 1, Fig. 1, no. 18.3). The *hi* translocation appeared both in homozygous and heterozygous condition, and the *ko* translocation – only in heterozygous condition. Metacentric *gm* which is characteristic of the Kiev race was not revealed in our sample.

At two sites to the north of Bobruisk, on the west and east banks of the Berezina, karyotypes with two metacentrics of the Białowieża race (*g/r*, *hn*, *ik*, *m/p*), *hn* and *ik*, were found (Table 1, Fig. 1, nos. 31, 32). Heterozygotes for the *hn* and *ik* translocations prevailed in the samples. Metacentrics *gr* and *mp*, specific of this chromosome race, were not revealed.

Discussion

The new results of the karyological study of *S. araneus* populations in eastern Belarus together with previously published data show a considerable variation in the frequency of Rb metacentrics characteristic of the Neroosa race in this area and the closest territories. The Neroosa race (*go*, *hi*, *kr*, *mn*, and *p/q*; Bulatova et al. 2000) is monomorphic for four Rb translocations throughout its large distribution range in the Oka and Don River basins (Bystrakova et al. 2007). The acrocentric chromosomes *g*, *o*, *k,* and *r* were noted at some sites in Ukraine (Mishta et al. 2000) and near the Belarusian boundary, in the vicinity of Novozybkov city (see Suppl. material 1) where 2NA varied from 19 to 25 (Bulatova et al. 2000, Sheftel and Krysanov 2002). It is supposed that homozygotes for acrocentric morphs *g*, *o* and *p*, *q* existed among individuals 2NA=25 described by Sheftel and Krysanov (2002) (unfortunately, the data on individual karyotypes were not shown). However, the registered karyotypes with 2NA=19 testify to the presence of all metacentrics specific of the Neroosa race in populations from Novozybkov. As for populations in the Gomel region, only one Rb translocation, *hi*, is fixed; chromosome arms *k*, *r* and *m*, *n* are presented by acrocentric and metacentric morphs, while arms *g*, *o* and *p*, *q* are presented exclusively by acrocentric morphs (Tables 1, 2, nos. 4–7).

A significant variation in the Rb translocation frequency was earlier described in populations of chromosome races West Dvina, Borisov, Kiev, and Białowieża in the Dnieper–Pripyat interfluve (Orlov and Borisov 2009, Borisov et al. 2010, Borisov et al. 2014, 2016). It should be noted that the West Dvina race (*gm*, *hk*, *ip*, *no*, *qr*; Bulatova et al. 2002), like the Neroosa race, is monomorphic for five Rb translocations throughout most part of its distribution range, from the Valdai Hills to the Smolensk Upland (Borisov et al. 2008). Acrocentric morphs of chromosomes *n*, *o* and *q*, *r* were found to the south of the Smolensk Upland (Fig. 1, no. 34; see Suppl. material 1). The Borisov race (a derivative of the West Dvina race) inhabiting the territory along the Berezina River (nos. 35–39) is polymorphic for the *gm*, *hk*, *no,* and *qr* translocations; however, chromosomes *i* and *p* appear only as acrocentrics (Orlov and Borisov 2009, Borisov et al. 2010). Karyotypes with two Rb translocations, *gm* and *hk*, and karyotypes with ten pairs

Table 2. Frequencies of race-specific metacentrics in populations of eight chromosome races of *S. araneus*. Numbers of sites are the same as in Table 1 and in Fig. 1; the frequency of Rb translocations is an average of studied specimens from all samples. S.s., sample size; two-three digits in column "S.s". are arranged in the same order as references in column "References".

Nos, Sites	References	S.s.	Metacentric frequencies				
			race Neroosa				
			go	*hi*	*kr*	*mn*	*pq*
1: Spas-Demensk	Bulatova et al. 2000	6	1.0	1.0	1.0	1.0	1.0
2: Novozybkov	Bulatova et al. 2000	3	0.33	1.0	1.0	0.66	0.50
3. Berezna	Mishta et al. 2000	2	1.0	1.0	1.0	1.0	1.0
4–6: Dobrush, Gomel, Chernoye	new data	34	0.0	1.0	0.17	0.39	0.0
7: Krasnoye	new data	14	0.0	1.0	0.13	0.21	0.0
			race Kiev				
			gm	*hi*	*ko*		
9–11: Yeslk, Leshnya, Mozyr'	Borisov et al. 2014, 2016	2, 29	0.08	0.65	0.16		
12, 13: Ozarichi, Rechitsa	Borisov et al. 2014, 2016	3, 16	0.0	0.72	0.53		
14, 15: Sosnovyi Bor, Svetlogorsk	Borisov et al. 2016	10	0.0	0.65	0.15		
16, 17: Zhlobin Parichi	Borisov et al. 2016	25	0.0	0.50	0.19		
18.1–18.3: Bobruisk)	Borisov et al. 2016, Mishta et al. 2000, new data	2, 1, 8	0.0	0.67	0.33		
			race Białowieża				
			gr	*hn*	*ik*	*mp*	
19: Bialystok	Banaszek et al. 2009	56	0.91	1.0	1.0	0.71	
20: Grodno	Borisov et al. 2014	2	1.0	1.0	1.0	1.0	
21: Lesnoe Ozero	Mishta et al. 2000	5	0.20	1.0	1.0	0.30	
22: Białowieża	Wójcik et al. 1996	87	0.99	1.0	1.0	0.95	
24: Ganzevichi	Borisov et al. 2014	2	0.50	1.0	1.0	0.50	
25: Chervonoye	Borisov et al. 2014	15	0.03	0.67	0.50	0.05	
26, 27: Turov, Khvoyensk	Borisov et al. 2014, 2016	23, 21	0.01	0.44	0.58	0.00	
29: Oktiabr'skiy	Borisov et al. 2014, 2016	22, 19	0.01	0.33	0.37	0.01	
30–32: Tatarka, Elizovo, Lyubonichi Borisov et al. 2014, 2016, new data	Borisov et al. 2014, 2016, new data	14, 18	0.0	0.71	0.56	0.0	
			race West Dvina				
			gm	*hk*	*ip*	*no*	*qr*
33: Kardymovo	Orlov Borisov 2009	2	1.0	1.0	1.0	1.0	1.0
34: Dubrovno	Orlov, Borisov 2009	3	0.5	1.0	0.75	1.0	0.75
			race Borisov				
			gm	*hk*	*ip*	*no*	*qr*
35: Smolyany	Orlov, Borisov 2009	2	0.5	1.0	0.0	1.0	0.5
36–39: Malyi Vyazok – Novaya Metcha	Orlov, Borisov 2009, Borisov et al. 2010	5, 33	0.84	0.99	0.0	0.38	0.09
			Polymorphic populations of unclear attribution				
40, 41: Leskovichi Mikhevichi	Borisov et al. 2010	7	0.64	0.86	0.0	0.0	0.0
42, 43: Berezino, Yedlino	Orlov, Borisov 2009, Borisov et al. 2010	2, 27	0.02	0.59	0.0	0.0	0.0

of acrocentrics (*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*) were revealed in populations southwards of the town of Borisov (Table 2, nos. 40–43). The analysis of additional samples is required to determine whether these polymorphic populations may be attributed to the Borisov race.

In the populations to the west of the Dnieper River, in the lower part of the Berezina River basin, two metacentrics of the Kiev race (*g/m*, *hi*, *k/o*, Mishta 1994), namely, *hi* and *ko* (Fig. 2), are distributed (Table 2, Fig. 1, nos. 12–18); metacentric *gm* was not found (Borisov et al., 2016). All three Rb metacentrics specific of the Kiev race were revealed on the south bank of the Pripyat (nos. 9 - 11), however, the frequency of the *gm* metacentric occurred to be lower than in the samples from the Ukraine (Mishta et al. 2000). Taking into account the new data, we note that distribution area of Rb metacentrics *hi* and *ko* includes type locality of the Bobruysk race (*g*, *h/i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*; Mishta et al. 2000) (Suppl. material 1, nos 18.1 – 18.3). Hence, the individuals carrying a single Rb translocation *hi* may be regarded as representatives of polymorphic race Kiev.

The shrews with Rb metacentrics of the Neroosa race (*kr* and *mn*) and the shrews with metacentric of the Kiev race (*ko*) were found in samples from Rechitsa and Bragin districts, and hybrid individuals with metacentrics of both the races (simple heterozygotes *hi*, *k/o*, *m/n*) were found in the vicinity of Rechitsa city (Suppl. material 1, nos. 6 and 7, and 13). Thus, the hybrid zone between the polymorphic populations of the Neroosa and Kiev races approximately passes along the Dnieper. Racial attribution of homozygotes for the *hi* translocation from Rechitsa and Bragin districts is unclear (Fig. 1, nos. 6 and 7).

The polymorphism for Rb translocations was earlier detected in the *S. araneus* population (a sample of 14 individuals; Table 1, no. 8) from the neighbourhood of Chernobyl; 2NA varied from 24 to 26 (Baker et al. 1996). The chromosomes were not identified by differential G-banding; the animals were not affiliated with any race. Previously known data of Bulatova et al. (2000) and Mishta et al. (2000) and our new results (Suppl. material 1, Fig. 1, nos. 2, 3, 6, 7, 9, 13) suggest that both the shrews of the Neroosa and shrews of the Kiev races, may live in the neighbourhood of Chernobyl'. It cannot be excluded that the population at site near Chernobyl belongs to the contact zone of these chromosome races.

In the populations inhabiting the southwestern territory of Belarus along the Ptich River and at some sites of the south bank of the Pripyat River, the metacentrics of the Białowieża race (*g/r*, *hn*, *ik*, *m/p*, Fredga and Nawrin 1977), *hn* and *ik*, were observed (Fig. 1, nos. 25–32; see Suppl. material 1). As for the *gr* and *mp* metacentrics, they were only revealed at three of the mentioned sites (frequency did not exceed 0.05; Table 2, nos. 26, 27, 29) and were not found at four sites (nos. 15, 30–32). For comparison, in eastern Poland, the *hn* and *ik* translocations are fixed, and the minimal frequencies of the *gr* and *mp* translocations are equal to 0.91 and 0.71, respectively (Table 2, 19, 22) (Wójcik et al. 1996, Banaszek et al. 2009).

Hybrid individuals with Rb metacentrics of the Kiev and Białowieża races: simple heterozygotes (*hn*, *ko; g/m*, *h/n, i/k*) and complex heterozygotes (metacentrics with monobrachial homology: *i/hi/hn/n* and *i/ik/ko/o*, are present in their karyotypes), were revealed along the Ptich River and on the south bank of the Pripyat River, close to

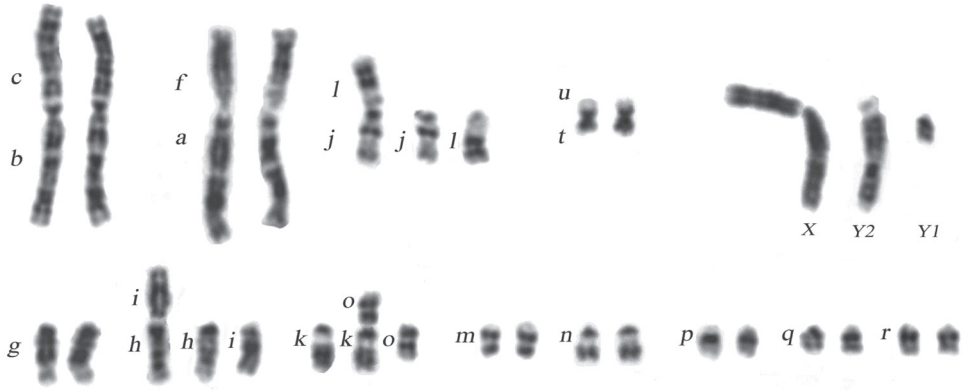


Figure 2. G-banded karyotype of male shrew of the Kiev race (Svetlogorsk vic., Belarus), *g, h/i, j/l, k/o, m, n, o, p, q, r* (2NA=26).

the confluence of the Pripyat and Uborot' Rivers (Suppl. material 1, nos. 10, 26, 27, and 29). The contact and hybrid zone between these races extends along the Ptich River and continues on the south bank of the Pripyat River. Karyotypes with ten pairs of acrocentrics were observed in some polymorphic populations of the Kiev and Białowieża races (Suppl. material 1, nos. 16, 17, 26–29.2, and 32).

There are two possible explanations of chromosome variation and a high frequency of acrocentrics in *S. araneus* populations in Belarus.

1) Hybridization between metacentric races which differ for the arm combinations of Rb metacentrics (*e.g.* chromosome arms *g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *r* are combined as metacentrics *gr*, *hn*, *ik*, *mp* in karyotype of the Białowieża race and as metacentrics *gm*, *hi*, *ko* in the karyotype of the Kiev race). The low fitness of hybrids (complex heterozygotes possessing metacentrics with monobrachial homology, *e.g. r/gr/gm/mp/p*, *n/hn/hi/ik/ko/o*) leads to the decrease of metacentric frequency. This phenomenon is called "acrocentric peak" (Searle 1986).

2) Hybridization between metacentric races and an acrocentric race that existed in the Dnieper basin in the past (Borisov et al. 2016).

Our hypothesis about the existence of acrocentric race in the present-day Belarus or neighbouring territory during the Last glacial maximum (LGM, 24–17 kyr BP) does not contradict the paleontological and paleobotanic data: fossil remains of the common shrew were found in the Middle Dnieper basin (Markova and Pusachenko 2008) and a forest refugium of LGM was revealed in this territory (Simakova and Pusachenko 2008). Proceeding from the data on the current distribution range of chromosome races and the data on the locations of Late Pleistocene forest refugia (Kozharinov 1994), we may come to the conclusion that the West Dvina, Neroosa, and Kiev races survived the most recent glacial period in the refugia of the Valdai Hills, the Middle Russian Upland, and the Carpathians (Orlov et al. 2008).

The karyotypic differentiation of *S. araneus* in the low Dnieper and Pripyat basin is determined by unequal spread of different Rb translocations and by fixation of acrocentric

variants of the particular chromosome arms. Each of four groups of the polymorphic populations possessing metacentrics, which are specific of the Neroosa, West Dvina, Kiev, and Białowieża races, consists of two subgroups: 1) polymorphic populations with all Rb metacentrics of the initial race, irrespective of their frequencies (Fig. 1, light figures); 2) polymorphic populations which lack 1-2 race-specific Rb metacentrics (Fig. 1, black figures). It should be stressed that the extent of distribution areas for the populations with fixed acrocentric morphs of definite chromosome arms exceeds 50 kilometres.

The subdivision of the populations of the *S. araneus* into chromosome races is a simplified methodic approach to describe the chromosomal polymorphism of this species (only the presence / absence of any Rb translocation is taken into consideration irrespective of its frequency; see Searle et al. 2003). According to the definition of a chromosome race as " … populations which share the same set of metacentrics and acrocentrics by descent" (Hausser et al. 1994), the populations that differ from neighbouring population owing to the presence or absence of a Rb translocation and occupy a definite area may be regarded as separate chromosome races. For example, the populations in Sweden that differ from the Abisko race (*g/m*, *h/n*, *i/p*, *k/q*, and *o/r*) by acrocentric variant of the arms *o* and *r* were recognized as the Ammarnas and Hattsjo races (Fredga 2007), and the population in the Rügen Island that differs from the mainland Danish race Jutland (*gm*, *hi*, *kq*, *no*) by acrocentric variant of arms *n* and *o* was recognized as the Rügen race (Brünner et al. 2002). Thus, the populations in the Dnieper – Pripyat interfluve which lack 1-2 race-specific Rb metacentrics, may be regarded as new chromosome races.

(1) The absence of metacentrics *go* and *pq*, that is, the fixation of acrocentric variants *g*, *o*, and *p*, *q* in *S. araneus* populations from the low Dnieper, Sozh and Pripyat basin (a total of 39 individuals from four sites; Tables 1 and 2, Fig. 1, nos. 4–7) allows them to be recognized as a race of its own called "Gomel' ".

Chromosome race: Gomel' (Gm)

Karyotype: XX/XY1Y2, *af*, *bc*, *g*, *hi*, *jl*, *k/r*, *m/n*, *o*, *p*, *q*, *tu*

Type locality: vicinity of Gomel' city, Belarus, 52°25'29"N, 30°52'31"E.

Distribution range: An area between Dnieper and Sozh Rivers, Gomel, and Rechitsa district; to the south of Rechitsa city to the latitude of Bragino city. The western boundary of the range lies along the west bank of the Dnieper River, approximately at the longitude of Rechitsa city. The other boundaries are not determined.

(2) All the three Rb metacentrics characteristic of the Kiev race occur only in populations to the south of the Pripyat River (Table 2, Fig. 1, nos. 9–11). We suppose that the polymorphic populations of the common shrew inhabiting the area between Dnieper and Pripyat Rivers (a total of 65 individuals from eight sites; Tables 1 and 2, Fig. 1, nos. 12–18), with fixed acrocentric morphs *g* and *m*, can be recognized as the Svetlogorsk race.

Chromosome race: Svetlogorsk (Sv)

Karyotype: XX/XY1Y2, *af*, *bc*, *g*, *h/i*, *j/l*, *k/o*, *m*, *n*, *p*, *q*, *r*, *tu* (see Fig. 2).

Type locality: vicinity of Svetlogorsk city, Belarus, 52°31'46"N, 29°34'49"E.

Distribution range: an area between Dnieper and Pripyat Rivers (Belarus, Gomel region); the low Berezina basin to the vicinity of Parichi and Zhlobin cities to the north, from the east bank of the Ptich River and to the west bank of the Dnieper River.

(3) The absence of metacentrics *gr* and *mp*, specific of the Białowieża race, in *S. araneus* populations inhabiting the territory along the Ptich River allows us to recognize these populations (a total of 104 individuals from nine sites) (Table 2; Fig. 1, nos. 25–27 and 29–32) as the Oktiabr'skiy race.

Chromosome race: Oktiabr'skiy (Ok)

Karyotype: XX/X Y1Y2, *af*, *bc*, *g*, *h/n*, *j/l*, *i/k*, *m*, *o*, *p*, *q*, *r*

Type locality: Rozhanov settlement, vicinity of Oktiabr'skiy town, Belarus 52°34'26"N, 28°44'37"E.

Distribution range: Southwestern Belarus, territory along the Ptich River, approximately to the latitude of Osipovichi city to the north. The western boundary is not determined. Easternmost site for shrews of this race is on the east bank of the Berezina River. The southern boundary extends along the south bank of the Pripyat River from Turov city to the confluence of the Ptich and Pripyat Rivers.

Now, 74 chromosome races of the common shrew (including 49 polymorphic ones) are known (Shchipanov and Pavlova 2016), however, the study of chromosomal polymorphism of this species is not yet completed. Equally with molecular-genetic and morphometric data, information on distribution and fixation of different Rb translocations in definite parts of the *S. araneus* range is very important for study of intraspecies structure of the *S. araneus*. Unequal spread of different Rb translocations and fixation of acrocentric variants of particular chromosome arms observed in each of these groups is an interesting example of karyotypic differentiation in populations of the common shrew.

Acknowledgements

The authors are grateful to Nikolay Alexander Shchipanov (DrSci senior researcher of Laboratory of Population Ecology, A.N. Severtsov Institute of Ecology and Evolution) for the assistance in capture of shrews in Bobruisk vic. in 2015. We wish to thank Robert Angus (scientific associate of Entomology Section, Natural History Museum, London, UK) for stylistic improvement of English. The study is supported by the Russian and the Belarusian Fund for Fundamental Research "RFBR-BRFFR" (http://www.rfbr.ru/rffi/ru/), Project No. 14-04-90006.

Reference

Baker RJ, Van Den Bussche RA, Wright AJ, Wiggins LE, Hamilton MJ, Reat EP, Smith MH, Lomakin MD, Chesser RK (1996) High levels of genetic change in rodents of Chernobyl. Nature 380(6576): 707–708. https://doi.org/10.1038/380707a0

Banaszek A (1994) The structure of the contact zone between the chromosome races Druzno and Łegucki Młyn in the common shrew (*Sorex araneus*) in north-eastern Poland. Folia Zoologica 43 (Suppl 1): 11–19.

Banaszek A, Taylor JRE, Ochocińska D, Chętnicki W (2009) Robertsonian polymorphism in the common shrew (*Sorex araneus* L.) and selective advantage of heterozygotes indicated by their higher maximum metabolic rates. Heredity 102: 155–162. https://doi.org/10.1038/hdy.2008.102

Bilton DT, Mirol PM, Mascheretti S, Fredga K, Zima J, Searle JB (1998) Mediterranean Europe as an area of endemism for small mammals rather than a source for northwards postglacial colonization. Proceedings of Royal Society London B 265(1402): 1219–1226. https://doi.org/10.1098/rspb.1998.0423

Borisov YuM, Kozlovsky AI, Balakirev AE, Demidova TB, Irchin SYu, Malygin VM, Okulova NM, Potapov SG, Shchipanov AN, Orlov VN (2008) Contact zones between chromosome races of the common shrew *Sorex araneus* L. (Insectivora, Mammalia) at the margins of the Veps Stage of the Valdai ice sheet. Sibirskii Ekologicheskii Zhurnal 15(5): 763–771. https://doi.org/10.1134/s1995425508050111 [In Russian with English translation in Contemporary Problems of Ecology 1(5): 583–589]

Borisov YuM, Cherepanova EV, Orlov VN (2010) A wide hybrid zone of chromosome races of the common shrew, *Sorex araneus* Linnaeus, 1758 (Mammalia), between the Dnieper and Berezina Rivers (Belarus). Comparative Cytogenetics 3(2): 195–201. https://doi.org/10.3897/compcytogen.v4i2.43

Borisov YuM, Kryshchuk IA, Cherepanova EV, Gajduchenko HS, Orlov VN (2014) Chromosomal polymorphism of populations of the common shrew, *Sorex araneus* L., in Belarus. Acta Theriology 59(2): 243–249. https://doi.org/10.1007/s13364-013-0160-y

Borisov YuM, Gaiduchenko HS, Cherepanova EV, Kryshchuk IA, Nikiforov ME, Orlov VN (2016) The clinal variation of metacentric frequency in the populations of the common shrew, *Sorex araneus* L., in the Dnieper and Pripyat interfluve. Mammal Research 61(2): 269–277. https://doi.org/10.1007/s13364-016-0272-2

Brünner H, Turni H, Kapischke HJ, Strubbe M, Vogel P (2002) New *Sorex araneus* karyotypes from Germany and the postglacial recolonization of Central Europe. Acta Theriologica 47(3): 277–293. https://doi.org/10.1007/BF03194147

Bulatova NSh, Searle JB, Bystrakova N, Nadjafova R, Shchipanov N, Orlov V (2000) The diversity of chromosome races in *Sorex araneus* from European Russia. Acta Theriologica 45(Suppl 1): 33–46. https://doi.org/10.4098/AT.arch.00-60

Bulatova NSh, Nadjafova RS, Krapivko TP (2002) Intraspecific phylogenetic relationships in *Sorex araneus* L.: the Southern Baltic Subgroup of chromosome races. Russian Journal of Genetics 38(1): 64–69. https://doi.org/10.1023/A:1013767828674 [In Russian with English translation in Genetika 38(1): 79–85]

Bulatova NSh, Jones RM, White TA, Shchipanov NA, Pavlova SV, Searle JB (2011) Natural hybridization between extremely divergent chromosome races of the common shrew (*Sorex araneus*, Soricidae, Soricomorpha): hybrid zone in European Russia. Journal of Evolutionary Biology 24: 573–586. https://doi.org/10.1111/j.1420-9101.2010.02191.x

Bystrakova N, Shchipanov NA, Bulatova N, Sheftel BI, Nadjafova RS, Pavlova SV, Demidova TB, Bobretsov AB, Alexandrov DYu, Kalinin AA, Kouptsov AV, Volkova AT, Oleinichenko

VYu, Searle JB (2007) New data on the geographic distribution of chromosome races of *Sorex araneus* (Soricidae, Eulipotyphla) in European Russia. Russian Journal of Theriology 6(1): 105–109.

Deffontaine V, Libois R, Kotlik P, Sommer R, Nieberding C, Paradis E, Searle JB, Michaux JR (2005) Beyond the Mediterranean peninsulas: evidence of central European glacial refugia for a temperate forest mammal species, the bank vole (*Clethrionomys glareolus*). Molecular Ecology 14: 1727–1739. https://doi.org/10.1111/j.1365-294X.2005.02506.x

Ford CE, Hamerton JL (1956) A colchicine hypothonic citrate, squash sequence for mammalian chromosomes. Stain Technology 31: 247–251. https://doi.org/10.3109/1052029560-9113814

Fredga K (2007) Reconstruction of the postglacial colonization of *Sorex araneus* into northern Scandinavia based on karyotype studies, and the subdivision of the Abisko race into three. Russian Journal of Theriology 6(1): 85–96.

Fredga K, Nawrin J (1977) Karyotype variability in *Sorex araneus* L. (Insectivora, Mammalia). Chromosomes Today 6: 153–161.

Hausser J, Fedyk S, Fredga K, Searl JB, Volobouev V, Wójcik JM, Zima J (1994) Definition and nomenclature of the chromosome races of *Sorex araneus*. Folia Zoologica 43(Suppl 1): 1–9.

Kozharinov AV (1994) Vegetation dynamic of Eastern Europe in late glaciation – Holocene. Autoreferat of Ph.D. Dissertation, Moscow, Russian Federation: Institute of Ecology and Evolution, Russian Academy of Sciences, 47 pp. [In Russian]

Lukáčová L, Pialec J, Zima J (1994) A hybrid zone between the Ulm and Drnholec karyotypic races of *Sorex araneus* in the Czech Republic. Folia Zoologica 43(Suppl 1): 37–42.

Markova AK, Pusachenko AYu (2008) Mammal assembles of the Last Glacial Maximum (LGM, 24 – 17 kyr. BP). In: Markova A, Van Kolfschoten T (Eds) Evolution of the European Ecosystems during Pleistocene-Holocene Transition (24 – 8 kyr. BP). KMK Scientific Press, Moscow, 91–116. [In Russian, with English summary]

Mishta A (1994) A karyological study of the common shrew, *Sorex araneus* L. (Insectivora, Soricidae) in Ukraine: first results. Folia Zoologica (Brno) 43(Suppl 1): 63–68.

Mishta AV, Searle JB, Wójcik JM (2000) Karyotypic variation of the common shrew *Sorex araneus* in Belarus, Estonia, Latvia, Lithuania and Ukraine. Acta Theriology 45(Suppl 1): 47–58. https://doi.org/10.4098/AT.arch.00-61

Narain Y, Fredga K (1996) A hybrid zone between the Hallefors and Uppsala chromosome races of *Sorex araneus* in central Sweden. Hereditas 125(2-3): 137–145. https://doi.org/10.1111/j.1601-5223.1996.00137.x

Orlov VN, Borisov YuM (2009) Phylogenetic relationships between the common shrew (*Sorex araneus* L., Insectivora) populations in Belarus according to karyological data. Zoologichesky Zhurnal 88(12): 1506–1514. [In Russian, with English summary]

Orlov VN, Kozlovskii AI, Balakirev AE, Borisov YuM (2008) Fixation of metacentric chromosomes in populations of the common shrew, *Sorex araneus* L., from Eastern Europe. Russian Journal of Genetics 44(5): 581–593. [Original Russian article: Genetika (2008) 44(5): 501–512]. PMID: 18672791.

Orlov VN, Borisov YuM, Cherepanova EV, Grigor'eva OO, Sycheva VB (2012) Narrow hybrid zone between Moscow and Western Dvina chromosome races and specific features of

population isolation in common shrew *Sorex araneus* (Mammalia). Russian Journal of Genetics 48(1): 70–78. [Original Russian article: Genetika 48(1): 80–88]. PMID: 22567857.

Seabright M (1971) A rapid banding technique for human chromosomes // lancet 11: 971– 972.

Searle J (1986) Factors responsible for a karyotypic polymorphism in the common shrew, *Sorex araneus*. Proceedings of Royal Society of London B 229: 277–298. https://doi.org/10.1098/rspb.1986.0086

Searle JB, Fedyk S, Fredga K, Hausser J, Volobouev VT (1991) Nomenclature for the chromosomes of the common shrew (*Sorex araneus*). Memoires de la Societe Vaudoise des Sciences Naturelles. 19: 13–22. [Reprinted in: Comparative Cytogenetics (2010) 47(1): 87–96 . https://doi.org/10.3897/compcytogen.v4i1.28]

Shchipanov NA, Pavlova SV (2016) Multi-level subdivision in the species group "*araneus*" of the genus *Sorex* . 1. Chromosomal differentiation. Zoologicheskii Zhurnal 95(2): 216–233. [In Russian with English summary]

Sheftel BI, Krysanov EY (2002) Chromosome polymorphism of the Neroosa race (*Sorex araneus*) in the territory with radioactive pollution after the Chernobyl accident. In: Volobuev V (Ed.) 6-th Meeting of the International *Sorex araneus* Cytogenetics Committee (ISACC). Paris, September 3-7, 2002, 29–30.

Simakova A, Puzachenko A (2008) The vegetation during the Last Glacial Maximum (LGM) (24.0–17.0 kyr BP) In: Markova A, Van Kolfschoten T (Eds) Evolution of the European Ecosystems during Pleistocene-Holocene Transition (24–8 kyr. BP). KMK Scientific Press, Moscow, 315–341. [In Russian, with English summary]

Szałaj KA, Fedyk S, Banaszek A, Chętnicki W, Ratkiewicz M (1996) A hybrid zone between two chromosome races of the common shrew, *Sorex araneus*, in eastern Poland. Preliminary results. Hereditas 125: 169–176. https://doi.org/10.1111/j.1601-5223.1996.00169.x

Wójcik JM, Borodin PM, Fedyk S, Fredga K, Hausser J et al (2003) The list of chromosome races of the common shrew, *Sorex araneus* (updated 2002). Mammalia 68: 169–179. https://doi.org/10.1515/mamm.2003.67.2.169

Wójcik JM, Ratkiewicz M, Searle JB (2002) Evolution of the common shrew *Sorex araneus*: chromosomal and molecular aspects. Acta Theriologica 47(Suppl 1): 139–167. https://doi.org/10.1007/BF03192485

Wójcik JM, Wójcik AM, Zalewska H (1996) Chromosome and allozyme variation of the common shrew, *Sorex araneus*, in different habitats. In: Fredga K, Searl JB (Eds) Evolution in the *Sorex araneus* Group. Cytogenetic and Molecular aspects. Proc of the ISAACC's 5th Intern Meeting. Hereditas, Offprint 125: 183–189. https://doi.org/10.1111/j.1601-5223.1996.00183.x

Zima J, Fedyk S, Fredga K, Hausser J, Mishta A, Searle JB, Volobouev VT, Wójcik JM (1996) The list of the chromosome races of the common shrew (*Sorex araneus*). In: Fredga K, Searl JB (Eds) Evolution in the *Sorex araneus* Group. Cytogenetic and Molecular aspects. Proc of the ISAACC's 5th Intern Meeting. Hereditas, Offprint 125: 97–107. https://doi.org/10.1111/j.1601-5223.1996.00097.x

Zima J, Slivková L, Tomášková L (2003) New data on karyotypic variation in the common shrew, *Sorex araneus*, from the Czech Republic: an extension of the range of the Laska race. Mammalia 68(2): 209–215. https://doi.org/10.1515/mamm.2003.67.2.209

Supplementary material 1

Collection sites, chromosome races and karyotypes of common shrews in the Dnieper and Pripyat river basins (Belarus) and neighboring areas

Authors: Yury M. Borisov, Iryna A. Kryshchuk, Helen S. Gaiduchenko, Elena V. Cherepanova, Svetlana V. Zadyra, Elena S. Levenkova, Dmitriy V. Lukashov, Victor N. Orlov

Data type: occurence

CompCytogen 11(2): 375–392 (2017)
doi: 10.3897/CompCytogen.11i2.11779
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

B chromosome in *Plantago lagopus* Linnaeus, 1753 shows preferential transmission and accumulation through unusual processes

Manoj K. Dhar[1], Gurmeet Kour[1], Sanjana Kaul[1]

1 *School of Biotechnology, University of Jammu, Jammu-180006, INDIA*

Corresponding author: *Manoj K. Dhar* (manojkdhar@rediffmail.com)

Academic editor: *Ilya Kirov* | Received 12 January 2017 | Accepted 13 March 2017 | Published 22 May 2017

http://zoobank.org/1605026B-DDE2-4D98-BC62-DBA79D1330F9

Citation: Dhar MK, Kour G, Kaul S (2017) B chromosome in *Plantago lagopus* Linnaeus, 1753 shows preferential transmission and accumulation through unusual processes. Comparative Cytogenetics 11(2): 375–392. https://doi.org/10.3897/CompCytogen.11i2.11779

Abstract

Plantago lagopus is a diploid (2n = 2x =12) weed belonging to family Plantaginaceae. We reported a novel B chromosome in this species composed of 5S and 45S ribosomal DNA and other repetitive elements. In the present work, presence of B chromosome(s) was confirmed through FISH on root tip and pollen mother cells. Several experiments were done to determine the transmission of B chromosome through male and female sex tracks. Progenies derived from the reciprocal crosses between plants with (1B) and without (0B) B chromosomes were studied. The frequency of B chromosome bearing plants was significantly higher than expected, in the progeny of 1B female × 0B male. Thus, the B chromosome seems to have preferential transmission through the female sex track, which may be due to meiotic drive. One of the most intriguing aspects of the present study was the recovery of plants having more chromosomes than the standard complement of 12 chromosomes. Such plants were isolated from the progenies of B chromosome carrying plants. The origin of these plants can be explained on the basis of a two step process; formation of unreduced gametes in 1B plants and fusion of unreduced gametes with the normal gametes or other unreduced gametes. Several molecular techniques were used which unequivocally confirmed similar genetic constitution of 1B (parent) and plants with higher number of chromosomes.

Keywords

Accumulation, drive, FISH, Molecular markers, Unreduced gametes

Introduction

B chromosomes are dispensable genetic elements that do not recombine with the chromosomes of the standard complement (A chromosomes). Generally, Bs differ morphologically from A chromosomes. B chromosomes have been observed to be heterochromatic in nature, composed mainly of repetitive or genetically inert DNA (Jones et al. 2008b, Kumke et al. 2016). In some cases heterochromatin content of B chromosomes has been reported to be similar to that of A chromosomes (Jones and Houben 2003). B chromosomes contain no genes with major function necessary for the growth and development of the plant. However, Carchilan et al. (2009) isolated 16 putative B chromosome-associated transcripts in rye by cDNA-AFLP, which constitute 0.7% of the total transcripts, thereby suggesting presence of some important genes on B chromosome. The mode of inheritance of B chromosomes is non-Mendelian, irregular and they follow their own evolutionary pathway. Bs persist in the populations which is exemplified by their widespread occurrence in plants; angiosperms, gymnosperms, some ferns, bryophytes and fungi besides, animals, including mammals (Jones and Rees 1982).

B chromosomes have generally been considered as nuclear parasites since their mode of inheritance is autonomous and drive ensures their survival in the population. Drive is the property that qualifies the B chromosomes as selfish elements. The various mechanisms of drive (reviewed by Chiavarino et al. 1998) include: i) the suppression of meiotic loss especially when only one B chromosome is present, ii) non- disjunction at the second pollen mitosis, and iii) higher competitive ability of B chromosome carrying pollen grain. The drive can occur at any stage of life cycle and has accordingly been classified as pre-meiotic, meiotic and post-meiotic (Camacho et al. 2000). In pre-meiotic drive, B chromosomes increase in number in the germline cells and when the latter enter meiosis to form gametes, the mean number of B chromosomes increases. This type of pre-meiotic accumulation has been observed in *Locusta migratoria* Linnaeus, 1758 (Viseras et al. 1990) and *Crepis capillaris* (Linnaeus) Wallr., 1840 (Parker et al. 1989, 1990). Meiotic drive depends on the functional symmetry of meiotic products. There are reports on existence of meiotic drive in some grasshopper species (Camacho 2005). Post-meiotic drive occurs immediately after meiosis during the development of the male and female gametophyte (Jones 1991). The molecular mechanisms involved in drive were not known for long, but recently Banaei-Moghaddam et al. (2012) showed meiotic drive to be due to non-disjunction of chromatids of B chromosome.

The B chromosomes show unstable meiotic behaviour, but have preferential segregation to the nuclei, which form gametes (Jones and Houben 2003). Also in rye, non-disjunction of Bs and unequal spindle formation at first pollen mitosis are responsible for the accumulation and transmission of B chromosomes at a higher rate to the next generation (Banaei-Moghaddam et al. 2012). Many B chromosomes have transmission rates clearly higher than 50%, which leads to their accumulation in the subsequent generations (Jones et al. 2008b). Accumulation of B chromosomes has been reported through female sex track in several plants and animals (Hewitt 1973).

In some cases the B chromosomes accumulate during male meiosis (Nur 1962), while in few organisms accumulation is through both the sexes.

Variation in transmission rate of B chromosomes is a common feature since these tend to be lost in some progenies while they increase in number in others. The genetic control of the transmission rate of B chromosomes has been demonstrated in some plant species (Bougourd and Plowman 1996, Puertas et al. 2000). It was suggested that in maize a single major gene located on A chromosome controls B chromosome transmission by acting in the haploid egg cell at the time of fertilization (Chiavarino et al. 2001). In rye, non-disjunction of Bs and unequal spindle formation at first pollen mitosis are responsible for the accumulation and transmission of B chromosomes at a higher rate to the next generation (Banaei-Moghaddam et al. 2012).

Plantago Linnaeus, 1753 is a large genus, of annual/perennial herbs and sub-shrubs, with a worldwide distribution. It is the only genus within the family Plantaginaceae and is based on about 200 species (Rahn 1996). *Plantago lagopus* is a small (about 30 cm tall), annual herb. It grows as a weed in the Mediterranean region. The diploid chromosome number of the species is 2n = 2x = 12. *P. lagopus* is genetically unstable which is reflected in the presence of aneuploidy (Dhar and Koul 1995). Dhar et al. (2002) reported a novel B chromosome in *Plantago lagopus*, whose main body is composed of 5S rDNA and has few 45S rDNA sequences at the ends. The authors presented the experimental evidence of *de novo* origin of novel B chromosome in *P. lagopus* through specific DNA sequence amplification. Using molecular cytogenetic techniques like FISH and Fiber-FISH, Kour et al. (2014) further characterized this chromosome and reported it to be a mixture of rDNA sequences and transposable elements.

In order to explore the mechanism of accumulation of B chromosome in *Plantago lagopus*, extensive crossing experiments were conducted. Based on the data so obtained, transmission of B chromosome through male and female sex tracks was calculated. These studies are expected to throw light on the existence of drive in B chromosome of *P. lagopus* besides, understanding the mechanism of perpetuation in the populations.

Materials and methods

The data on male and female transmission of B chromosome was collected over a period of 5 years (2005–2010). Seeds of *Plantago lagopus* were sown in earthen pots during October, every year. Generally, the seeds germinated within 4-5 days. After about 2 months, the seedlings were transplanted to the experimental beds in the Jammu University Botanic Garden.

Before transferring to the soil, young seedlings were uprooted from the pots and root tips excised. The root tips were used for cytological studies to determine the presence of B chromosome(s). The root tips were stained with Feulgen stain and squashed in 1% acetocarmine. For meiotic studies, young floral buds were used for cytology. Slides were prepared using anthers from freshly fixed buds squashed in 1% acetocarmine. Three cytotypes were used for the present investigation; plants with standard

complement of 12 chromosomes (0B), plants with one B chromosome in addition to the standard complement (1B) and plants with two B chromosomes in addition to the standard complement (2B). For Fluorescence in situ hybridization (FISH) the protocol of Dhar et al. (2002) was followed. Probes for 45S (pTa71) (Gerlach and Bedbrook 1979) and 5S ribosomal DNA (pPov1) (Dhar et al. 2002) were used for FISH. The signals were visualized using a Zeiss Axioskop microscope equipped with phase contrast and epifluorescence.

Transmission

The progenies raised after selfing and crossing were analyzed to determine the mode of transmission of B chromosomes. To work out the transmission of B chromosomes through male and female sex tracks, crosses were attempted between the 0B, 1B and 2B plants. The seeds obtained from different crosses were sown in the pots during October every year. The chromosome number of progeny plants was determined from the seedlings.

Single stranded DNA conformation polymorphism (SSCP) analysis

For SSCP analysis, PCR was performed using the 5S rDNA specific primers. PCR product (5µl) was denatured by adding the loading dye consisting of 95% formamide, 10% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) followed by its immediate quenching on ice. The samples were electrophoresed on 6% polyacrylamide gel in 0.5X TBE. For staining polyacrylamide gels, silver staining protocol of Bassam et al. (1991) was followed.

Sequence specific amplified polymorphism (SSAP) analysis

SSAP analysis was performed as per the protocol of Pearce et al. (1999). The primers selected for the analysis were MKD-4, MKD-5, MKD-9, MDK-11, ASW-8, ASW-9 and ASW-10 (Kour et al. 2009). A total of twelve different combinations were made using these primers (Suppl. material 1: Table 1). The PCR amplified fragments were electrophoresed on 6% polyacrylamide denaturing gel in 0.5X TBE followed by silver staining. Only reproducible bands were considered for scoring.

Simple Sequence Repeat (SSR) analysis

For SSR amplification four primer pairs were used as given by Squirrell and Wolff (2001) for *Plantago major*, and ten other primers were tried (Suppl. material 1: Table 2).

PCR amplification of SSR loci was performed in 20µl reaction mixture containing 20ng DNA, 1X PCR buffer, 2.5mM $MgCl_2$, 0.5µmol each primer, 200µM dNTPs, 1U *Taq* polymerase (Fermentas, USA). The thermal cycling conditions were as follows: initial denaturation at 94°C for 4min followed by 35 cycles of 94°C for 30sec, 55-65°C for 30sec, extension at 72°C for 1min and final extension for 10min at 72°C. The amplification products were resolved on 10% polyacrylamide denaturing gel in 0.5X TBE and subsequently visualized by silver staining. For each microsatellite locus, size of the alleles was estimated by comparison with standard size. After scoring the allelic bands, other bands were also scored.

Results

During the present investigation, about 531 plants were screened cytologically for the presence of the B chromosome (Fig. 1a, 1b), since it is not possible to distinguish plants with and without B chromosomes, morphologically. In *P. lagopus* the size of the B chromosome is almost equal to one of the A chromosomes, therefore, we used FISH with 5S rDNA probe, to identify the B chromosome(s) in 1B and 2B plants (Fig. 1c–f). As has been demonstrated in our earlier study (Dhar et al. 2002), the B chromosome gets completely painted when 5S rDNA is used as a probe (Fig. 1d). Therefore, in a 0B plant 5S rDNA signals were found only on two chromosomes (Fig. 1c). Similarly, 45S rDNA probe clearly identified the B chromosome, as the FISH signals were observed at the two ends of the B chromosome, besides the signals on the pair of NOR bearing chromosomes (Fig. 1e). In 2B plants, FISH with 5S rDNA probe clearly identified B chromosomes at somatic pro-metaphase and metaphase (Fig. 2a–b).

Preferential transmission to progeny

The transmission of B chromosomes was ascertained through male and female sex tracks by attempting various reciprocal crosses. When 1B plant was used as a male, transmission rate was in accordance with the Mendelian expected ratio of 1:1 ($p=0.05$; $\chi^2= 0.058$) (Table 1). In the reciprocal crosses, when 1B plant was used as female parent, progeny based on a total of 187 plants was screened for chromosome number (Table 2); the ratio between the plants with and without B chromosome(s) was 3.1:1. Thus, it is clear that frequency of B chromosome bearing plants was higher than the Mendelian expected value ($p < 0.05$, $\chi^2=50.30$) when 1B chromosome plant was used as female parent. The deviation obtained from the Mendelian ratio was significant. These results clearly indicate that the B chromosome has preferential transmission through the female sex track.

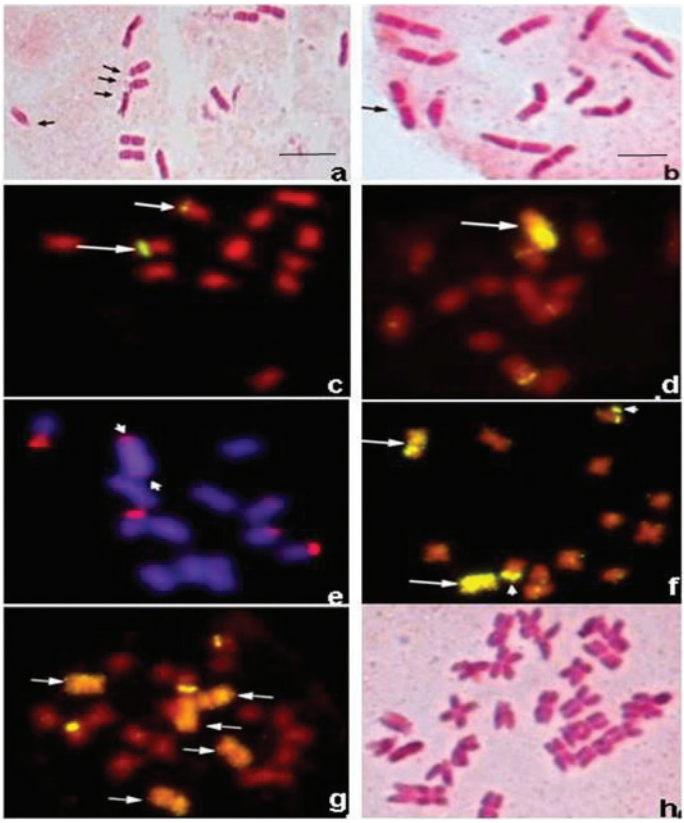


Figure 1. Root tip mitosis in different plants. **a** Metaphase spread showing 12 standard (A) chromosomes of *Plantago lagopus*. NOR bearing chromosomes have been marked **b** Metaphase spread showing 12 A and one B chromosome. Note the B chromosome is indistinguishable **c** Mapping of 5S rDNA sequences in 0B plant using FISH. Note the presence of 5S rDNA signals on two A chromosomes **d** Painting of B chromosome with 5S rDNA probe, besides signals of 5S rDNA on two A chromosomes **e** A metaphase spread containing one B chromosome probed with 45S rDNA, showing two additional NOR sites (arrow heads) **f** 5S rDNA probed metaphase spread showing 12 A and two B chromosomes **g** FISH of a metaphase spread of 23-chromosome plant revealing the presence of 5 B chromosomes **h** Metaphase spread showing 28 chromosomes. Scale bars: 10μm.

Meiotic behaviour of B chromosome

To understand the meiotic behaviour of B chromosome in 1B plants, a total of 1450 pollen mother cells from 145 plants were scanned at anaphase I and II. At metaphase-I the B chromosome remained as a univalent and at anaphase-I, in 1160 (80%) cells the B chromosome was present at one of the poles. In majority of the cases, the B chromosome seemed to have reached the pole earlier than the A chromosomes. At anaphase-II, the B chromosome divided into chromatids, which segregated to the poles. Similarly, in 2B plant at metaphase-I of meiosis, B chromosomes existed either as a bivalent (Fig. 2c) or two univalents (Fig. 2d). During anaphase-I the two chromosomes moved

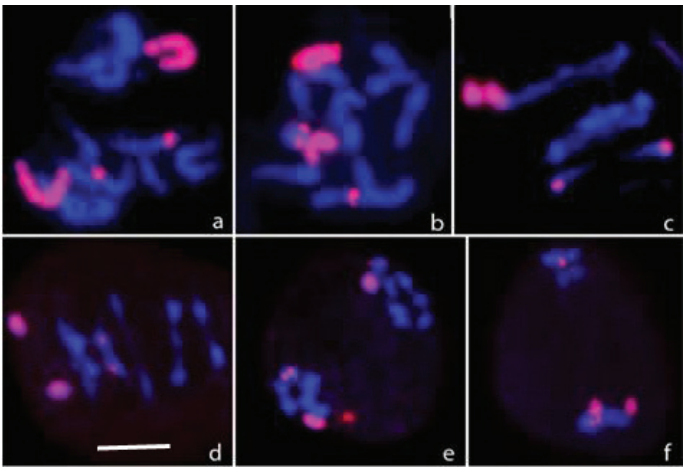


Figure 2. FISH on cells having 2 B chromosomes. Root tip metaphase cells at prometaphase (**a**) and metaphase (**b**) showing two B chromosomes. Segregation pattern of B chromosomes during meiosis (**c–f**). Note the two B chromosomes at the same pole (**f**). Scale bar: 10μm.

Table 1. Data on crosses among 0B (female) × 1B (male) plants.

Parent plants (0B × 1B)	Progeny plants			Total plants		Ratio
	0B	1B	2B	0B	B	0B:B
36-9-07 × 36-1-07	06	09	06	06 (28.5)	15 (71.4)	1:1.2
36-9-07 × 36-2-07	09	06	03	09 (50.0)	09 (50.0)	1:1
40-6-07 × 40-11-07	18	09	09	18 (50.0)	18 (50.0)	1:1
40-6-07 × 40-13-07	27	21	09	27 (47.3)	30 (52.6)	1:1.1
1M-4-06 × 1M-1-06	15	06	0	15 (71.4)	06 (28.5)	2.5:1
TOTAL	75	51	27	75 (49.01)	78 (50.9)	1:1.04

Percentage in parenthesis.

Table 2. Data on crosses among 1B (female) × 0B (male) plants.

Parent plants (1B × 0B)	Progeny plants			Total plants		Ratio
	0B	1B	2B	0B	B	0B: B
36-1-07 × 36-9-07	06	24	09	06 (15.4)	33 (84.6)	1:5.5
36-2-07 × 36-9-07	03	09	06	03 (16.6)	15 (83.33)	1:5
40-11-07 × 40-6-07	03	04	03	03 (30.0)	07 (70.0)	1:2.3
40-13-07 × 40-607	21	24	12	21 (36.8)	36 (63.2)	1:1.7
1M-1-06 × 1M-4-06	06	21	06	06 (18.2)	27 (81.8)	1:4.5
35-2-06 × 35-5-07	06	18	06	06 (20.0)	24 (80.0)	1:4
TOTAL	45	100	42	45 (24.06)	142 (75.93)	1:3.1

Percentage in parenthesis.

to the opposite or to the same pole (Fig. 2e, f). In 2B plants, 670 pollen mother cells were scanned, of which 490 cells showed B chromosomes at the poles earlier than the A chromosomes.

Accumulation of B chromosomes

In some progenies of 1B plants, rarely, plants with very high number of chromosomes were observed, which included plants having 23, 26 and 28 chromosomes (Fig. 1g, h). FISH analysis of 23-chromosome plant with 5S rDNA probe revealed its chromosome constitution as 18A + 5B chromosomes. Similarly, plants with 26 and 28 chromosomes had 24A + 2B chromosomes and 24A + 4B chromosomes, respectively (figures not given). In other words, the B chromosomes were found in addition to triploid and tetraploid states.

Origin of plants with 26 and 28 chromosomes

In order to trace the origin of plants with 2n = 26 and 2n = 28 chromosomes, three molecular markers were tried. The details are presented below:

SSCP analysis

As shown in our earlier study (Dhar et al. 2002) and the present study, B chromosome is mainly composed of 5S rDNA sequences, therefore, SSCP analysis was carried out by targeting these sequences. For this purpose, DNA isolated from the selfed progeny plants of 1B and 2B parents (separately), including plants with 26 and 28 chromosomes, respectively, was used. The banding pattern of 1B (mother plant) and 26 chromosome progeny plant, 2B (mother plant) and 28 chromosome progeny plant showed 100% similarity. The remaining plants in the two progenies composed of 0B, 1B and 2B plants showed variable banding pattern (Suppl. material 3: Fig. S2).

SSAP analysis

The SSAP exploits the insertional polymorphism of long terminal repeats (LTR retrotransposons) in the genome. In the present case SSAP analysis was used with twelve different primer combinations. It was observed that plant with 26 chromosomes showed 100% similarity with the 1B mother plant while other progeny plants showed polymorphism. Similarly, progeny plant with 28 chromosomes recovered from the selfed progeny of 2B plant showed 100% similarity with its mother plant as compared to other progeny plants.

SSR analysis

For the present investigation we used 14 SSR primers. From SSR data generated demonstrated that 1B and 2B mother plants showed 100% similarity with the 26 (Suppl.

material 4: Fig. S3a, b) and 28 (Suppl. material 4: Fig. S3c, d) progeny plants respectively, while differences were detected in other progeny plants.

The results obtained from the above three molecular markers clearly establish the maternal origin of 26 and 28 chromosome plants.

Discussion

Structure and behavior of B chromosome

In *Plantago lagopus* Dhar et al. (2002) demonstrated that the B chromosome is mainly composed of 5S rDNA sequences. Using FISH and reverse GISH techniques, the entire chromosome was found to get painted with 5S rDNA probe, while 45S rDNA sequences were localized at the two ends, just below the telomeric sequences. There are several reports on identification of B chromosomes using FISH (reviewed in Jones et al. 2008a). Most of the B chromosomes reported in different organisms are heterochromatic mainly due to the presence of repetitive DNA sequences made of satellite DNA, ribosomal DNA and transposable elements (Martis et al. 2012). Since 0B, 1B and 2B plants are indistinguishable morphologically (Dhar et al. 2002), therefore, in order to confirm the chromosomal status of the parents and the progeny plants, FISH with 5S rDNA probe was used in the present investigation.

The B chromosome does not pair or recombine with any A chromosome. B chromosomes, in general, have been reported to follow non-Mendelian mode of inheritance (Jones and Rees 1982) attributed to their irregular mitotic and meiotic behavior (Jones 1991). The meiotic behavior of the B chromosome in the pollen mother cells was very interesting. During anaphase, the B chromosome generally reached the poles earlier than A chromosomes. Thus, the B chromosome showed meiotic drive, which ensures its segregation and subsequent inclusion in the microspore mother cells.

Transmission of the B chromosome

In the present case, transmission of the B chromosome was ascertained through male and female sex tracks by following the progenies of the reciprocal crosses. Interestingly, when 1B plant was used as a male, transmission rate was in accordance with the expected Mendelian ratio. The differences in segregation ratio observed among various cross combinations can be attributed to the heterozygous nature of *P. lagopus* - being a cross-pollinated species. On the other hand, when 1B plant was used as a female, there was significant deviation from 1:1 ratio; frequency of B chromosome bearing plants was higher than the Mendelian expected value. These results clearly indicate preferential transmission of B chromosome through the female sex track. According to Houben and Carchilan (2012), variation in transmission rate is a common feature of B inheritance, such that the Bs tend to distort Mendelian expectation in their favor. Their

rate of transmission can be irregular with different levels of meiotic and post-meiotic drive or drag (Beukeboom 1994, Rusche et al. 1997). The drive through the female sex track can be explained on the basis of the fact that during female meiosis in plants, only one out of the four meiotic products survives. Therefore, in order to ensure inclusion in the surviving megaspore/egg, the B chromosome must be hosting a meiotic drive locus, as has been shown in *Mimulus* Linnaeus, 1753 - a phenomenon called female meiotic drive (Fishman and Saunders 2008). These authors have demonstrated that selfish chromosomal drive can be an important fitness determinant in natural populations. It can therefore be concluded that the B chromosome of *P. lagopus* is perpetuated preferentially due to drive, expressed during both male and female meiosis. The same is true of rye, where the drive happens in both male and female sex tracks (Jones and Houben 2003).

Accumulation of B chromosomes

One of the important characteristics of B chromosomes is their accumulation in selfed or outcrossed progenies. In some species the accumulation is mainly due to non-disjunction of B chromosomes during pollen mitosis. The present case is perhaps the first in plants where the entire complement of the species gets duplicated in presence of a B chromosome. Earlier, Bidau (1987) reported the presence of macrospermatids (>diploid chromosome number) in B containing individuals of a grasshopper, *Dichroplus pratensis* Bruner, 1900. The formation of macrospermatids was attributed to nuclear fusion. In the present case, the plants with 23, 26 and 28 chromosomes were obtained from the selfed progenies of B chromosome bearing plants. The origin of these plants can be attributed to formation of unreduced gametes, followed by their fusion with other gametes or their endoreduplication and parthenogenetic development of the plant. Earlier, it has been postulated by Sharma et al. (1985) that in *P. lagopus* unreduced egg of the trisomic mother plant may have developed parthenogenetically giving rise to plants with 13 chromosomes. The authors reported formation of aneutriploid individuals with 19 chromosomes (Sharma et al. 1985) in the progeny of a cross between a trisomic and disomic plants. Similarly, Bhan et al. (1990) reported an autotetraploid in *P. lagopus* isolated from an experimental population, which could be the result of fusion of unreduced gametes.

Formation of unreduced gametes has been reported in many plants and has been proposed as an important mechanism for origin of polyploids (Mason et al. 2011, Mason and Pires 2015).

Molecular markers in tracing origin of 26 and 28 chromosome plants

To substantiate the proposed mode of origin of plants with 26 and 28 chromosomes, recovered from selfed progeny of B chromosome plants, recourse was taken to mo-

lecular markers namely SSCP, SSAP and SSR. For SSCP analysis we targeted those sequences, which are present on the B chromosome. The technique was used to check whether Single Nucleotide Polymorphism exists among 1B and 2B mother plants and their progeny plants, including 26 and 28 chromosome plants. The SSCP pattern of 1B (mother) and 26 (progeny) plant showed 100% similarity, as compared to rest of the progeny plants. Similarly 2B (mother) and 28 chromosome progeny plants showed monomorphic pattern of bands, while in other 14 chromosome plants polymorphic pattern was observed. Thus SSCP analysis of 5S rDNA showed that the mother plants and the higher chromosome plants are 100% similar, thereby suggesting that they must have originated from the maternal genome. The experiments were repeated 3-4 times, however, similar SSCP pattern was observed in the mother plant and the offsprings with 26 and 28 chromosomes. SSCP technique is known to detect variation due to SNPs. Recombination is known to affect the SNPs; the SNP variation is less in regions of low recombination while it is more in high recombination regions (Brumfield et al. 2003). Recombination frequencies vary due to several genetic and non-genetic factors such as sex, the genetic background, genes and structures involved in meiotic recombination, age, irradiation, chemicals, nutrient salts and antibiotics (Barth et al. 2000). Occurrence of such processes more frequently during sexual reproduction in comparison to asexual reproduction, will lead to generation of SNPs in the former. This gets exemplified in the present case, by detection of large number of SNPs among progeny plants bearing 1B and 2B chromosomes than in 26 or 28 chromosome plants.

SSAP has also been used for the recombination studies in the selfed progeny plants of *Pisum* Linnaeus, 1753 (Jing et al. 2007). The marker has been used to detect the variation at genic level due to recombination and the combination of male and female genome mutation. Similarly, the inheritance of the B chromosome at the genome level has been analyzed using the SSR markers in *Brassica* sp. Linnaeus, 1753 (Navabi et al. 2011). SSAP and SSR used in the present case have also shown the same pattern in case of mother and the progeny plant having higher chromosome number. The molecular data does not support occurrence of recombination events in the origin of 26 and 28 chromosome plants, therefore, it can be presumed that the latter have originated through a two step process; formation of unreduced gametes in the parent, followed by parthenogenetic development of unreduced gametes, as has been proposed by Sharma et al. (1985).

Unreduced gametes and parthenogenetic development

Apomixis, or clonal propagation by seed, has been reported in many genera of higher plants following the gametophytic apomixis (Carman 1997). Plants arising from apomixis retain the maternal genotype. The main components of apomixis include unreduced gamete formation and parthenogenetic development (Koltunow and Grossniklaus 2003), which are also exemplified by the plants studied in present investigation. The genes and pathways involved in gametophytic apomixis have not been discovered

as yet and are the subject of intense research (Hand and Koltunow 2014). Polyploidy is the common feature of almost all apomicts (Roche et al. 2001). B chromosomes have been documented in apomictic species (Jones and Rees 1982), however, there is no report on apomixis gene(s) necessarily residing on a B-chromosome (Roche et al. 2001). In an animal species, B-chromosomes were found in polyploid individuals reproducing by pseudogamous parthenogenesis but were conspicuously absent in the diploid sexual individuals (Beukeboom et al. 1998).

Preferential transmission of B chromosomes and occurrence of plants with high chromosome numbers (2n =23, 26, 28) can have serious implications in the evolution of *Plantago lagopus* genome and the speciation. Recently, in *Arabidopsis thaliana* (Linneaus) Heynh., 1842, unreduced gamete-producing mutants, on account of defects in the meiotic cell cycle machinery, have been identified which has further advanced our understanding of the mechanisms behind unreduced gamete formation (Brownfield and Kohler, 2011). In the present case, plants with 23, 26 and 28 chromosomes were isolated in the progenies of the plants carrying B chromosome(s), therefore, it can be presumed that some DNA element located on B chromosome is activating the gene(s) promoting unreduced gamete formation, which is (are) located on A chromosome. Similar observations have been made in rye (Carchilan et al. 2009). However, more intense molecular studies need to be conducted to identify the gene/genes responsible in *P. lagopus*.

Funding

This work was supported by Department of Science and Technology, Government of India (No. SR/SO/PS-07/2001), DST-DAAD (No. INT/FRG/DAAD/P-186/2009) and CSIR (No. 9/100(0169/2K12-EMR-I).

Acknowledgements

The authors are grateful to Prof. Andreas Houben (IPK, Gatersleben, Germany) for his generous support and valuable suggestions.

References

Banaei-Moghaddam AM, Schubert V, Kumke K, Weiss O, Klemme S, Nagaki K, Macas J, Gonzalez-Sanchez M, Heredia V, Gomez-Revilla D, Gonzalez-Garcia M, Vega JM, Puertas MJ, Houben A (2012) Nondisjunction in favor of a chromosome: the mechanism of rye B chromosome drive during pollen mitosis. Plant Cell 24: 4124–4134. https://doi.org/10.1105/tpc.112.105270

Barth S, Melchinger AE, Devezi-Savula B, Lübberstedt T(2000) A high-throughput system for genome-wide measurement of genetic recombination in *Arabidopsis thaliana* based

on transgenic markers. Functional and Integrative Genomics 1: 200–206. https://doi.org/10.1007/s101420000030

Bassam BJ, Anolles GC, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Analytical Biochemistry 196: 80–83. https://doi.org/10.1016/0003-2697(91)90120-I

Beukeboom LW (1994) Bewildering Bs: an impression of the 1st B-chromosome conference. Heredity 73: 328–336. https://doi.org/10.1038/hdy.1994.140

Beukeboom LW, Sharbel TF, Michiels NK (1998) Reproductive modes, ploidy distribution, and supernumerary chromosome frequencies of the flatworm *Polycelis nigra* (Platyhelminthes: Tricladida). Hydrobiologia 383: 277–285. https://doi.org/10.1023/A:1003460132521

Bhan A, Dhar MK, Langer A (1990) Genetic diversity in Plantagos. XX. A tetraploid *Plantago lagopus* L. Cytologia 55: 511–517. https://doi.org/10.1508/cytologia.55.511

Bidau CJ (1987) Influence of a rare unstable B-chromosome on chiasma frequency and non-haploid sperm production in *Dichroplus pratensis* (Melanoplinae, Acrididae). Genetica 73: 201–210. https://doi.org/10.1007/BF00055275

Bougourd SM, Plowman AB (1996) The inheritance of B chromosomes in *Allium schoenoprasum* L. Chromosome Research 4: 151–158. https://doi.org/10.1007/BF02259709

Brownfield L, Köhler C (2011) Unreduced gamete formation in plants: mechanisms and prospects. Journal of Experimental Botany 62: 1659–1668. https://doi.org/10.1093/jxb/erq371

Brumfield RT, Beerli P, Nickerson DA, Edwards SV (2003) The utility of single nucleotide polymorphisms in inference of population history. Trends in Ecology and Evolution 18: 249–256. https://doi.org/10.1016/S0169-5347(03)00018-1

Camacho JPM (2005) B chromosomes. In: Gregory TR (Ed.) The Evolution of the Genome. Elsevier, San Diego, 223–286. https://doi.org/10.1016/b978-012301463-4/50006-1

Camacho JPM, Sharbel TF, Beukeboom LW (2000) B-chromosome evolution. Philosophical Transactions of the Royal Society of London. Series B, Biological Science 355: 163–178. https://doi.org/10.1098/rstb.2000.0556

Carchilan M, Kumke K, Mikolajewski S, Houben A (2009) Rye B chromosomes are weakly transcribed and might alter the transcriptional activity of A chromosome sequences. Chromosoma 118: 607–616. https://doi.org/10.1007/s00412-009-0222-8

Carman JG (1997) Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. Biological Journal of the Linnean Society 61: 51–94. https://doi.org/10.1111/j.1095-8312.1997.tb01778.x

Chiavarino AM, Gonzalez-Sanchez M, Poggio L, Puertas MJ, Rosato M, Rosi P (2001) Is maize B chromosome preferential fertilization controlled by a single gene? Heredity 86: 743–748. https://doi.org/10.1046/j.1365-2540.2001.00894.x

Dhar MK, Koul AK (1995) Four novel teloaneuploids of *Plantago lagopus* L. Journal of Heredity 86: 243–245. https://doi.org/10.1093/oxfordjournals.jhered.a111571

Dhar MK, Friebe B, Koul AK, Gill BS (2002) Origin of an apparent B chromosome by mutation, chromosome fragmentation and specific DNA sequence amplification. Chromosoma 111: 332–340. https://doi.org/10.1007/s00412-002-0214-4

Fishman L, Saunders A (2008) Centromere-associated female meiotic drive entails male fitness costs in monkeyflowers. Science 322: 1559–1562. https://doi.org/10.1126/science.1161406

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Research 7: 1869–1885. https://doi.org/10.1093/nar/7.7.1869

Hand ML, Koltunow AM (2014) The genetic control of apomixis: asexual seed formation. Genetics 197: 441–450. https://doi.org/10.1534/genetics.114.163105

Hewitt GM (1973) Variable transmission rates of a B chromosome in *Myrmeleotettix maculates* (Thunb.) (Acrididae: Orthoptera). Chromosoma 40: 83–106. https://doi.org/10.1007/BF00319837

Houben A, Carchilan M (2012) Plant B chromosomes: what makes them different? In: Bass HW, Birchler JA (Eds) Plant cytogenetics: genome structure and chromosome function. Plant genetics and genomics: Crops and models. Springer Verlag, New York, 59–77. https://doi.org/10.1007/978-0-387-70869-0_3

Jing R, Johnson R, Seres A et al. (2007) Gene-based sequence diversity analysis of field pea (*Pisum*). Genetics 177: 2263–2275. https://doi.org/10.1534/genetics.107.081323

Jones RN (1991) B-chromosome drive. The American Naturalist 137: 430–442. https://doi.org/10.1086/285175

Jones RN, Houben A (2003) B chromosomes in plants: escapees from the A chromosome genome? Trends in Plant Science 8: 417–423. https://doi.org/10.1016/S1360-1385(03)00187-0

Jones RN, Rees H (1982) B chromosomes. Academic Press, London.

Jones RN, Viegas W, Houben A (2008a) A century of B chromosomes in plants: so what? Annals of Botany 101: 767–775. https://doi.org/10.1093/aob/mcm167

Jones RN, Gonzalez-Sanchez M, Gonzalez-Garcia M, Vega JM, Puertas MJ (2008b) Chromosome with a life of their own. Cytogenetics and Genome Research 120: 265–280. https://doi.org/10.1159/000121076

Koltunow AM, Grossniklaus U (2003) Apomixis: A developmental perspective. Annual Review of Plant Physiology 54: 547–74. https://doi.org/10.1146/annurev.arplant.54.110901.160842

Kour G, Kour B, Kaul S, Dhar MK (2009) Genetic and epigenetic instability of amplification-prone sequences of a novel B chromosome induced by tissue culture in *Plantago lagopus* L. Plant Cell Reports 28: 1857–1867. https://doi.org/10.1007/s00299-009-0789-9

Kour G, Kaul S, Dhar MK (2014) Molecular characterization of repetitive DNA sequences from B chromosome in *Plantago lagopus* L. Cytogenetic and Genome Research 142: 121–128. https://doi.org/10.1159/000356472

Kumke K, Macas J, Fuchs J, Altschmied L, Kour J, Dhar MK, Houben A (2016) *Plantago lagopus* B chromosome is enriched in 5S rDNA-derived satellite DNA. Cytogenetics and Genome Research 148: 68–73. https://doi.org/10.1159/000444873

Martis MM, Klemme S, Banaei-Moghaddam AM, Blattner FR, Macas J, Schmutzer T, et al. (2012) Selfish supernumerary chromosome reveals its origin as a mosaic of host genome and organellar sequences. Proceedings of the National Academy of Sciences USA 109: 13343–13346. https://doi.org/10.1073/pnas.1204237109

Mason AS, Pires JC (2015) Unreduced gametes: meiotic mishap or evolutionary mechanism? Trends in Genetics 31: 5–10. https://doi.org/10.1016/j.tig.2014.09.011

Mason AS, Nelson MN, Yan G, Cowling WA (2011) Production of viable male unreduced gametes in *Brassica* interspecific hybrids is genotype specific and stimulated by cold temperatures. BMC Plant Biology 11: 103. https://doi.org/10.1186/1471-2229-11-103

Navabi ZK, Stead KE, Pires JC, Xiong Z, Sharpe AG, Parkin IA, Rahman MH, Good AG (2011) Analysis of B-genome chromosome introgression in interspecific hybrids of *Brassica napus* × *B. carinata*. Genetics 187: 659–673. https://doi.org/10.1534/genetics.110.124925

Nur U (1962) A supernumerary chromosome with an accumulation mechanism in the lecanoid genetic system. Chromosoma 13: 249–271. https://doi.org/10.1007/BF00577042

Parker JS, Jones GH, Edgar L, Whitehouse C (1989) The population cytogenetics of *Crepis capillaris* II. The stability and inheritance of B-chromosomes. Heredity 63: 19–27. https://doi.org/10.1038/hdy.1989.71

Parker JS, Jones GH, Edgar L, Whitehouse C (1990) The population cytogenetics of *Crepis capillaris* III. B-chromosomes effect on meiosis. Heredity 67: 377–385. https://doi.org/10.1038/hdy.1990.47

Puertas MJ, Jiménez G, Manzanero S, Chiavarino AM, Rosato M, Naranjo CA, Poggio L (2000) Genetic control of B chromosome transmission in maize and rye. In: Olmo E, Redi CA (Eds) Chromosomes Today. Birkhäuser Verlag: Switzerland. Vol. 13: 79–92. https://doi.org/10.1007/978-3-0348-8484-6_7

Pearce SR, Stuart-Rogers C, Kumar A, Flavell AJ (1999) Rapid isolation of plant Ty1-copia group retrotransposons LTR sequences for molecular marker studies. Plant Journal 19: 1–7. https://doi.org/10.1046/j.1365-313x.1999.00556.x

Rahn KA (1996) Phylogenetic study of the Plantaginaceae. Botanical Journal of the Linnean Society 120: 145–198. https://doi.org/10.1111/j.1095-8339.1996.tb00484.x

Roche D, Hanna WW, Ozias-Akins P (2001) Is supernumerary chromatin involved in gametophytic apomixis of polyploid plants? Sexual Plant Reproduction 13: 343–349. https://doi.org/10.1007/s004970100094

Rusche ML, Mogensen HL, Shi L, Keim P, Rougier M, Chaboud A, Dumas C (1997) B chromosome behavior in maize pollen as determined by a molecular probe. Genetics 147: 1915–1921.

Sharma PK, Langer A, Koul AK (1985) Genetic diversity among Plantagos V. Transmission of the additional chromosome in a triplo 4 individual of *Plantago lagopus* L. Genetica 67: 131–135. https://doi.org/10.1007/BF02424419

Squirrell J, Wolff K (2001) Isolation of polymorphic microsatellite loci in *Plantago major* and *P. intermedia*. Molecular Ecology Notes 1: 179–181. https://doi.org/10.1046/j.1471-8278.2001.00068.x

Viseras E, Camacho JPM, Cano MI, Santos JL (1990) Relationship between mitotic instability and accumulation of B chromosomes in males and females of *Locusta migratoria*. Genome 33: 23–29. https://doi.org/10.1139/g90-005

Supplementary material 1

Tables S1 and S2

Authors: Manoj K. Dhar, Gurmeet Kour, Sanjana Kaul

Data type: Nucleotide sequences

Explanation note: Table S1: Sequences of primers used for SSR analysis.
Table S2: The twelve different primer combinations used in the study.

Supplementary material 2

Figure S1

Authors: Manoj K. Dhar, Gurmeet Kour, Sanjana Kaul

Data type: Images

Explanation note: SSCP profile of 5S rDNA amplified from various plants. M = 100bp ladder. Lane1: 1B mother plant. Lane 2: progeny plant with 2n = 26 chromosomes. Lanes 3, 4: progeny plants (1B). Lane 5: 2B mother plant. Lane 6: progeny plant with 2n = 28 chromosomes. Lanes 7-9: progeny plants (2B). The band pattern of higher chromosome plants completely matches that of the mother plants as indicated by arrows.

Supplementary material 3

Figure S2

Authors: Manoj K. Dhar, Gurmeet Kour, Sanjana Kaul

Data type: Images

Explanation note: **S2a:** SSAP profile of 1B mother plant and progeny plants. M = 100bp ladder. Lane1: 1B mother plant. Lane 2: progeny plant with 2n = 26 chromosomes. Lanes 3-8: 1B progeny plants. **S2b**: SSAP profile of 2B mother plant and progeny plants. M = 100bp ladder. Lane 1: 2B mother plant. Lane 2: progeny plant with 2n = 28 chromosomes. Lanes 3-8: 2B progeny plants.

Supplementary material 4

Figure S3

Authors: Manoj K. Dhar, Gurmeet Kour, Sanjana Kaul

Data type: Images

Explanation note: **S3a, b:** SSR profile of 1B mother and its selfed progeny plants. M = 100bp ladder. Lane1: 1B mother plant. Lane 2: progeny plant with 2n = 26 chromosomes. Lanes 3- 26: 1B chromosome containing progeny plants. **S3c, d**: SSR profile of 2B and its selfed progeny plants. M = 100bp ladder. Lane1: 2B mother plant. Lane 2: progeny plant with 2n = 28 chromosomes. Lanes 3- 14: 2B chromosome containing progeny plants. Note exactly similar band pattern of mother and higher chromosome progeny plants.

CompCytogen 11(2): 393–403 (2017)
doi: 10.3897/CompCytogen.v11i2.12784
http://compcytogen.pensoft.net

SHORT COMMUNICATION

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

B chromosome dynamics in *Prochilodus costatus* (Teleostei, Characiformes) and comparisons with supernumerary chromosome system in other *Prochilodus* species

Silvana Melo[1,2], Ricardo Utsunomia[2], Manolo Penitente[3], Patrícia Elda Sobrinho-Scudeler[2], Fábio Porto-Foresti[3], Claudio Oliveira[2], Fausto Foresti[2], Jorge Abdala Dergam[1]

1 *Departamento de Biologia Animal, Campus Universitário, Universidade Federal de Viçosa, 36570-000, Viçosa, Minas Gerais, Brazil* **2** *Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista, Distrito de Rubião Junior, s/n, 18618-689, Botucatu, São Paulo, Brazil* **3** *Departamento de Ciências Biológicas, Faculdade de Ciências, Universidade Estadual Paulista, Campus de Bauru, 17033-360 Bauru, São Paulo, Brazil*

Corresponding author: *Silvana Melo* (silvanamelo90@gmail.com)

Academic editor: *N. Bulatova* | Received 18 March 2017 | Accepted 13 April 2017 | Published 1 June 2017

http://zoobank.org/1AA32135-9D3D-4B35-A523-D16FA4676ECB

Citation: Melo S, Utsunomia R, Penitente M, Sobrinho-Scudeler PE, Porto-Foresti F, Oliveira C, Foresti F, Dergam JA (2017) B chromosome dynamics in *Prochilodus costatus* (Teleostei, Characiformes) and comparisons with supernumerary chromosome system in other *Prochilodus* species. Comparative Cytogenetics 11(2): 393–403. https://doi.org/10.3897/CompCytogen.v11i2.12784

Abstract

Within the genus *Prochilodus* Agassiz, 1829, five species are known to carry B chromosomes, i.e. chromosomes beyond the usual diploid number that have been traditionally considered as accessory for the genome. Chromosome microdissection and mapping of repetitive DNA sequences are effective tools to assess the DNA content and allow a better understanding about the origin and composition of these elements in an array of species. In this study, a novel characterization of B chromosomes in *Prochilodus costatus* Valenciennes, 1850 (2n=54) was reported for the first time and their sequence complementarity with the supernumerary chromosomes observed in *Prochilodus lineatus* (Valenciennes, 1836) and *Prochilodus argenteus* Agassiz, 1829 was investigated. The hybridization patterns obtained with chromosome painting using the micro B probe of *P. costatus* and the satDNA SATH1 mapping made it possible to assume homology of sequences between the B chromosomes of these congeneric species. Our results suggest that the origin of B chromosomes in the genus *Prochilodus* is a phylogenetically old event.

Keywords
additional chromosomes, genome, Prochilodontidae

Introduction

Supernumerary or B chromosomes are dispensable genomic elements found in approximately 15% of eukaryotes (Camacho et al. 2000). Usually, these elements are assumed to have derived from standard genomic elements (e.g. A or sex chromosomes) from the same (intraspecific origin) or a different (interspecific origin) species (reviewed in Camacho et al. 2000). As a result of their reduced recombination rates, these elements are prone to accumulate several types of repetitive DNA sequences during their evolution (Camacho 2005). For instance, ribosomal and histone clusters, snDNA genes and satellite DNAs have been extensively found on B chromosomes of several species and provided evidence for the origin of these elements in different organisms (Teruel et al. 2009, Silva et al. 2014, 2016, Menezes-de-Carvalho et al. 2015, Utsunomia et al. 2016).

Prochilodus is the most species-rich genus within family Prochilodontidae and its species exhibit a well-preserved karyotypic macrostructure, a diploid number of 54 chromosomes and karyotypic formula of 40m + 14sm (Pauls and Bertollo 1983, 1990, Venere et al. 1999, Oliveira et al. 2003). However, prominent intra- and interspecific differences have been reported regarding the frequency and occurrence of B chromosomes. To date, supernumerary elements were reported in five species: *Prochilodus lineatus* (Valenciennes, 1836), *Prochilodus brevis* Steindachner, 1874, *Prochilodus nigricans* Agassiz, 1829, *Prochilodus mariae* Eigenmann, 1922, and *Prochilodus argenteus* Agassiz, 1829 (Pauls and Bertollo 1983, 1990, Venere et al. 1999, Oliveira et al. 2003, Penitente et al. 2015); however, information regarding the origin, molecular content, and populational dynamics of these B chromosomes are restricted to *P. lineatus* (Maistro et al. 2000, Jesus et al. 2003, Artoni et al. 2006, Voltolin et al. 2010, 2013a, Penitente et al. 2013).

In a previous study, Jesus et al. (2003) isolated two satellite DNA families from the *P. lineatus* genome, SATH1 and SATH2, mainly located in the pericentromeric region of chromosomes. Remarkably, in this species the SATH1 satDNA was associated with both A and B chromosomes, suggesting an intra-specific origin of these elements (Artoni et al. 2006, Vicari et al. 2010). However, no information related to the chromosomal location of SATH1 in congeneric species is available, which could be useful in understanding the origin and dynamics of B chromosomes in this genus.

Prochilodus costatus Valenciennes, 1850 is an endemic species of the São Francisco River basin and previous cytogenetic analyses did not reveal the presence of B chromosomes in this species (Pauls and Bertollo 1990, Galetti 1991, Voltolin et al. 2013a, 2013b). In this study, we described the occurrence of B chromosomes in *P. costatus* for the first time and performed a comparative analysis with other B chromosome systems found in *P. lineatus* and *P. argenteus* using chromosome painting and mapping of SATH1 satDNA.

Methods

Prochilodus costatus specimens (N=23) were collected in three distinct sites along São Francisco River basin (Table 1), Minas Gerais, Brazil, with SISBIO14975-1 permission. One sample of *Prochilodus argenteus* was collected near the Três Marias Dam, from the São Francisco River basin and one sample of *Prochilodus lineatus* was collected in Volta Grande Dam - MG, from the Grande River basin.

Before analysis, the animals were anesthetized and euthanized with a 300 mg L^{-1} clove oil aqueous solution (Lucena et al. 2013) in accordance with the Universidade Federal de Viçosa Animal Welfare Committee authorization #35/2014. The mitotic chromosomes were obtained from cell suspensions from the anterior kidney (Bertollo et al. 1978) and C-banding technique was carried out according to Sumner (1972). The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), modified from Levan et al. (1964).

Because the B chromosome frequency was variable among cells within the same individual, an analysis of mitotic instability causing this variation was performed. For this purpose, we used a mitotic instability index previously developed in a migratory locust (Pardo et al. 1995) that is based on the assumption that the median number of B chromosomes in the adult represents the number of B chromosomes in the zygotic stage. This mitotic instability index (MI) estimates the sum of deviations in B numbers in a sample of cells with respect to the median, normalized per B chromosome.

Microdissection was performed in an Eppendorf TransferMan NK2 micromanipulator attached to a Zeiss Axiovert 100 microscope. Ten B chromosomes were microdissected from the same *P. costatus* specimen (JD5483) carrying one B chromosome. The microdissected DNAs were placed in 9 µl of DNase-free ultrapure water and then fragmented and amplified using the GenomePlex Single Cell Whole Genome Amplification Kit (wga4-Sigma) (Gribble et al. 2004). After the initial amplification, we obtained a B chromosome DNA probe (BPC probe) labeled with digoxigenin-11-dUTP (Roche Applied Science) using the GenomePlex Whole Genome Amplification Reamplification Kit (wga3-Sigma), following the manufacturer's protocol. This BPC probe was hybridized on metaphase plates of *P. costatus*, *P. lineatus* and *P. argenteus.*

Considering that SATH1 satDNA was described to occur on the B chromosomes of *P. lineatus* (Jesus et al. 2003, Artoni et al. 2006), the distribution of this probe in different *Prochilodus* species would be relevant for understanding evolutionary aspects of this B chromosome system. Thus, three SATH1 sequences (AF363731.1, AF363732.1 and AF363734.1) were retrieved from GenBank and aligned using MUSCLE algorithm (Edgar 2004). Subsequently, the convergent primers SATH1-F 5'-GCTGCAGCAAAAACCCTACC- 3' and SATH1-R 5'-AGTGGGAGCTAGGGTTAGGG-3' were designed on conserved regions to yield a 563bp PCR product, suitable for FISH (Suppl. material 1). The reactions were performed in 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 U of *Taq* polymerase (Invitrogen), 0.1 µM each primer and 5 ng of DNA. The PCR products were checked in 2% agarose gels (Suppl. material 2). After amplification, PCR products were labeled with digoxigenin-11-dUTP.

Table 1. B chromosome frequency and Mitotic Instability index (MI) of somatic cells in *P. costatus*.

Sample	Locality	Geographic coordinates	Number of B per cell		MB	N	MI
			0B	*1B*			
JD5480	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	12	–	0B	12	0
JD5481	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	29	1	0B	30	0.01
JD5482	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	28	2	0B	30	0.01
JD5483	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	–	30	1B	30	0
JD5486	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	15	–	0B	15	0
JD5490	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	27	3	0B	30	0.01
JD5497	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	29	1	0B	30	0.01
JD5502	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	17	1	0B	18	0.11
JD5604	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	27	3	0B	30	0.01
JD5605	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	27	3	0B	30	0.01
JD5619	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	30	–	0B	30	0
JD5620	São Francisco River – Iguatama	20°09'50"S, 45°43'08"W	16	1	0B	17	0.01
JD5517	Pandeiros River – Januária	15°23'28"S, 44°53'37"W	30	–	0B	30	0
JD5531	Pandeiros River – Januária	15°23'28"S, 44°53'37"W	27	3	0B	30	0.01
JD5562	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	25	5	0B	30	0.1
JD5563	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	11	–	0B	11	0
JD5565	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	15		0B	15	0
JD5566	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	15	1	0B	16	0.04
CT4639	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	21	6	0B	27	0.01
CT4640	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	23	1	0B	24	0.04
CT4644	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	10	–	0B	10	0
CT4645	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	10	–	0B	10	0
CT4647	São Francisco River – Três Marias Dam	18°08'47"S, 45°13'37"W	10	–	0B	10	0
							X_{MI}=0,0292

MB: modal number of B chromosomes; N: number of metaphases analyzed; MI: Mitotic Instability index; X_{MI}: MI average among individuals with B chromosomes.

Fluorescent *in situ* hybridization (FISH) was performed under high stringency conditions using the method described by Pinkel et al. (1986) with modifications. Pre-hybridization: slides were incubated with 0,005% pepsin/10mM HCl for 10 min at 37 °C and the chromosomal DNA was denatured in 70% formamide/2xSSC for 5 min at 70 °C. For each slide, 30 µl of hybridization solution (containing 200 ng of labeled probe, 50% formamide, 2xSSC and 10% dextran sulphate) was denatured for 10 min at 95 °C, then dropped onto the slides and allowed to hybridize overnight at 37 °C in a moist chamber. Post hybridization: slides were washed in 0,2xSSC/15% formamide for 20 min at 42 °C, followed by a second wash in 0,1xSSC for 15 min at 60 °C and a final wash at room temperature in 4xSSC/0,5% Tween for 10 min. Probe detection was carried out with anti-digoxigenin-rhodamine (Roche), and the chromosomes were counterstained with DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories) and analyzed using an optical photomicroscope (Olympus BX61). Images were captured with an Olympus DP70 digital camera using the IMAGE PRO PLUS 6.0 software (Media Cybernetics). From each individual, a minimum of five cells was analyzed for FISH.

Results and discussion

The analyzed samples of *P. costatus* showed the expected karyotypic macrostructure of 54 chromosomes (40m + 14sm) (Fig. 1), a conserved trait among Prochilodontidae, indicating that large chromosome rearrangements are apparently unusual in this fish group (Feldberg et al. 1987, Pauls and Bertollo 1983, 1990, Venere et al. 1999, Oliveira et al. 2003, Voltolin et al. 2013, Penitente et al. 2015).

Mitotically unstable B chromosomes were observed in 14 of 23 *P. costatus* specimens analyzed, ranging from 0 to 1 B chromosome per cell, characterizing the sixth species within *Prochilodus* carrying these elements (Table 1). Although chromosome numbers and karyotype structure are highly stable in *Prochilodus* species, the frequency of different B chromosomes in distinct species/populations is remarkable (Cavallaro et al. 2000, Artoni et al. 2006, Penitente et al. 2015). For instance, two populations of *P. costatus* were analyzed until now and none of them showed B chromosomes in their cells (Pauls and Bertollo 1990, Voltolin et al. 2013a, 2013b). In fact, supernumerary chromosomes in fish are usually highly dynamic elements, and the existence of B-lacking and B-carrying populations is quite common and largely known (Oliveira et al. 2009).

The Mitotic Instability calculation resulted in an average index of 0.0292 (Table 1). This MI index is considered low and indicates that this elements may be testifying a process of stabilization on *P. costatus* populations, as suggested for populations of *P. lineatus* (Cavallaro et al. 2000) and *P. argenteus* (Penitente et al. 2015).

C-banding revealed small pericentromeric heterochromatic regions in all chromosomes (Fig. 1b), with a narrow band in the metacentric chromosome pair number 2, as reported for other species of *Prochilodus*, except in *P. lineatus* (Artoni et al. 2006, Vicari et al. 2006, Voltolin et al. 2013a). Additionally, the supernumerary chromosomes were entirely C-band positive (Fig. 1b; arrowhead).

Cross-species chromosome painting showed that the BPC probe hybridized on the B chromosomes of *P. costatus*, *P. lineatus*, and *P. argenteus* (Fig. 2). Notably, this hybridization pattern evidenced that all B chromosomes analyzed in this study shared anonymous sequences, as already reported for *P. lineatus* and *P. nigricans* (Voltolin et al. 2013b). The identical hybridization pattern generated throughout chromosome painting between different species or populations allow us to suggest that these B chromosomes show a high degree of homology. Notably, such hypothesis should be better tested in the future since chromosome painting is not a conclusive method when studying B chromosomes origin in closely related species (Silva et al. 2016). However, one must say that the association of this technique with known repetitive sequences mapping by FISH may provide additional information about the DNA content and sequence homology in supernumerary chromosomes of different species.

FISH experiments revealed large clusters of SATH1 satDNA in the pericentromeric regions of many A-chromosomes in the three studied species. Remarkably, the largest metacentric chromosome of *P. lineatus* exhibited a strong signal in the pericentromeric region, differently from *P. costatus* and *P. argenteus* (Fig. 3; asterisks), characterizing an interesting chromosomal marker. In addition, our results evidenced that

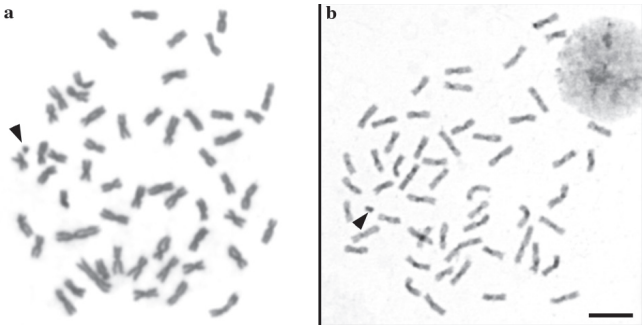


Figure 1. Metaphases of *P. costatus* with conventional staining (**a**) and C-banding (**b**). Arrowheads indicate the supernumerary chromosomes. Bar = 5 μm.

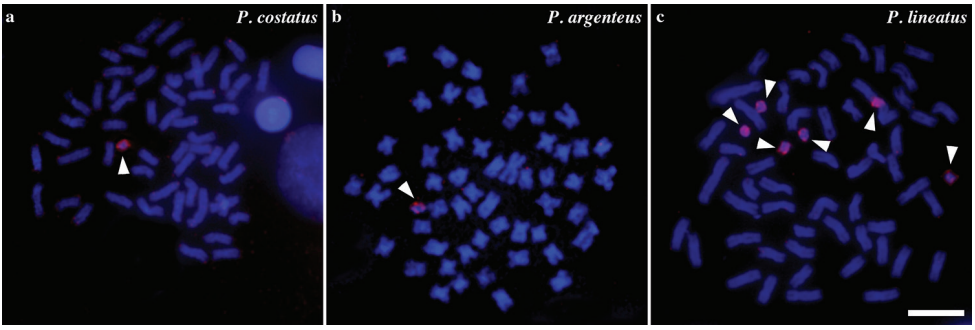


Figure 2. Chromosome painting with BPC probe on metaphases of *P. costatus* (**a**), *P. argenteus* (**b**) and *P. lineatus*. Arrowheads indicate the supernumerary chromosomes. Bar = 10 μm.

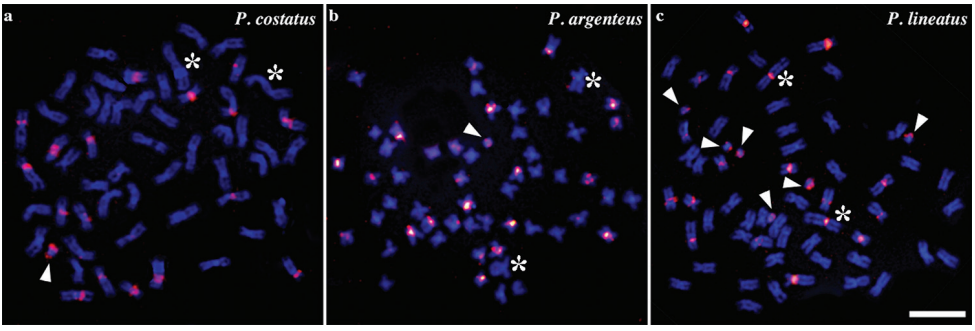


Figure 3. Metaphases of *P. costatus* (**a**), *P. argenteus* (**b**) and *P. lineatus* hybridized with SATH1 probe. The asterisks indicate the first pair of metacentric chromosomes and the arrowheads indicate the supernumerary chromosomes. Bar = 10 μm.

the supernumerary chromosomes of *P. costatus* (one B chromosome), *P. argenteus* (one B chromosome) and *P. lineatus* (six B chromosomes) carry the SATH1 satDNA sequences (Fig. 3; arrowheads). Notably, SATH1 sequences were also extensively spread

over several A chromosomes in all species, whereas the BPC and all previously microdissected B-probes of *Prochilodus* (Voltolin et al. 2013b) revealed signals only on the B chromosomes. Such deviation might be caused by a possible bias of amplification in the GenomePlex reaction. Thus, several different repetitive DNA elements might be located on these B chromosomes and deserve further investigation.

Cytogenetic data show a conservative trend within the family Prochilodontidae, with a diploid number of 2n=54 biarmed chromosomes (Pauls and Bertollo 1983, 1990, Feldberg et al. 1987, Voltolin et al. 2013, Nirchio-Tursellino et al. 2016). However, *Semaprochilodus* Fowler, 1941 and *Prochilodus* genera exhibit the presence of sex related and supernumerary chromosomes, respectively, different from *Ichthyoelephas* Posada Arango, 1909, in which these elements are absent. Accordingly, recent molecular phylogenetic analyses (Melo et al. 2016) proposed the monophily of Prochilodontidae and placed *Ichthyoelephas* as a sister group of the *Semaprochilodus* + *Prochilodus* clade. The absence of sex related and supernumerary chromosomes, together with differences on the location of the repetitive 5S and 18S rDNA, suggest a plesiomorphic position of the *Ichthyoelephas* karyotype (Nirchio-Tursellino et al. 2016), involving at least two rearrangements events in the common ancestor of the Prochilodontidae.

Only eight out of the 13 valid species of *Prochilodus* have been karyotyped and the presence of B chromosomes was reported for six species. In this sense, B chromosomes are present in most species, except for the *P. vimboides* and the trans-Andean clade. However, our results suggest that these chromosomes may be absent in some populations, or their low frequency may demand higher sampling efforts. The hybridization patterns of both SATH1 satDNA and chromosome painting with the B-specific probe suggested an old and intraspecific origin of B chromosomes within this genus.

Acknowledgements

This work was supported by the Companhia Energética de Minas Gerais (CEMIG) project GT455, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp).

References

Artoni RF, Vicari MR, Endler AL, Cavallaro ZI, Jesus CM, Almeida MC, Moreira-Filho O, Bertollo LAC (2006) Banding pattern of A and B chromosome of *Prochilodus lineatus* (Characiformes, Prochilodontidae), with comments on B chromosome evolution. Genetica 127: 277-284. https://doi.org/10.1007/s10709-005-4846-1

Bertollo LAC, Takahashi CS, Moreira-Filho O (1978) Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). Brazilian Journal of Genetics 1: 103–120.

Camacho JPM, Sharbel TF, Beukboom LW (2000) B-chromosome evolution. Philosophical Transaction of the Royal Society B 355(1394): 163–178. https://doi.org/10.1098/rstb.2000.0556

Camacho JPM (2005) B chromosomes. In: Gregory TR (Ed.) The evolution of the genome. Elsevier, San Diego, 223–286. https://doi.org/10.1016/b978-012301463-4/50006-1

Cavallaro ZI, Bertollo LAC, Perfectti F, Camacho JPM (2000) Frequency increase and mitotic stabilization of a B chromosome in fish *Prochilodus lineatus*. Chromosome Research 8: 627–634. https://doi.org/10.1023/A:1009242209375

Edgar RC (2004) Muscle: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5: 113. https://doi.org/10.1186/1471-2105-5-113

Feldberg E, Bertollo LAC, Almeida-Toledo L, Moreira-Filho O (1987) Biological aspects of Amazonian fishes. IX. Cytogenetic studies in two species of the genus *Semaprochilodus* (Pisces, Prochilodontidae). Genome 29: 1–4. https://doi.org/10.1139/g87-001

Galetti PM (1991) Chromosome complement of induced hybrids of the fish *Prochilodus marggravii* and *Prochilodus affinis* (Prochilodontidae, Characiformes). Revista Brasileira de Genética 14(1): 203–207.

Gribble S, Ng BL, Prigmore E, Burford DC, Carter NP (2004) Chromosome paints from single copies of chromosomes. Chromosome Research 12: 143–151. https://doi.org/10.1023/B:CHRO.0000013167.12527.f0

Jesus CM, Galetti Jr PM, Valentini SR, Moreira-Filho O (2003) Molecular characterization and chromosomal localization of two families of satellite DNA in *Prochilodus lineatus* (Pisces, Prochilodontidae), a species with B chromosomes. Genetica 118(1): 25-32. https://doi.org/10.1023/A:1022986816648

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–204. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Lucena CAS, Calegari BB, Pereira EHL, Dallegrave E (2013) O uso de óleo de cravo na eutanásia de peixes. Boletim Sociedade Brasileira de Ictiologia 105: 20-24.

Maistro EL, Oliveira C, Foresti F (2000) Cytogenetic analysis of A- and B-chromosomes of *Prochilodus lineatus* (Teleostei, Prochilodontidae) using different restriction enzyme banding and staining methods. Genetica 108: 119–125. https://doi.org/10.1023/A:1004063031965

Menezes-de-Carvalho NZ, Palacios-Gimenez OM, Milani D, Cabral-de-Mello DC (2015) High similarity of U2 snDNA sequence between A and B chromosomes in the grasshopper *Abracris flavolineata*. Molecular Genetics and Genomics 290: 1787–1792. https://doi.org/10.1007/s00438-015-1033-7

Melo BF, Sidlauskas BL, Hoekzema K, Frable BW, Vari RP, Oliveira C (2016) Molecular phylogenetics of the Neotropical fish family Prochilodontidae (Teleostei: Characiformes). Molecular Phylogenetics and Evolution 102: 189–201. https://doi.org/10.1016/j.ympev.2016.05.037

Nirchio Tursellino M, Silva DMZA, Quezada AC, Moreira BWA, Sánchez ROR, Oliveira C (2016) First cytogenetic analysis of *Ichthyoelephas humeralis* (Günther, 1860) by conventional and molecular methods with comments on the karyotypic evolution in Prochilodontidae. Comparative Cytogenetics 10(4): 615–624. https://doi.org/10.3897/CompCytogen.v10i4.9858

Oliveira C, Nirchio M, Granado A, Levy S (2003) Karyotypic characterization of *Prochilodus mariae*, *Semaprochilodus kneri* and *S. laticeps* (Teleostei: Prochilodontidae) from Caicara del Orinoco, Venezuela. Neotropical Ichthyology 1: 47–52. https://doi.org/10.1590/S1679-62252003000100005

Oliveira C, Foresti F, Hilsdorf AWS (2009) Genetics of neotropical fish: from chromosomes to populations. Fish Physiology Biochemistry 35: 81–100. https://doi.org/10.1007/s10695-008-9250-1

Pardo MC, López-León MD, Viseras E, Cabrero J, Camacho JPM (1995) Mitotic instability of B chromosomes during embryo development in *Locusta migratoria*. Heredity 74: 164–169. https://doi.org/10.1038/hdy.1995.24

Pauls E, Bertollo LAC (1983) Evidence for a system of supernumerary chromosomes in *Prochilodus scrofa* Steidachner, 1881 (Pisces, Prochilodontidae). Caryologia 36: 307–314. https://doi.org/10.1080/00087114.1983.10797671

Pauls E, Bertollo LAC (1990) Distribution of a supernumerary chromosome system and aspects of karyotypic evolution in the genus *Prochilodus* (Pisces, Prochilodontidae). Genetica 81: 117–123. https://doi.org/10.1007/BF00226450

Penitente M, Voltolin TA, Senhorini JA, Bortolozzi J, Foresti F, Porto-Foresti F (2013) Transmission rate variation among three B chromosome variants in the fish *Prochilodus lineatus* (Characiformes, Prochilodontidae). Annals of the Brazilian Academy of Sciences 85(4): 1371–1377. https://doi.org/10.1590/0001-3765201387611

Penitente M, Foresti F, Porto-Foresti F (2015) B chromosomes in the species *Prochilodus argenteus* (Characiformes, Prochilodontidae): morphological identity and dispersion. Comparative Cytogenetics 9(1): 79–87. https://doi.org/10.3897/CompCytogen.v9i1.8587

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences USA 83: 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Silva DMZA, Pansonato-Alves JC, Utsunomia R, Araya-Jaime C, Ruiz-Ruano FJ, Daniel SN, Hashimoto DT, Oliveira C, Camacho JPM, Porto-Foresti F, Foresti F (2014) Delimiting the Origin of a B Chromosome by FISH Mapping, Chromosome Painting and DNA Sequence Analysis in *Astyanax paranae* (Teleostei, Characiformes). PLoS ONE 9(4): e94896. https://doi.org/10.1371/journal.pone.0094896

Silva DMZA, Daniel SN, Camacho JPMC, Utsunomia R, Ruiz-Ruano FJ, Penitente M, Pansonato-Alves JC, Hashimoto DT, Oliveira C, Porto-Foresti F, Foresti F (2016) Origin of B chromosomes in the genus *Astyanax* (Characiformes, Characidae) and the limits of chromosome painting. Molecular Genetics and Genomics 291: 1407–1418. https://doi.org/10.1007/s00438-016-1195-y

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

Teruel M, Cabrero J, Montiel EE, Acosta MJ, Sánchez A, Camacho JPM (2009) Microdissection and chromosome painting of X and B chromosomes in *Locusta migratoria*. Chromosome Research 17: 11–18. https://doi.org/10.1007/s10577-008-9001-2

Utsunomia R, Silva DMZA, Ruiz-Ruano FJ, Araya-Jaime C, Pansonato-Alves JC, Scacchetti PC, Hashimoto DT, Oliveira C, Trifonov VA, Porto-Foresti F, Camacho JPM, Foresti F

(2016) Uncovering the Ancestry of B Chromosomes in *Moenkhausia sanctaefilomenae* (Teleostei, Characidae). PLoS ONE 11(3): e0150573. https://doi.org/10.1371/journal.pone.0150573

Venere PC, Miyazawa CS, Galetti Junior, PM (1999) New cases of supernumerary chromosomes in characiform fishes. Genetics and Molecular Biology 22: 345–349. https://doi.org/10.1590/S1415-47571999000300010

Vicari MR, Nogaroto V, Noleto RB, Cestari MM, Cioffi MB, Almeida MC, Moreira-Filho O, Bertollo LAC, Artoni RF (2010) Satellite DNA and chromosomes in neotropical fishes: methods, applications and perspectives. Journal of Fish Biology 76: 1094–1116. https://doi.org/10.1111/j.1095-8649.2010.02564.x

Voltolin TA, Laudicina A, Senhorini JA, Oliveira O, Foresti F, Porto-Foresti F (2010) Origin and molecular organization of supernumerary chromosome of *Prochilodus lineatus* (Characiformes, Prochilodontidae) obtained by DNA probes. Genetica 138: 1133–1139. https://doi.org/10.1007/s10709-010-9502-8

Voltolin TA, Penitente M, Mendonça BB, Senhorini JA, Foresti F, Porto-Foresti F (2013a) Karyotypic conservatism in five species of *Prochilodus* (Characiformes, Prochilodontidae) disclosed by cytogenetic markers. Genetics and Molecular Biology 36(3): 347–352. https://doi.org/10.1590/S1415-47572013000300008

Voltolin TA, Pansonato-Alves JC, Senhorini JA, Foresti F, Camacho JPM, Porto-Foresti F (2013b) Common descent of B chromosomes in two species of the fish genus *Prochilodus* (Characiformes, Prochilodontidae). Cytogenetic and Genome Research 141: 206–211. https://doi.org/10.1159/000354987

Supplementary material 1

Alignment of three SATH1 satDNA sequences from *Prochilodus lineatus* genome

Authors: Silvana Melo, Ricardo Utsunomia, Manolo Penitente, Patrícia Elda Sobrinho-Scudeler, Fábio Porto-Foresti, Claudio Oliveira, Fausto Foresti, Jorge Abdala Dergam

Data type: molecular data

Explanation note: Alignment of three SATH1 satDNA sequences from *Prochilodus lineatus* genome (accession no. AF363731.1, AF363732.1 and AF363734.1). Designed primers are boxed, evidencing a 563bp amplicon size.

Supplementary material 2

B chromosome microdissection process and SATH1 satDNA amplification checked in 2% agarose gel

Authors: Silvana Melo, Ricardo Utsunomia, Manolo Penitente, Patrícia Elda Sobrinho-Scudeler, Fábio Porto-Foresti, Claudio Oliveira, Fausto Foresti, Jorge Abdala Dergam

Data type: molecular data

Explanation note: Amplification patterns of the B chromosome after WGA (B-probe lane) and the SATH1 satDNA (SATH1 lane) from *P. costatus* genome. L: 1 Kb Plus DNA Ladder.

CompCytogen 11(2): 405–420 (2017)
doi: 10.3897/CompCytogen.v11i2.12824
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Cytogenetic maps of homoeologous chromosomes A_h01 and D_h01 and their integration with the genome assembly in *Gossypium hirsutum*

Yuling Liu[1,2], Zhen Liu[1], Renhai Peng[1], Yuhong Wang[2], Zhongli Zhou[2], Xiaoyan Cai[2], Xingxing Wang[2], Zhenmei Zhang[2], Kunbo Wang[2], Fang Liu[2]

1 *Anyang Institute of Technology, Anyang, Henan, 455000, China* **2** *State Key Laboratory of Cotton Biology / Institute of Cotton Research of Chinese Academy of Agricultural Science, Anyang, Henan, 455000, China*

Corresponding authors: *Kunbo Wang* (wkbcri@163.com); *Fang Liu* (liufcri@163.com)

Academic editor: *A. Belyayev* | Received 21 March 2017 | Accepted 22 May 2017 | Published 19 June 2017

http://zoobank.org/2B0C3311-F6E1-49C9-B7AF-F769422AA1D7

Citation: Liu Y, Liu Z, Peng R, Wang Y, Zhou Z, Cai X, Wang X, Zhang Z, Wang K, Liu F (2017) Cytogenetic maps of homoeologous chromosomes A_h01 and D_h01 and their integration with the genome assembly in *Gossypium hirsutum*. Comparative Cytogenetics 11(2): 405–420. https://doi.org/10.3897/CompCytogen.v11i2.12824

Abstract

Cytogenetic maps of *Gossypium hirsutum* (Linnaeus, 1753) homoeologous chromosomes A_h01 and D_h01 were constructed by fluorescence *in situ* hybridization (FISH), using eleven homoeologous-chromosomes-shared bacterial artificial chromosomes (BACs) clones and one chromosome-specific BAC clone respectively. We compared the cytogenetic maps with the genetic linkage and draft genome assembly maps based on a standardized map unit, relative map position (RMP), which allowed a global view of the relationship of genetic and physical distances along each chromosome, and assembly quality of the draft genome assembly map. By integration of cytogenetic maps with sequence maps of the two chromosomes (A_h01 and D_h01), we inferred the locations of two scaffolds and speculated that some homologous sequences belonging to homoeologous chromosomes were removed as repetitiveness during the sequence assembly. The result offers molecular tools for cotton genomics research and also provides valuable information for the improvement of the draft genome assembly.

Keywords

cotton, BAC, FISH, physical map, draft genome assembly

Introduction

The genus *Gossypium* (Linnaeus, 1753) includes approximately 47 diploid species (2n = 2x = 26) that are divided into eight genome groups, named as A-G and K genome (Endrizzi et al. 1985, Wendel et al. 2012). Ancient hybridization between A and D diploids resulted in a new allopolyploid (AD) (2n = 4x = 52) lineage approximately 1–2 million years ago (Wendel 1989, Li et al. 2015, Zhang et al. 2015, Liu et al. 2015, Yuan et al. 2015). As the most important natural fiber crop in the world, four *Gossypium* species were independently domesticated for their long, spinnable, epidermal seed trichomes, which include *G. hirsutum* (Linnaeus, 1753) (AD_1), *G. barbadense* (Linnaeus, 1753) (AD_2), *G. herbaceum* (Linnaeus, 1753) (A_1) and *G. arboreum* (Linnaeus, 1753) (A_2). Among the four species, *G. hirsutum* (AD_1) provides more than 90% of the world's cotton fiber production (Wendel and Cronn 2003). Moreover, as a typical polyploid species, cotton is a model system for studying polyploidization. So dissecting the cotton genome is important for facilitating advances in crop germplasm development and utilization, as well as understanding of other polyploid crops. At present, sequencing *Gossypium* species genomes is ongoing in full swing with successively draft maps of whole genome in wild and cultivated cotton species (Paterson et al. 2012, Wang et al. 2012, Li et al. 2014, 2015, Zhang et al. 2015, Liu et al. 2015,Yuan et al. 2015). It is expected that new genome assemblies will soon became available. However, a high level of sequence conservation between homoeologous genomic regions makes it difficult to annotate and assemble whole-genome sequences in allotetraploid species including cotton and wheat (Wang et al. 2010), which may result in many gaps and blurred chromosome scaffolds in the draft genome, and access to high-quality assembly sequence still has a long way to go. Therefore, it is necessary to carry out the relevant basic research work on cotton genome research to help for genome sequence assembly.

The uneven distribution of recombination events on chromosomes results in divergence between genetic distance and physical distance, which limits the application of genetic map in guiding genome sequence assembly and map-based cloning (Sun et al. 2013). A cytogenetic map, which can integrate genetic loci into physical location of chromosome, has great potential to help in the assembly of genome sequence. Fluorescence *in situ* hybridization (FISH), which allows direct mapping of DNA sequence on chromosome, has been widely used in the study of different plants as an important tool for constructing cytogenetic maps (Jiang and Gill 2006). At present, physical maps based on high resolution FISH in many crops have been reported, such as maize (Figueroa and Bass 2012), rice (Cheng et al. 2001, Kao et al. 2006), *Brassica* (Linnaeus, 1753) (Xiong et al. 2010), tomato (Koo et al. 2008, Szinay et al. 2008), potato (Tang et al. 2008), bean (Fonsêca et al. 2010), cucumber (Han et al. 2011, Sun et al. 2013).

Tetraploid cotton contains too many chromosomes (2n = 4x = 52) and it is difficult to prepare chromosomes due to large amounts of secondary metabolites in cells. So research on cotton cytogenetic maps has lagged behind other crops. Moreover, previous cotton FISH mapping was mainly limited to the use of repetitive DNA (Hanson

et al. 1996, Ji et al. 2007), the chromosome-specific bacterial artificial chromosomes (BACs) (Wang et al. 2007). To date, there have been only a few cotton cytogenetic maps (Wang et al. 2010, Cui et al. 2015).

Structure analysis of homoeologous chromosomes in allotetraploid cotton plays an important guiding role in sequence assembly, map-based cloning, and so on. Xu et al. (2008) selected homoeologous chromosomes Chr.12 and Chr.26 (12A and 12D) in allotetraploid cotton, which contain important genes related to fiber fuzz, gland development, and male sterility, and constructed their physical maps using the BAC contigs, which provided an important platform for the clone mapping of the important genes. Wang et al. (2010) constructed cytogenetic maps of homoeologous chromosomes 12A and 12D using BAC-FISH, which had guided the next genome sequence assembly to a certain extent (Zhang et al. 2015). Chr.01 and Chr.15 (i.e. A_h01 and D_h01) in upland cotton linkages have been shown to be homoeologous chromosomes based on genetic markers, which contain many genes or QTLs related to stress tolerance, fiber development, fiber yield and quality (Said et al. 2013). In this study, the cytogenetic maps of homoeologous chromosomes A_h01 and D_h01 of *G. hirsutum* were constructed by FISH using marker-anchored BACs. By using similar relative map position (RMP) units, which was the percentage distance of a locus from the end of the short arm along a given chromosome, we made a comparative analysis between the cytogenetic, the genetic linkage, and draft genome assembly maps of *G. hirsutum* homoeologous chromosomes A_h01 and D_h01 preliminarily.

Material and methods

Plant materials and BAC library

G. hirsutum (Linnaeus, 1753) accession TM-1 was used for cytological studies. BACs used for FISH mapping were identified by screening two genomic BAC libraries derived from *G. herbaceum* (Linnaeus, 1753) *var. africenum* (Gao et al. 2013) and *G. barbadense* (Linnaeus, 1753) Pima 90-53 (kindly provided by Prof. Zhiying Ma of Hebei Agricultural University). The chromosome-specific BAC clones for *G. hirsutum* A_h01/D_h01 were kindly provided by Prof. Tianzhen Zhang of Nanjing Agricultural University, The simple sequence repeat (SSR) markers used for BAC screening were selected from a whole genome marker map (WGMM) (Wang et al. 2013) and a genetic map (Yu et al. 2011).

BAC library screening

The screening was performed using bacteria liquid-PCR according to the protocol previously described (Cheng et al. 2012).

Chromosome preparation and FISH

Chromosome preparation and FISH were conducted according to the previous protocols (Gan et al. 2011). In order to reduce the interference from the background signals, heat-shock-interrupted (1.5 mL Eppendorf tube filled with 100 μl genome DNA was placed in sterilization pot with 105°C for 8 min) cotton genome DNA fragments with size from 200 bp to 800 bp were used as blocking DNA. BAC-DNA used to label probes was isolated using Plasmid Miniprep Kit (Biomiga) according to the handbook. Biotin- and digoxigenin-labeled probes were detected using rhodamine-conjugated anti-digoxigenin and fluorescein-conjugated avidin (Roche Diagnostics, USA), respectively. Chromosomes were counter-stained with 4, 6-diamidino-2-phenylindole (DAPI, Sigma, USA) and antifade (Vector, USA) under a cover-slip.

Image analysis

Slides were examined under a Zeiss Imager M1 microscope. Images were captured and merged using MetaSystems isis software with a CCD camera (MetaSystems CoolCube 1) attached to a Zeiss Imager M1 microscope. To determine physical positions of signals, only chromosomes without apparent morphological distortion were introduced and their physical positions of signals were measured using MetaSystems isis. Final image adjustments were performed using Adobe Photoshop CS3 software.

Comparative mapping using standardized map units

The RMP unit was used as standardized map unit for comparative analysis between different types of maps. The RMP values for the SSR linkage map were the percentage from the genetic location (cM) of each locus along the total length (cM) of the corresponding linkage group. The RMP values of the cytogenetic map were the percentage of the distance (μm) from the FISH signal site to the end of the short arm showed relative to the total length of the chromosome (μm) (Sun et al. 2013). In order to determine the genomic locations (bp) of each BAC clones, the primer sequences of BACs-corresponding SSR markers were obtained from the database Cotton Marker Database (http://www.cottonmarker.org/), then according to Electronic PCR command line tools (Version 2.3.12), e-PCR was run against the *G. hirsutum* (AD_1) genome NAU-NBI Assembly (https://www.cottongen.org/organism/*Gossypium*/*hirsutum*) according to the default parameters. The RMP values for the *G. hirsutum* draft genome assembly map were calculated from the genomic location (bp) of each locus along the physical length of chromosomes A_h01 and D_h01. These RMP values were used to produce the comparative map alignments.

Results

Screening of SSR markers

To construct the cytogenetic maps of chromosomes A_h01 and D_h01 of *G. hirsutum*, an initial set of 47 SSR markers shared by both chromosomes of A_h01 and D_h01 from a whole genome marker map (WGMM) (Wang et al. 2013, Rong et al. 2004) and a genetic map (Yu et al. 2011) were used to screen two BAC libraries of *G. herbaceum var. africenum* and *G. barbadense* Pima 90-53. Based on the WGMM, the SSR markers were distributed along the linkage group of chr.15 (D_h01) from 0.6 cM (CIR009) to 176.3 cM (CIR110) (Table 1). In total, 84 positive BAC clones were identified based on the result of BAC libraries screening (Table 2). Due to abundance of repetitive sequence in cotton genome, by dual-color FISH with the chromosome-specific BAC clones 52D06 (A1) and 48F11 (D1) as controls, only 12 BAC clones were selected for FISH mapping which produced little or no background signal when hybridized to *G. hirsutum* chromosomes with the aid of blocking DNA.

FISH identification

By dual-color FISH on mitotic chromosomes, the order of the two BACs was determined along the chromosomes based on the genetic positions of their corresponding SSR markers. Results showed, among the 12 positive BAC clones, 11 BAC clones were

Table 1. Information of selected SSR markers based on the WGMM*[1].

SSR	BAC	Loc. in D-genome sequence			Chr.15 cM	RMP (%)*[2]	Loc. in tetraploid	
		Chr.	Start bp	End bp				
NAU2015	305A19	Chr02	61962135	61962910	12.6	7.14	Chr.01	Chr.15
NAU3254	348I20	Chr02	60694684	60699332	29.1	16.49	Chr.01	Chr.15
NAU2474	144E04	Chr02	59155451	59156001	39.5	22.39	Chr.01	Chr.15
NAU3433	64M24	Chr02	55462914	55463585	53.6	30.38	Chr.01	Chr.15
BNL2921	400N03	Chr02	27353761	27353982	73.3	41.55	Chr.01	Chr.15
NAU4891	118G12	Chr02	15429614	15428837	86.2	48.86	Chr.01	Chr.15
NAU3135	85P13	Chr02	11717323	11717890	90.2	51.13	Chr.01	Chr.15
BNL3888b	164I21	Chr02	11188812	11189229	90.3	51.19	Chr.01	Chr.15
BNL3580	421E24	Chr02	7879846	7880283	93.4	52.94	Chr.01	Chr.15
NAU4044	400L15	Chr02	2312144	2313542	111.5	63.20	Chr.01	Chr.15
HAU076	378J07	--*3	--	--	--	--	Chr.01	--
TMB0062	423C18	--*3	--	--	--	--	Chr.01	--

Note: *[1], WGMM, whole-genome marker map. *[2], RMP, relative map position, it refers to the percentage of marker's cM value accounting for chromosome's total cM value. *[3], SSR derived from a tetraploid genetic map (Yu et al. 2011).

Table 2. BAC clones screened from two BAC libraries.

SSR markers	BAC library	Screened BAC clones
HAU2861	1*	22K17; 22L15; 22L18; 67J23; 75D24; 75E24; 108E08; 108E24; 130M09; 151C24; 151E18
NAU3433	1*	41J08; 41K08; 46K02; 64M20; 64M24; 78G20; 78H20
NAU3053	1*	22K18; 22L17; 67I12; 75C23; 75E24;107P10; 107P24
NAU4891	1*	50H19; 51C14; 51H12; 56J17; 118G11; 118G12
Gh649	1*	99L01; 136O19; 136P17
NAU2095	1*	52B01
Gh216	1*	50P23; 57I23; 79A06; 79A12; 79B07; 101K10; 101K12; 146P05
NAU5163	2*	141H01; 158M07; 158N09; 158L08; 159L07; 159L08
BNL3888b	2*	164I21; 164I22
NAU3254	2*	348I18; 348I20; 348I21; 348H17; 348J19
CIR049	2*	256N07
BNL2921	2*	400N03; 400L02
BNL3580	2*	421E24
NAU2015	2*	305A19
NAU4044	2*	400L15
NAU2474	2*	144E04; 165B11
NAU3135	2*	85P13; 377G04; 377H05; 247P16; 247P17; 325M09; 325M10
TMB0062	2*	298N21; 403A13; 423C18; 423C19; 424A12
HAU076	2*	249G03; 249G04; 249I5; 325N10; 378J07; 398J05; 398H05; 249G05

Note: 1* BAC library *G. herbaceum var. africenum*
2* BAC library *G. barbadense* Pima 90-53

homoeologous-specific BACs because they generated signals on both chromosomes of A_h01 and D_h01, indicating sequence homology between these BACs retained in A_h01 and D_h01 (Fig. 1a-k). One BAC clone 378J07, derived from SSR HAU076, only had one pair of FISH signals on chromosome A_h01, which had collinearity with the chromosome A_h01-specific BAC clone A1 (52D06) (Fig. 1l). Based on these results, the relative position of all probes can be preliminarily distinguished along the mitotic metaphase chromosomes.

Construction of the cytogenetic maps

The genetic distances of SSR markers associated with the corresponding BACs were also converted into the relative positions in the corresponding linkage map (Fig. 2a). In order to confirm the physical position of each clone, FISH signal of each BAC clone was measured in 5-8 cells with clear chromosome spreads and the RMP of FISH signals were computed (Table 3). Based on the data, the cytogenetic maps of the ho-

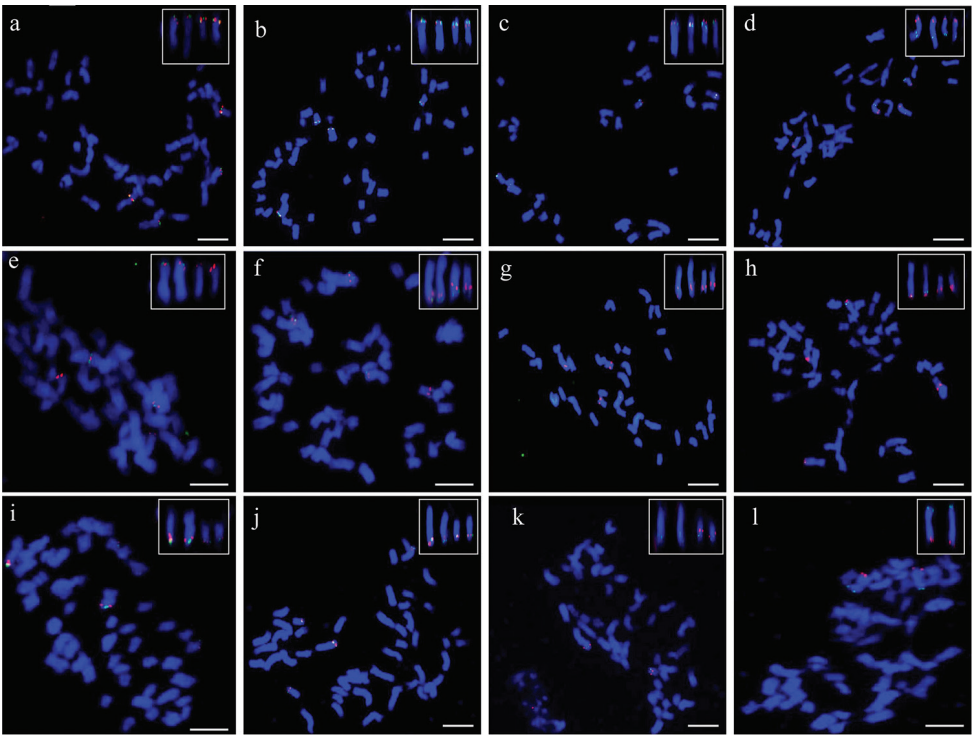


Figure 1. The order of two BACs on metaphase chromosome of *G. hirsutum* (AD_1) TM-1 using Dual-color-FISH. **a** 305A19(green)/348I20(red) **b** 305A19 (green)/64M24(red) **c** 144E04 (red)/64M24(green) **d** 64M24 (red)/400N03(green) **e** 305A19 (red)/378J07(green) **f** 118G12(red)/164I24(green) **g** 423C18 (red)/400L15(green) **h** 85P13 (green)/400L15(red) **i** 85P13 (red)/421E24(green) **j** 85P13 (red)/164I24(green) **k** D1 (red)/118G12(green) **l** 378J07 (green)/ A1 (red). Bar = 5 μm.

moeologous chromosomes D_h01 and A_h01 were constructed (Fig. 2b, c). The order of individual BACs along the chromosome was generally collinear with the order of the corresponding SSR markers along the linkage map, except for a few closely linked loci, 144E04 (NAU2474) and 348I20 (NAU3253), 118G12 (NAU4891) and 400N03 (BNL2921), which displayed changes in the order between the genetic markers and BAC locations (Fig. 2a, b). Moreover, the BACs showed better concordance in the orders and positions between the two cytogenetic maps of the homoeologous chromosomes A_h01 and D_h01, except for 400N03 (BNL2921) (Fig. 2b, c), which suggests a rearrangement between the A_h01 and D_h01 homoeologous chromosomes in the process of evolution. A significant difference between the two types of maps was viewed, that is, the markers flanking the middle region were separated by short genetic distance but long physical distance. For example, the genetic distance between markers NAU3433 and BNL2921 is 11.2% of total genetic distance of chromosome 15 (D_h01), but the physical distances between these two markers is 59.4% of the total length of the chromosome D_h01 (Fig. 2a, b).

Table 3. Physical locations of FISH-mapped BACs in *G. hirsutum* draft genome assembly and cytogenetic map.

BAC	SSR marker	Loc. in AD_1 *1 draft genome				Loc. in AD_1 Cytogenetic map *3	
		No. of chromosome	Start (bp)	End (bp)	RMP(%)*2	D_h01 RMP(%)	A_h01 RMP(%)
305A19	NAU2015	D_h01	60681011	60681490	1.26	3.00±0	4.51±0.41
378J07	HAU076	A_h01	96488204	96488397	3.40	/	8.01±0.48
144E04	NAU2474	D_h01	57722851	57723034	6.07	4.33±0.47	/
		scaffold183_A01	19925	20108	/	/	9.01±1.25
348I20	NAU3254	D_h01	59322542	59322834	3.47	8.33±0.47	10.02±0.51
64M24	NAU3433	A_h01	90268406	90268610	9.63	/	15.00±0.47
		D_h01	53813626	53813830	12.44	11.33±1.24	/
400N03	BNL2921	A_h01	40133025	40133182	59.82	84.66±0.47	61.99±0.94
423C18	TMB0062	A_h01	17562250	17562499	82.42	70.66±5.24	74.11±0.36
118G12	NAU4891	A_h01	17991434	17991731	81.99	79.33±4.49	84.01±1.10
85P13	NAU3135	A_h01	11722545	11722728	88.26	/	88.07±0.19
		D_h01	9387192	9387374	84.73	85.33±0.47	/
D1	BNL3902*4	D_h01	26803236	26803427	56.38	69.66±0.94	/
164I21	BNL3888b	A_h01	11084705	11084886	88.90	88.66±1.69	90.98±0.27
421E24	BNL3580	A_h01	7078093	7078309	92.91	89.00±1.41	92.99±0.65
400L15	NAU4044	A_h01	2245730	2245951	97.75	/	96.01±1.19
		scaffold3710_D01	109956	110177	/	90.33±1.24	/

Note: *1, the AD_1-NBI draft genome (Zhang et al. 2015); *2, RMP: relative map position, in cytogenetic map, it refers to the percentage of the distance (μm) from the FISH signal site to the end of the one arm accounting for the total length of the chromosome; in sequence map, it refers to the percentage of the sequence location of the corresponding SSRs of BACs accounting for the total length of the chromosome (A_h01 = 99884700 bp, D_h01 = 61456009 bp); *3, 5-8 cells were used for measurement; *4 corresponding SSR of D_h01-specific BAC.

Integration and analysis of BACs positions across the cytogenetic and genome assembly maps

To compare our cytogenetic maps directly to the draft genome assembly map (Zhang et al. 2015), the corresponding SSR primers of the BAC clones were mapped to the draft genome sequence by e-PCR, and the relative positions of the SSRs were calculated according to the e-PCR results (Table 3). Based on the above data, we integrated the cytogenetic maps with the genome sequence maps of the homoeologous chromosomes A_h01 and D_h01 to compare their distributions (Fig. 3). The alignments allowed a global view of the relations between the chromosomal positions and physical positions in draft genome map of the BAC clones. The number of BACs mapped on each pseudo-chromosome in the draft genome assembly map was significantly less than that on the corresponding cytogenetic maps (six to twelve on D_h01, nine to twelve on A_h01) (Fig. 3). Of the eleven homoeologous-chromosomes-shared BACs based on cytogenetic maps, four BACs' corresponding SSR markers (NAU3433, NAU3135, NAU2474 and NAU4044) were simultaneously mapped on the two corresponding chromosomes in *G. hirsutum* draft genome assembly. The others were only mapped on one of the

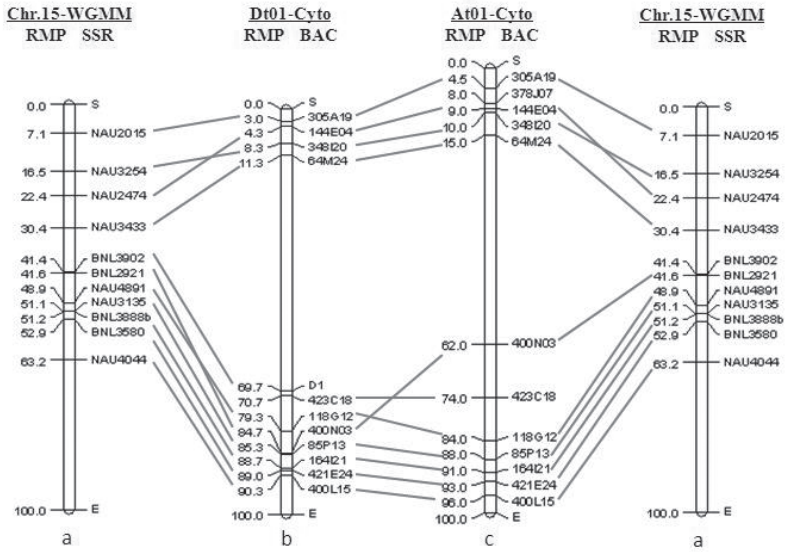


Figure 2. Comparison of positions of BACs in cytogenetic maps of *G. hirsutum* A_h01/D_h01 with genetic positions of SSR markers **a** Positions of SSR markers based on WGMM; **c, b** Cytogenetic maps of *G. hirsutum* A_h01/D_h01.

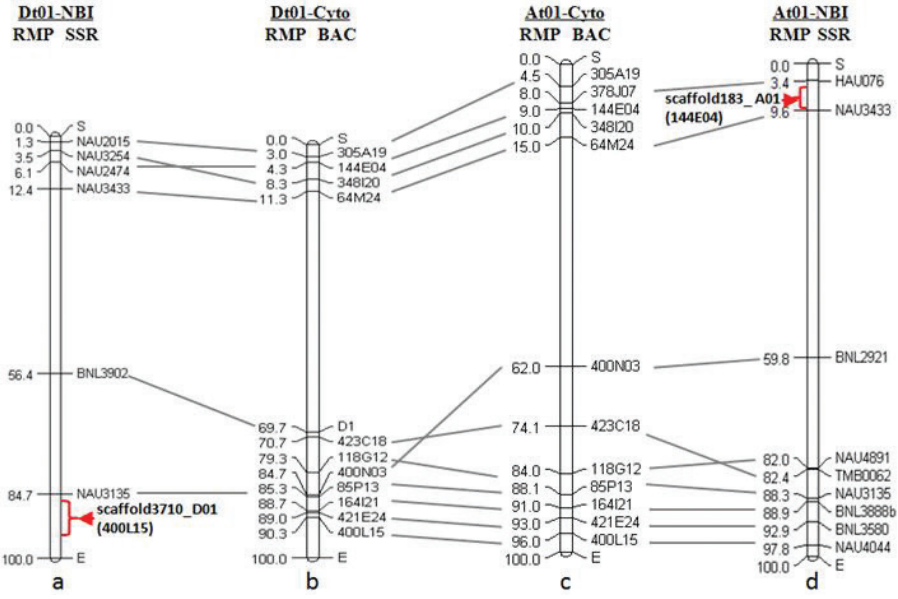


Figure 3. Integrated cytogenetic /genome assembly maps of *G. hirsutum* A_h01/D_h01. **a** Relative map position of BACs mapping to D_h01 of the AD_1-NBI draft genome **b** Cytogenetic map of *G. hirsutum* Chromosome D_h01 based on 12 BAC clones **c** Cytogenetic map of *G. hirsutum* A_h01 based on 12 BAC clones **d** Relative map position of BACs mapping to A_h01 of the AD_1-NBI draft genome. Arrow-head in a and d represent the locations of scaffold3710_D01 and scaffold183_A01 in the draft genome (AD_1-NBI) respectively.

chromosome A_h01 or D_h01 respectively. NAU2474 was mapped on the chromosome D_h01 and scaffold183_A01 of the draft genome assembly by e-PCR. Its corresponding BAC clone 144E04 was FISH mapped on chromosome A_h01 (RMP 9.01%) and D_h01 (RMP 4.33%) in cytogenetic maps. NAU4044 was mapped on the chromosome A_h01 and scaffold3710_D01 of the draft genome assembly by e-PCR. Its corresponding BAC clone 400L15 was FISH mapped on chromosome A_h01 (RMP 96.01%) and D_h01 (RMP 90.33%) in cytogenetic maps. Based on these comparison results, the locations of the two scaffolds in the draft genome assembly were determined approximately. That is, scaffold183_A01 (size 55529 bp) located between the SSR markers HAU076 and NAU3433 on the chromosome A_h01, i.e., the relative position between 3.4% and 9.6% (sequence loci from 90268610 bp to 96488204 bp) (shown by arrow Fig. 3d). Scaffold3710_D01 (size 191022 bp) locates near the end of the chromosome D_h01, i.e., the outer of the relative position 84.7% (sequence loci from 6145600 bp to 9387374 bp) (shown by arrow Fig. 3a).

Discussion

Integration of the genetic and cytogenetic maps of homoeologous chromosomes A_h01 and D_h01

In cotton, more than 30 genetic maps have been published, including several integrated maps with higher marker density (Yu et al. 2010, Yu et al. 2011, Blenda et al. 2012), and a whole-genome marker map (WGMM) by integrating publicly available sequence tagged DNA markers with the cotton D-genome sequence (Wang et al. 2013). Undeniably, they are a foundational tool and resources for marker-assisted selection and genomic studies. But the linkage maps provide little information about physical locations, distributions, distances, and sometimes orientations of genetic markers. Cytogenetic maps encompassing the information from both genetic maps and cytological maps, can relate the markers mapped across linkage groups to cytological position on chromosomes. Using a set of marker-anchored BACs, we developed the cytogenetic maps of homoeologous chromosomes Ah01 and Dh01 in *G. hirsutum*. The comparative map alignments revealed a significant disproportion between genetic and physical distances in the pericentromeric region, such as, the distance between markers NAU3433 and BNL2921 with 11.2 RMP(Fig. 2a) but on the cytogenetic map with 59.4 RMP (Fig. 2b). The reduction of recombination around the chromosome centromere is a common feature and the region of recombination suppression correlates directly with sizes of centromeric heterochromatic regions (Sun et al. 2013). So this implies larger region of suppressed recombination was detected in the pericentromeric region of chromosome D_h01. Moreover, the orders of most genetic markers are collinear with corresponding BAC locations although several closely linked loci in D_h01 display inconsistent orders or locations compared with those in BAC FISH maps.

In total, the integrated genetic and cytogenetic maps can serve as a template to facilitate sequence assembly, because the maps provided information on the distribution of genetic markers across chromosomes and the linkage gaps derived from recombination suppression.

Homologous relationships between chromosomes A_h01 and D_h01

As a typical allotetraploid, which contains two sub-genomes originating from related ancestor species with different genome sizes, *G. hirsutum* has been studied on its homoeologous chromosomes for a long time. Results revealed that fragment additivity (Liu et al. 2001), the independence of evolution of duplicated genes (Cronn et al. 1999), conservation in gene content, order, and spacing (Grover et al. 2004, 2007) between the homoeologous chromosomes, as well as the potential mechanisms for genome-size variation in the homoeologous chromosomes (Wang et al. 2010). Here, we constructed the cytogenetic maps of homoeologous chromosomes A_h01 and D_h01 using shared-markers-anchored BACs. By comparison analysis of BACs' positions, consistent orders of FISH signals were viewed in both homoeologous chromosomes, except for one BAC clone 400N03, which showed obvious location discrepancy in the homoeologous chromosomes (RMP 62% in A_h01 and 84.7% in D_h01). The discrepancy may be caused by a chromosomal rearrangement in this region during a certain period of polyploidization. In addition, better collinearity of ten of eleven shared BACs between the homoeologous chromosomes suggests that there remains a generally high level of sequence conservation between homoeologous chromosomes A_h01 and D_h01, though polyploidization occurred about 2 MYA (Cronn et al. 2002, Seelanan et al. 1997, Wendel 1989).

Integration of the cytogenetic maps and the cytogenetic and genome assembly maps

The e-PCR can be used to search for sub-sequences that closely match the primers of SSRs, which can help to identify the genome positions of SSRs within the reference genome sequence (McCouch et al. 2002, Li et al. 2015). In this study, we identified the genome positions of thirteen SSRs using e-PCR. Results showed the length and position of the target sequence for each pair of primers against the reference genome sequence were consistent with the initial selection, which ensured the accuracy of the next relative position calculation and comparative analysis.

Mis-assemblies are common when draft genome sequences have been generated by de novo assembly of sequences obtained with NGS technologies (Meader et al. 2010, Alkan et al. 2011). Since the assembly of *G. hirsutum* was done using the SOAPdenovo software, the final assembly comprised 265,279 contigs and 40,407 scaffolds (Zhang et al. 2015), so mis-assembled scaffolds may exist in the draft genome. On the other hand, there are a generally high level of sequence conservation between homoeolo-

gous genomic regions in allotetraploid species including cotton and wheat (Zhao et al. 2012, Brenchley et al. 2012), it is difficult to annotate and assemble whole-genome sequences. Since the cytogenetic map can reflect the true position of the DNA sequence in the chromosome, so it has some significance for verification and correction of the genome assembly. In the process of genome sequencing and sequence assembly, the cytogenetic map plays a role in filling the sequencing gaps, correcting assembly errors, evaluating the quality of assembly, achieving more scaffolds and contigs chromosomal localization and orientation. Wang et al. mapped 32 BAC clones to some of the homologous chromosomes 12A and 12D of upland cotton by FISH, and constructed the high resolution cytogenetic map of the two chromosomes (Wang et al. 2010). Through the integration of genetic loci and physical sites, considerable variations in the composition, structure and size of the two homoeologous chromosomes were viewed, which play an important role in the sequencing and sequence assembly of *G. hirsutum* (Wang et al. 2010; Zhang et al. 2015). By comparison of the distributions of fosmid clones on the cucumber draft genome assembly map and cytogenetic map, the accuracy and coverage of the draft genome assembly map were evaluated (Sun et al. 2013).

Here, we constructed the cytogenetic maps of homoeologous chromosomes A_h01 and D_h01 using shared-BACs. By integration of cytogenetic maps and the cytogenetic and genome assembly maps, we identified the positions of two scaffolds in chromosome (Fig. 3a, d). Among the eleven shared-BACs in the cytogenetic maps of chromosomes A_h01 and D_h01, only four (accounting for 36.36%) had hits both in two corresponding pseudo-chromosome in the draft genome assembly map, the others were only mapped on one of the chromosome A_h01 or D_h01 respectively. It may be that some homologous sequences were removed as repeats, and only partial sequences information with homology were assembled on one of the two homoeologous chromosomes during the assembly process.

Conclusions

We demonstrated concordant orders and RMP of markers between the sequence map and physical map based on FISH. By integration of cytogenetic maps with sequence maps of the two chromosomes, we inferred the locations of the two scaffolds, and speculated some homologous sequences belonging to homoeologous chromosomes were removed as repetitiveness during the process of sequence assembly. Our study not only offers molecular tools for cotton genomics research, but also provides valuable information for the improvement of the draft genome assembly.

Acknowledgements

We deeply thank Prof. Tianzhen Zhang (Nanjing Agricultural University, China) for providing the chromosome-specific BAC clones, Prof. Zhiying Ma (Heibei Agricul-

tural University, China) for supplying the BAC library. The research was sponsored by a grant from the National Natural Science Foundation of China (No. 31471548), PhD Scientific Research Fund of Anyang Institute of Technology (No. BSJ2016005), State Key Laboratory of Cotton Biology Open Fund (No. CB2017A06), State Key Laboratory of Cotton Biology Open Fund (No. CB2015A21).

References

Alkan C, Sajjadian S, Eichler EE (2011) Limitations of next-generation genome sequence assembly. Natture Methods 8: 61–65. https://doi.org/10.1038/nmeth.1527

Blenda A, Fang DD, Rami JF, Garsmeur O, Luo F, Lacape JM (2012) A high density consensus genetic map of tetraploid cotton that integrates multiple component maps through molecular marker redundancy check. PLoS ONE 7(9): e45739. https://doi.org/10.1371/journal.pone.0045739

Brenchley R, Spannagl M, Pfeifer M, Barker GL, D'Amore R, Allen AM, et al. (2012) Analysis of the bread wheat genome using whole-genome shotgun sequencing. Nature 491(7426): 705–710. https://doi.org/10.1038/nature11650

Cheng H, Peng RH, Zhang XD, Liu F, Wang CY, Wang KB (2012) A rapid method to screen BAC library in cotton. Biotechnology 22: 55–57. [In Chinese]

Cheng ZK, Presting GG, Buell CR, Wing RA, Jiang JM (2001) High-resolution pachytene chromosome mapping of bacterial artificial chromosomes anchored by genetic markers reveals the centromere location and the distribution of genetic recombination along chromosome 10 of rice. Genetics 157(4): 1749–1757.

Cronn RC, Small RL, Haselkorn T, Wendel JF (2002) Rapid diversification of the cotton genus (*Gossypium*: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. American Journal of Botany 89(4): 707–725. https://doi.org/10.3732/ajb.89.4.707

Cronn RC, Small RL, Wendel JF (1999) Duplicated genes evolve independently after polyploid formation in cotton. Proceedings of the National Academy of Sciences USA 96(25): 14406–14411. https://doi.org/10.1073/pnas.96.25.14406

Cui XL, Liu F, Liu Y, Zhou ZL, Zhao Y, Wang CY, Wang XX, Cai XY, Wang YH, Meng F, Peng RH, Wang KB (2015) Construction of cytogenetic map of *Gossypium herbaceum* chromosome 1 and its integration with genetic maps. Molecular Cytogenetics 8(1): 2. https://doi.org/10.1186/s13039-015-0106-y

Endrizzi JE, Turcotte EL, Kohel RJ (1985) Genetics, cytogenetics, and evolution of *Gossypium*. Advance in Genetics 23: 271–375. https://doi.org/10.1016/S0065-2660(08)60515-5

Figueroa DM, Bass HW (2012) Development of pachytene FISH maps for six maize chromosomes and their integration with other maize maps for insights into genome structure variation. Chromosome Research 20(4): 363-380. https://doi.org/10.1007/s10577-012-9281-4

Fonsêca A, Ferreira J, Dos Santos TR, Mosiolek M, Bellucci E, Kami J, Gepts P, Geffroy V, Schweizer D, Dos Santos KG, Pedrosa-Harand A (2010) Cytogenetic map of common bean (*Phaseolus vulgaris* L.). Chromosome Research 18(4): 487–502. https://doi.org/10.1007/s10577-010-9129-8

Gan YM, Chen D, Liu F, Wang CY, Li SH, Zhang XD, Peng RH, Wang KB (2011) Individual chromosome assignment and chromosomal collinearity in *Gossypium thurberi*, *G. trilobum* and D subgenome of *G. barbadense* revealed by BAC-FISH. Genes & Genetic Systems 86(3): 165–174. https://doi.org/10.1266/ggs.86.165

Gao HY, Wang HF, Liu F, Peng RH, Zhang Y, Cheng H, Ma XY, Wang KB (2013) Construction of the bacterial artificial chromosome library of *G. herbaceum var. africanum*. Chinese Science Bulletin 58(26): 3199–3201. https://doi.org/10.1007/s11434-013-5864-5

Grover CE, Kim H, Wing RA, Paterson AH, Wendel JF (2007) Microcolinearity and genome evolution in the AdhA region of diploid and polyploid cotton (*Gossypium*). The Plant Journal 50(6): 995–1006. https://doi.org/10.1111/j.1365-313X.2007.03102.x

Grover CE, Kim H, Wing RA, Paterson AH, Wendel JF (2004) Incongruent patterns of local and global genome size evolution in cotton. Genome Research 14(8):1474–1482. https://doi.org/10.1101/gr.2673204

Han YH, Zhang ZH, Huang SW, Jin WW (2011) An integrated molecular cytogenetic map of *Cucumis sativus* L. chromosome 2. BMC Genetics 12: 18. https://doi.org/10.1186/1471-2156-12-18

Hanson RE, IslamFaridi MN, Percival EA, Crane CF, Ji YF, McKnight TD, Stelly DM, Price HJ (1996) Distribution of 5S and 18S-28S rDNA loci in a tetraploid cotton (*Gossypium hirsutum* L) and its putative diploid ancestors. Chromosoma 105: 55–61. https://doi.org/10.1007/BF02510039

Ji Y, Zhao X, Paterson AH, Price HJ, Stelly DM (2007) Integrative mapping of *Gossypium hirsutum* L. by meiotic fluorescent in situ hybridization of a tandemly repetitive sequence (B77). Genetics 176(1): 115–123. https://doi.org/10.1534/genetics.107.071738

Jiang JM, Gill BS (2006) Current status and the future of fluorescence in situ hybridization (FISH) in plant genome research. Genome 49(9): 1057–1068. https://doi.org/10.1139/g06-076

Kao FI, Cheng YY, Chow TY, Chen HH, Liu SM, Cheng CH, Chung MC (2006) An integrated map of *Oryza sativa* L. chromosome 5. Theoretical and Applied Genetics 112(5): 891–902. https://doi.org/10.1007/s00122-005-0191-0

Koo DH, Jo SH, Bang JW, Park HM, Lee S, Choi D (2008) Integration of cytogenetic and genetic linkage maps unveils the physical architecture of tomato chromosome 2. Genetics 179(3): 1211–1220. https://doi.org/10.1534/genetics.108.089532

Li F, Fan G, Lu C, Xiao G, Zou C, Kohel RJ, et al. (2015) Genome sequence of cultivated Upland cotton (*Gossypium hirsutum* TM-1) provides insights into genome evolution. Nature Biotechnology 33(5): 524–530. https://doi.org/10.1038/nbt.3208

Li FG, Fan GY, Wang KB, Sun FM, Yuan YL, Song GL, et al. (2014) Genome sequence of the cultivated cotton *Gossypium arboreum*. Nature Genetics 46(6): 567–572. https://doi.org/10.1038/ng.2987

Li JC, Xie CH, Tian ZD, Lindqvist-Kreuze H, Bonierbale M, Liu J (2015) SSR and e-PCR provide a bridge between genetic map and genome sequence of potato for marker development in target QTL region. American Journal of Potato Research 92: 312–317. https://doi.org/10.1007/s12230-015-9432-1

Liu B, Brubaker CL, Mergeai G, Cronn RC, Wendel JF (2001) Polyploid formation in cotton is not accompanied by rapid genomic changes. Genome 44(3): 321–330. https://doi.org/10.1139/g01-011

Liu X, Zhao B, Zheng HJ, Hu Y, Lu Gang, Yang CQ, et al. (2015) *Gossypium barbadense* genome sequence provides insight into the evolution of extra-long staple fiber and specialized metabolites. Science Report 5: 14139. https://doi.org/10.1038/srep14139

McCouch S, Teytelman L, Xu Y, Lobos KB, Clare K, Walton M, Fu B, Maghirang R, Li Z, Xing Y, Zhang Q, Kono I, Yano M, Fjellstrom R, DeClerck G, Schneider D, Cartinhour S, Ware D, Stein L (2002) Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). DNA Research 9(6): 199–207.

Meader S, Hillier LW, Locke D, Ponting CP, Lunter G (2010) Genome assembly quality, assessment and improvement using the neutral indel model. Genome Research 20(5): 675–684. https://doi.org/10.1101/gr.096966.109

Paterson AH, Wendel JF, Gundlach H, Guo H, Jenkins J, Jin D, et al. (2012) Repeated polyploidization of Gossypium genomes and the evolution of spinnable cotton fibres. Nature 492(7429): 423–427. https://doi.org/10.1038/nature11798

Rong J, Abbey C, Bowers JE, Brubaker CL, Chang C, Chee PW, et al. (2004) A 3347-locus genetic recombination map of sequence-tagged sites reveals features of genome organization, transmission and evolution of cotton (*Gossypium*). Genetics 166(1): 389–417. https://doi.org/10.1534/genetics.166.1.389

Said JI, Lin ZX, Zhang XL, Song MZ, Zhang JF (2013) A comprehensive meta QTL analysis for fiber quality, yield, yield related and morphological traits, drought tolerance, and disease resistance in tetraploid cotton. BMC Genomics 14: 776. https://doi.org/10.1186/1471-2164-14-776

Seelanan T, Schnabel A, Wendel JF (1997) Congruence and consensus in the cotton tribe. Systematic Botany 22: 259–290. https://doi.org/10.2307/2419457

Sun JY, Zhang ZH, Zong X, Hang SW, Li ZY, Han YH (2013) A high-resolution cucumber cytogenetic map integrated with the genome assembly. BMC Genomics,14: 461. https://doi.org/10.1186/1471-2164-14-461

Szinay D, Chang SB, Khrustaleva L, Peters S, Schijlen E, Bai Y, Stiekema WJ, Van Ham RC, De Jong H, Klein Lankhorst RM (2008) High-resolution chromosome mapping of BACs using multi-colour FISH and pooled-BAC FISH as a backbone for sequencing tomato chromosome 6. The Plant Journal 56(4): 627–637. https://doi.org/10.1111/j.1365-313X.2008.03626.x

Tang X, Szinay D, Lang C, Ramanna MS, van der Vossen EA, Datema E, Lankhorst RK, De Boer J, Peters SA, Bachem C, Stiekema W, Visser RG, De Jong H, Bai Y (2008) Cross-species BAC-FISH painting of the tomato and potato chromosome 6 reveals undescribed chromosomal rearrangements. Genetics 180(3): 1319–1328. https://doi.org/10.1534/genetics.108.093211

Wang K, Guo WZ, Zhang TZ (2007) Development of one set of chromosome specific microsatellite containing BACs and their physical mapping in *Gossypium hirsutum* L. Theoretical and Applied Genetics 115(5): 675–682. https://doi.org/10.1007/s00122-007-0598-x

Wang K, Guo WZ, Yang ZJ, Hu Y, Zhang WP, Zhou BL, Stelly DM, Chen ZJ, Zhang TZ (2010) Structure and size variations between 12A and 12D homoeologous chromosomes based on high-resolution cytogenetic map in allotetraploid cotton. Chromosoma 119(3): 255–266. https://doi.org/10.1007/s00412-009-0254-0

Wang KB, Wang ZW, Li FG, Ye WW, Wang JY, Song GL, et al. (2012) The draft genome of a diploid cotton *Gossypium raimondii*. Nature Genetics 44(10): 1098–1103. https://doi.org/10.1038/ng.2371

Wang ZN, Zhang D, Wang XY, Tan X, Guo H, Paterson AH (2013) A whole-genome DNA marker map for cotton based on the D-genome sequence of *Gossypium raimondii* L. Genes Genome Genetics 3(10): 1759–1767. https://doi.org/10.1534/g3.113.006890

Wendel JF, Cronn RC (2003) Polyploidy and the evolutionary history of cotton. Advances in Agronomy, 78: 139–186. https://doi.org/10.1016/S0065-2113(02)78004-8

Wendel JF, Flagel L, Adams KL (2012) Jeans, genes, and genomes: cotton as a model for studying polyploidy. In: Soltis PS, Soltis DE (Eds) Polyploidy and Genome Evolution. Berlin: Springer, 181–207. https://doi.org/10.1007/978-3-642-31442-1_10

Wendel JF (1989) New World tetraploid cottons contain Old World cytoplasm. Proceedings of the National Academy of Sciences, USA, 86(11): 4132–4136. https://doi.org/10.1073/pnas.86.11.4132

Xiong Z, Kim JS, Pires JC (2010) Integration of genetic, physical, and cytogenetic maps for *Brassica rapa* chromosome A7. Cytogenetic and Genome Research 129(1–3): 190–198. https://doi.org/10.1159/000314640

Xu ZY, Kohel RJ, Song GL, Cho J, Yu J, Yu SX, Tomkins J, Yu JZ (2008) An integrated genetic and physical map of homoeologous chromosomes 12 and 26 in upland cotton (*G. hirsutum* L.). BMC Genomics 9: 108. https://doi.org/10.1186/1471-2164-9-108

Yu J, Kohel RJ, Smith CW (2010) The construction of a tetraploid cotton genome wide comprehensive reference map. Genomics 95(4): 230–240. https://doi.org/10.1016/j.ygeno.2010.02.001

Yu Y, Yuan D, Liang S, Li X, Wang X, Lin Z, Zhang X (2011) Genome structure of cotton revealed by a genome-wide SSR genetic map constructed from a BC1 population between *Gossypium hirsutum* and *G. barbadense*. BMC Genomics 12: 15. https://doi.org/10.1186/1471-2164-12-15

Yuan DJ, Tang ZH, Wang MJ, Gao WH, Tu LL, Jin X , et al. (2015) The genome sequence of Sea-Island cotton (*Gossypium barbadense*) provides insights into the allopolyploidization and development of superior spinnable fibres. Science Report 5: 17662. https://doi.org/10.1038/srep17662

Zhang TZ, Hu Y, Jiang WK, Fang L, Guan XY, Chen JD, et al. (2015) Sequencing of allotetraploid cotton (*Gossypium hirsutum* L. acc. TM-1) provides a resource for fiber improvement. Nature Biotechnology 33(5): 531–537. https://doi.org/10.1038/nbt.3207

Zhao L, Lv YD, Cai CP, Tong XC, Chen XD, Zhang W, Du H, Guo XH, Guo WZ (2012) Toward allotetraploid cotton genome assembly: integration of a high-density molecular genetic linkage map with DNA sequence information. BMC Genomics 13: 539. https://doi.org/10.1186/1471-2164-13-539

CompCytogen 11(2): 421–430 (2017)
doi: 10.3897/CompCytogen.v11i2.13494
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Study of male-mediated gene flow across a hybrid zone in the common shrew (*Sorex araneus*) using Y chromosome

Andrei V. Polyakov[1], Viktor V. Panov[2]

1 *Institute of Cytology and Genetics, the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia*
2 *Institute of Systematics and Ecology of Animals, the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia*

Corresponding author: *Andrei Polyakov* (polyakov@bionet.nsc.ru)

Academic editor: *Jan Zima* | Received 1 May 2017 | Accepted 22 May 2017 | Published 19 June 2017

http://zoobank.org/4D6BB777-7C07-4074-9B3E-976D77C2D67F

Citation: Polyakov AV, Panov VV (2017) Study of male-mediated gene flow across a hybrid zone in the common shrew (*Sorex araneus*) using Y chromosome. Comparative Cytogenetics 11(2): 421–430. https://doi.org/10.3897/CompCytogen.v11i2.13494

Abstract

Despite many studies, the impact of chromosome rearrangements on gene flow between chromosome races of the common shrew (*Sorex araneus* Linnaeus, 1758) remains unclear. Interracial hybrids form meiotic chromosome complexes that are associated with reduced fertility. Nevertheless comprehensive investigations of autosomal and mitochondrial markers revealed weak or no barrier to gene flow between chromosomally divergent populations.

In a narrow zone of contact between the Novosibirsk and Tomsk races hybrids are produced with extraordinarily complex configurations at meiosis I. Microsatellite markers have not revealed any barrier to gene flow, but the phenotypic differentiation between races is greater than may be expected if gene flow was unrestricted. To explore this contradiction we analyzed the distribution of the Y chromosome SNP markers within this hybrid zone. The Y chromosome variants in combination with race specific autosome complements allow backcrosses to be distinguished and their proportion among individuals within the hybrid zone to be evaluated. The balanced ratio of the Y variants observed among the pure race individuals as well as backcrosses reveals no male mediated barrier to gene flow. The impact of reproductive unfitness of backcrosses on gene flow is discussed as a possible mechanism of the preservation of race-specific morphology within the hybrid zone.

Keywords

Sorex araneus, phenotypic evolution, hybrid zone, gene flow, Y chromosome

Introduction

The common shrew (*Sorex araneus* Linnaeus, 1758) is assumed to be a promising model species for evolutionary studies because of the remarkable diversity of its karyotype. Ten chromosome arms joined together in various Robertsonian fusions form dozens of chromosome races (Wójcik et al. 2003) – "groups of geographically contiguous populations that share the same set of metacentrics and acrocentrics by descent" (Hausser et al. 1994). Ranges of the races do not overlap but parapatric races can establish geographic contacts in narrow zones of intergradation where they hybridize and produce interracial hybrids. At meiosis I of these hybrids, chromosomes form multivalents of different complexity following the pattern of arm homology. These multivalents are associated with reduced fertility of hybrids due to aberrations in chromosome pairing, recombination and segregation, which in turn may lead to germ cell death or/and generation of unbalanced gametes (Searle 1993). The decline in fertility can act as a mechanism to impede gene flow, contributing, thus, to speciation (King 1993). Nevertheless, comprehensive studies of protein, autosomal and mitochondrial DNA markers revealed weak or no divergence between chromosomally divergent populations [(Wójcik et al. 2002 (for review of previous works), Andersson et al. 2004, 2005, Jadwiszczak et al. 2006, Lundqvist et al. 2011, Moska et al. 2011, Horn et al. 2012)]. However, in some rare cases races inhabiting adjacent areas exhibit clear morphological distinction within the zones of intergradation (Chętnicki et al. 1996, Polyakov et al. 2002, Polly et al. 2013), providing an excellent opportunity to clarify the details of interracial contact.

The Novosibirsk and Tomsk races occupy the whole territory of West Siberia (Polyakov et al. 1996, 2000) and form there a hybrid zone approximately 8.5 kilometers in width (Polyakov et al. 2011). Characteristic chromosomes of the Novosibirsk race comprise six metacentric autosomes *go, hn, ik, jl, mp, qr*, whereas the Tomsk race is characterized by metacentrics *gk, hi, jl, mn* and acrocentrics *o, p, q, r*. An italicized letter of the alphabet indicates here a chromosome arm, which can either be unattached as an acrocentric or attached to another chromosome arm as a metacentric (Searle et al. 1991).

Interracial hybrids form a complex multivalent (a chain of nine chromosomes) *o/og/gk/ki/ih/hn/nm/mp/p* (Polyakov et al. 2004) that is expected to cause substantially reduced fertility compared to pure race individuals. This assumption is supported by the observation of a wide variety of chromosome pairing abnormalities in hybrid males, in which the overall proportion of cells with synaptic abnormalities was 13 times higher than in homozygotes (Borodin 2008).

The Novosibirsk and Tomsk races apparently evolved in allopatry during the last glacial maximum in Ural and Altai refugia, respectively (Polyakov et al. 2003). They are well differentiated for morphological traits (Yudin 1989) and DNA markers (Polyakov et al. 2009). Interracial differences in morphology remain significant even within the zone where races meet and hybridize (Polyakov et al. 2002, Polly et al. 2013). The estimated duration of hybridization is at least several hundreds of generations

(Polyakov et al. 2011) and preservation of race-specific morphological features within the hybrid zone for such a long period would only be possible if the barrier to gene flow between populations is very strong (Polly et al. 2013). If this barrier arises due to the influence of chromosomal rearrangements on fertility of hybrids, the fecundity of these hybrids should be very low.

Surprisingly, analysis of microsatellites has revealed low level of differentiation within this hybrid zone, which implies a free flow of genes (Horn et al. 2012). This contradicts the results of morphological studies and requires an additional consideration. It is necessary to mention however, that analysis based on microsatellites may underestimate the values of differentiation because of high variability of these markers (Balloux et al. 2000).

To explore the contradiction between the microsatellites and morphology, it might be useful to re-examine the fertility of hybrids with an additional set of markers. If their reproductive potential is low enough to impede the introgression of morphological traits, then microsatellites can be considered an inappropriate marker system for such analyses. The impact of chromosome rearrangements on gene flow in this case will be proved. Otherwise, the mechanism of restriction of gene flow needs to be revised.

In order to estimate a contribution of males - hybrids F1 in reproduction we identified two variants of a new SNP marker in the Y chromosome intron UTY11 and examined their frequencies within the hybrid zone and at the adjacent territory of the Novosibirsk race. In this article we focus particularly on descendants of the hybrid males. The Y chromosome variants in combination with race specific autosome complements allow backcrosses to be distinguished, i.e. individuals that have the Y chromosome from one race together with autosome complement of another parental race. This combination can only occur if the Y chromosome is transmitted through the F1 male. This study is the first that examines the fitness of hybrids directly according to the presence of their descendants in population. All previous studies were based on the assessment of the level of meiotic aberrations and the width of the zones of introgression.

The methodological approach of the presented study was based on the following reasoning:

1. Balanced gametes in hybrids have either the full Novosibirsk or the full Tomsk complement of autosomes. Therefore, only three variants of karyotype - Novosibirsk homozygotes, Tomsk homozygotes and Novosibirsk/Tomsk heterozygotes, occur within the hybrid zone.
2. The Y chromosome does not recombine and thus its alleles retain their racial specificity.
3. A Y chromosome allele of one race can occur in another race only if it has been transmitted through the F1 male.

If the fertility of hybrids is so low that provides a barrier to gene flow, the expected number of backcrosses will be close to zero.

Material and methods

The variability of intron UTY11 of the Y-chromosome was studied among 39 males from the centre of the hybrid zone between the Novosibirsk and Tomsk chromosome races of the common shrew (Figure 1). Of these males, 25 were homozygous for the Novosibirsk race and 14 for the Tomsk race chromosome complements. Trapping and karyotyping were performed in previous studies (Polyakov et al. 2011). Additional 32 individuals from two localities situated within the distribution range of the Novosibirsk race (27 from Akademgorodok and 5 from Chemskoy Bor) were examined. Shrews of these localities are monomorphic for the Novosibirsk race karyotypes (Král and Radjabli 1974, Polyakov et al. 1997).

Intron UTY11 of the Y chromosome was amplified following the protocol of Hellborg and Ellegren (2003). Sequencing was performed in both directions and analyzed using an ABI Prism 3100 genetic analyzer (Applied Biosystems) in the SB RAS Genomics Core Facility (Novosibirsk, Russia).

Student's t-test statistics was used to assess the difference in the ratio of the Y haplotypes between races. The level of linkage disequilibrium between the Y haplotypes (Y) and autosome complements (A) was quantified by the coefficient of linkage disequilibrium $D_{YA} = p_{YA} - p_Y p_A$.

Results

Two haplotypes of intron UTY11 with cytosine/thymine substitution at position 585 (C-haplotype/T-haplotype, respectively) were identified among the studied shrews (GenBank (www.ncbi.nlm.nih.gov/Genbank) accession numbers KY652093 and KY652094). Table 1 shows the distribution of these haplotypes in the studied races. The haplotype C was detected in the shrews trapped in Akademgorodok, Chemskoy Bor and in the hybrid zone. The haplotype T was detected in the hybrid zone only.

In the hybrid zone the frequency of C-haplotype (0.77) is greater than the frequency of T-haplotype (0.23), however the ratio of the Y haplotypes between shrews with the Novosibirsk and Tomsk autosome complements does not differ statistically ($t_d = 0.59$, $P > 0.05$).

We did not detect linkage disequilibrium between the Novosibirsk- and Tomsk-derived autosomes and the Y chromosome variants ($D = 0.02$, $\chi 2 = 0.37$, $P > 0.05$).

Table 1. Frequency of the Y chromosome variants in the studied races.

Localities	Autosomal complement	n	n of T-haplotype	Frequency of T-haplotype	SE
	Novosibirsk	25	5	0.20	0.08
Hybrid zone	Tomsk	14	4	0.29	0.12
	Total	39	9	0.23	0.07
Akademgorodok	Novosibirsk	27	0	0	
Chemskoy Bor	Novosibirsk	5	0	0	
	Total	32	0	0	

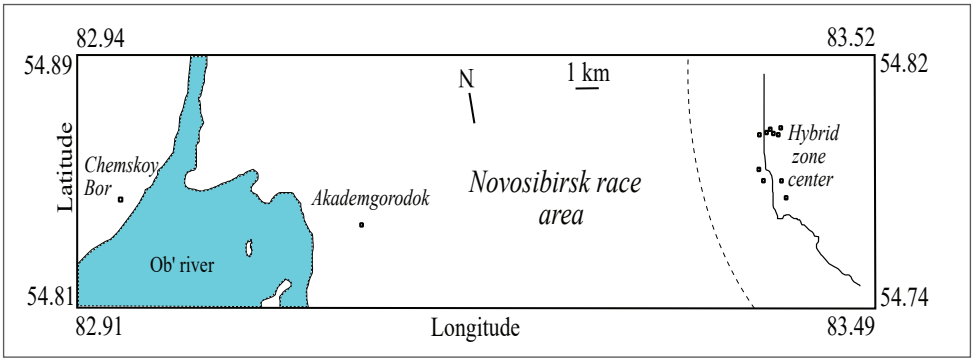


Figure 1. Location of sampling sites. Dotted line marks limits of introgression of the Tomsk autosomes complement; firm curved line determines the centre of the hybrid zone according to Polyakov et al. (2011).

Discussion

Akademgorodok and Chemskoy Bor are situated within the distribution range of the Novosibirsk race. Only the C-haplotype of the Y chromosome was found among shrews from both localities. Thus, we may suggest that the Novosibirsk race is monomorphic for this haplotype.

In the hybrid zone the frequency of C-haplotype is greater than the frequency of T-haplotype. This may indicate that both haplotypes of the Y chromosome are present in the Tomsk race. Alternatively, this could reflect a shift of the Y-chromosomal cline towards the Tomsk race area. The latter explanation is consistent with the results of previous morphological studies, where the clines in medial and lateral mandible sizes were centered at the Tomsk race side of the hybrid zone (Polly et al. 2013).

Backcrosses with the T-haplotype and autosomes of the Novosibirsk race are present in the hybrid zone. They would not be there, if the hybrid males were sterile. Nearly equal number of the T-haplotype in combination with both autosome complements implies a continuous flow of Y chromosome from the Tomsk to Novosibirsk race. This observation suggests that even if the hybrid males suffer from reduced fertility, it does not provide an insurmountable barrier to gene flow between the contacting populations.

Hybridization between divergent populations begins with the production of F1 and subsequent backcrossing. Repeated generations of backcross individuals result in introgression of mutations, collected by populations in allopatry (Maheshwari and Barbash 2011). Introgression can be prevented if hybrid incompatibilities reduce the fitness of the F1 or/and backcross generations (Turelli and Orr 2000).

Poor reproductive performance of hybrid shrews with chromosomal multivalents can be related not only to aberrations in generative tissues and gametes. The other cause can be the failure in competition for mating or low viability of their offspring. Our results indicate that none of this happens and the F1 hybrids are adequately involved in reproduction. The balanced ratio of Y variants among the pure race individuals and backcrosses in the Novosibirsk/Tomsk hybrid zone suggests that the F1 produce viable

progeny. It does not explain the distinct differentiation of shrews in morphological traits. However, if this differentiation is facilitated by a barrier to gene flow, and if this barrier is determined by hybrid incompatibilities, the results of the present study make the list of possible incompatibilities shorter. Indeed, after the rehabilitation of the F1, low fertility of backcrosses remains the only thing that can be suspected to influence gene flow. Certainly, this assumption requires careful consideration. Below we discuss some issues related to the possible impact of low fertility of backcrosses on gene flow.

The inheritance of morphological traits is defined by many loci with additive effect (Kemper et al. 2012). In a study of the inheritance of body size, crosses between strains of laboratory mice with different size have been made. In these experiments the F1 and F2 means were halfway between the parents and the backcross means were halfway between the F1 and respective parents (Butler 1952, Chai 1956). Similar crosses occur among shrews within the hybrid zone. In evaluation of morphological traits of shrews with consideration of their karyotypes, all the homozygous individuals with the Novosibirsk race karyotype were significantly smaller than the Tomsk homozygous individuals (Polyakov et al. 2002). This difference enabled differentiation of two separate morphotype groups, and the Novosibirsk shrews never grouped with the Tomsk shrews and vice-versa (see Figure 1 in Polyakov et al. 2002). Morphological variability of the heterozygotes was much broader and overlapped the extent of variation found in both homozygotes. Figure 2 illustrates the segregation of morphology and karyotypes in the hybrid zone as it may be expected following the experiments of Butler (1952) and Chai (1956). In this figure the relationship between the karyotypes and morphological types at stages the F1 and the first-generation backcrosses is in a good agreement with the experimental data from the hybrid zone of shrews. Parents and homozygous first-generation backcrosses form two distinct morphological groups, while karyotypically similar F1 and heterozygous first-generation backcrosses show variation that overlaps both homozygous groups. The appearance of the second-generation backcrosses, that combine the homozygous karyotypes of one race with a morphotype of the other race, would bring discrepancy in this concordance. However, no discrepancy between the karyotypes and morphological types was observed in experimental studies of the Novosibirsk/Tomsk hybrid zone, and it can be assumed that the second-generation backcrosses do not appear in this case. The reason of the absence of the second-generation backcrosses is difficult to explain unequivocally. We can only assume that if the first-generation backcrosses had been involved in reproduction, unlimited introgression could have been expected: foreign alleles would have accumulated on both sides of the hybrid zone and phenotypic differences would have become blurred after several generations. However, although hybridization between the Novosibirsk and Tomsk races has been lasting for much longer than several generations, none of this has happened.

Even a strong barrier to gene flow, based on low fertility of backcrosses, is not incompatible with the lack of differentiation of the autosomal markers including microsatellites. The populations in contact may have clear differentiation for these markers outside of the hybrid zone. If the sampling is carried out in the zone of hybridization, backcrosses will be collected together with pure race specimens. Recombination in the F1 shuffles

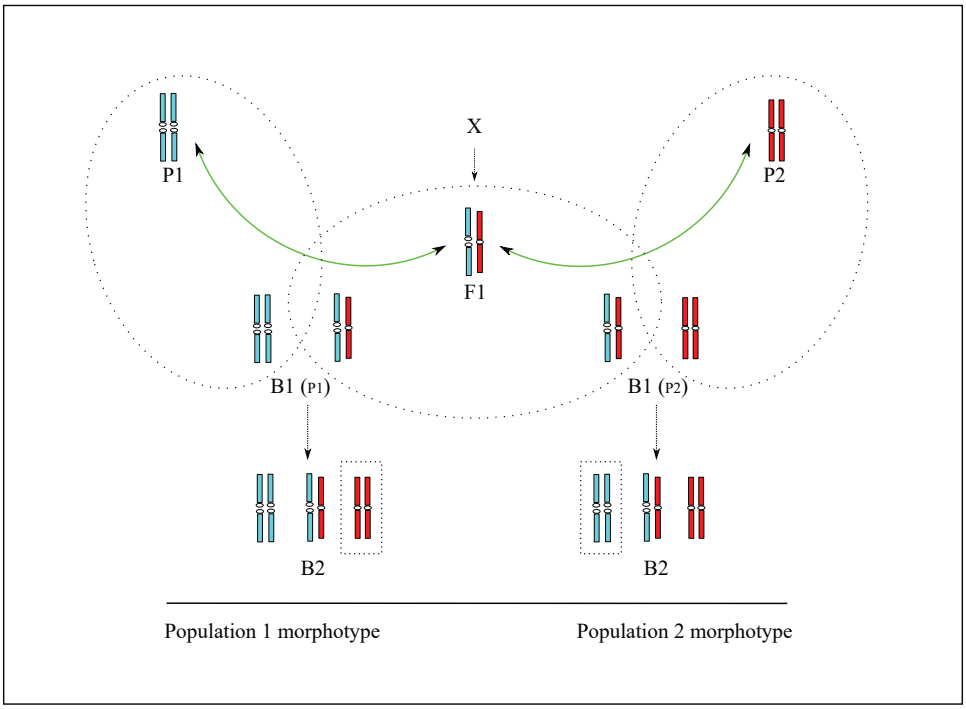


Figure 2. Segregation of karyotypes and morphological traits in the hybrid zone between two chromosome races. Positions of karyotypes reflect their morphological state: individuals of pure parental type (P1 and P2) with the most pronounced morphological differences occupy rightmost and leftmost positions, F1 – intermediate between P1 and P2 and the first-generation backcrosses – intermediate between F1 and respective parents ($B1_{P1}$ and $B1_{P2}$). The second-generation backcrosses (B2) contain karyotypes that do not correspond to the expected morphotypes (marked with squared frames). Round frames mark karyotypically indistinguishable parents, F1 and B1 (see text for details).

mutations between the race specific chromosome complements and backcrosses inherit alleles from both races. The karyotypes of homozygous backcrosses are indistinguishable from the karyotypes of the pure race individuals. Appearance of these backcrosses in the same group with the pure race individuals may significantly reduce the observed differentiation. Evaluation of samples collected within the zone of hybridization may thus explain the failure of previous studies to demonstrate a distinct differentiation.

Low reproductive ability of the generation following the F1 can become a promising hypothesis for further studies of barriers to gene flow between the chromosome races of the common shrew.

Conclusion

Aberrations in pairing, recombination and segregation of chromosomes in hybrids with complex meiotic configurations are a generally assumed barrier to gene flow among the

karyotypically divergent chromosome races of the common shrew (Searle 1993). The presented study suggests that gene incompatibilities in backcrosses may have more substantial influence on gene flow than erroneous behaviour of chromosomes at meiosis. Apparently, the Novosibirsk and Tomsk races have not yet reached the final stage of divergence, when hybridization does not go beyond the F1 production; however, the poor reproductive performance of the first-generation backcrosses may preserve the adaptive genetic architecture from assimilation and thus contribute to further divergence, promoting the progress of speciation.

Acknowledgments

We are grateful to Jan Zima and anonymous referee for the valuable comments made on the manuscript. This work was supported by the RF Basic Project No. 0324-2015-0004 and research grant from Russian Foundation for Basic Research № 13-04-00316.

References

Andersson A-C, Narain Y, Tegelström H, Fredga, K (2004) No apparent reduction of gene flow in a hybrid zone between the West and North European karyotypic groups of the common shrew, *Sorex araneus*. Molecular Ecology 13: 1205–1215. https://doi.org/10.1111/j.1365-294X.2004.02146.x

Andersson A-C, Alström-Rapaport C, Fredga K (2005) Lack of mitochondrial DNA divergence between chromosome races of the common shrew, *Sorex araneus*, in Sweden. Implications for interpreting chromosomal evolution and colonization history. Molecular Ecology 14: 2703–2716. https://doi.org/10.1111/j.1365-294X.2005.02584.x

Balloux F, Brünner H, Lugon-Moulin N, Hausser J, Goudet J (2000) Microsatellites can be misleading: an empirical and simulation study. Evolution 54(4): 1414–1422. https://doi.org/10.1111/j.0014-3820.2000.tb00573.x

Borodin P (2008) Chromosomes and Speciation. In: Dobretsov N, Kolchanov N, Rozanov A, Zavarzin G (Eds) Biosphere Origin and Evolution. Springer, US, 315–325. https://doi.org/10.1007/978-0-387-68656-1_23

Butler L (1952) A study of size inheritance in the house mouse. Canadian Journal of Zoology 30: 154–171. https://doi.org/10.1139/z52-014

Chai CK (1956) Analysis of quantitative inheritance of body size in mice. II**.** Gene action and segregation. Genetics 41: 165–178.

Chętnicki W, Fedyk S, Banaszek A, Szalaj K, Ratkiewicz M (1996) Morphometrical characteristic of the common shrew (*Sorex araneus* L.) from interracial hybrid zones. Hereditas 125: 201–207. https://doi.org/10.1111/j.1601-5223.1996.00201.x

Hausser J, Fedyk S, Fredga K, Searle JB, Volobouev VT, Wójcik JM, Zima J (1994) Definition and nomenclature of chromosome races of Sorex araneus. Folia Zoologica 43(1): 1–9.

Hellborg L, Ellegren H (2003) Y chromosome conserved anchored tagged sequences (YCATS) for the analysis of mammalian male specific DNA. Molecular Ecology 12: 283–291. https://doi.org/10.1046/j.1365-294X.2003.01702.x

Horn A, Basset P, Yannic G, Banaszek A, Borodin PM, Bulatova NS et al (2012) Chromosomal rearrangements do not seem to affect the gene flow in hybrid zones between karyotypic races of the common shrew (*Sorex araneus*). Evolution 66(3): 882–889. https://doi.org/10.1111/j.1558-5646.2011.01478.x

Jadwiszczak K, Ratkiewicz M, Banaszek A (2006) Analysis of molecular differentiation in a hybrid zone between chromosomally distinct races of the common shrew Sorex araneus (Insectivora: Soricidae) suggests their common ancestry. Biological Journal of the Linnean Society 89: 79–90. https://doi.org/10.1111/j.1095-8312.2006.00659.x

Kemper KE, Visscher PM, Goddard ME (2012) Genetic architecture of body size in mammals. Genome Biology 13(4): 244. https://doi.org/10.1186/gb-2012-13-4-244

King M (1993) Species evolution: the role of chromosome change. Cambridge University Press, Cambridge, 322 pp.

Král B, Radjabli SI (1974) Banding patterns and Robertsonian fusion in the Western Siberian population of *Sorex araneus* (Insectivora, Soricidae). Folia Zoologica 23: 217–227.

Lundqvist A-C, Alström-Rapaport C, Tegelström H (2011) Fennoscandian phylogeography of the common shrew *Sorex araneus*. Postglacial recolonization-combining information from chromosomal variation with mitochondrial DNA data. Acta Theriologica 56: 103–116. https://doi.org/10.1007/s13364-010-0022-9

Maheshwari S, Barbash DA (2011) The genetics of hybrid incompatibilities. Annual Review of Genetics 45: 331–355. https://doi.org/10.1146/annurev-genet-110410-132514

Moska M, Wierzbicki H, Macierzyńska A, Strzała T, Maślak R, Warchałowski M (2011) A microsatellite study in the Łegucki Młyn/Popielno hybrid zone reveals no genetic differentiation between two chromosome races of the common shrew (*Sorex araneus*). Acta Theriologica 56: 117–122. https://doi.org/10.1007/s13364-011-0029-x

Polly PD, Polyakov AV, Ilyashenko VB, Onischenko SS, White TA, Shchipanov NA et al (2013) Phenotypic variation across chromosomal hybrid zones of the common shrew (*Sorex araneus*) indicates reduced gene flow. PLOS ONE 8(7): e67455. https://doi.org/10.1371/journal.pone.0067455

Polyakov AV, Volobouev VT, Borodin PM, Searle JB (1996) Karyotypic races of the common shrew (Sorex araneus) with exceptionally large ranges: the Novosibirsk and Tomsk races of Siberia. Hereditas 125: 109–115. https://doi.org/10.1111/j.1601-5223.1996.00109.x

Polyakov AV, Chadova NB, Rodionova MI, Panov VV, Dobrotvorsky AK, Searle JB et al. (1997) Novosibirsk revisited 24 years on: chromosome polymorphism in the Novosibirsk population of the common shrew *Sorex araneus* L. Heredity 79: 172–177. https://doi.org/10.1038/hdy.1997.140

Polyakov AV, Zima J, Banaszek A, Searle JB, Borodin PM (2000) New chromosome races of the common shrew *Sorex araneus* from Eastern Siberia. Acta Theriologica 45(1): 11–18. https://doi.org/10.4098/AT.arch.00-57

Polyakov AV, Onischenko SS, Ilyashenko VB, Searle JB, Borodin PM (2002) Morphometric difference between the Novosibirsk and Tomsk chromosome races of *Sorex araneus* in a zone of parapatry. Acta Theriologica 47: 381–387. https://doi.org/10.1007/BF03192464

Polyakov AV, Volobouev VT, Aniskin VM, Zima J, Searle JB, Borodin PM (2003) Altitudinal partitioning of two chromosome races of the common shrew (*Sorex araneus*) in West Siberia. Mammalia 67: 201–207. araneus) in West Siberia. Mammalia 67: 201–207. https://doi.org/10.1515/mamm.2003.67.2.201

Polyakov AV, Bukina MS, Borodin PM (2004) Karyotype evolution and formation of mammals diversity. Contemporary problems of ecology 5: 629–634. [In Russian]

Polyakov AV, Ilyashenko VB, Onischenko SS, Panov VV, Borodin PM (2009) AFLP diversity between the Novosibirsk and Tomsk chromosome races of the common shrew (*Sorex araneus*). Comparative Cytogenetics 3: 85–89. https://doi.org/10.3897/compcytogen.v3i2.14

Polyakov AV, White TA, Jones RM, Borodin PM, Searle JB (2011) Natural hybridization between extremely divergent chromosomal races of the common shrew (*Sorex araneus*, Soricidae, Soricomorpha): hybrid zone in Siberia. Journal of Evolutionary Biology 24(7): 1393–1402. https://doi.org/10.1111/j.1420-9101.2011.02266.x

Searle JB, Fedyk S, Fredga K, Hausser J, Volobouev V (1991) Nomenclature for the chromosomes of the common shrew (Sorex araneus). Mémoires de la Société Vaudoise des Sciences Naturelles 19: 13–22.

Searle JB (1993) Chromosomal hybrid zones in eutherian mammals. In: Harrison RG (Ed.) Hybrid Zones and Evolutionary Process. Oxford University Press, New York, 309–353.

Turelli M, Orr HA (2000) Dominance, epistasis and the genetics of postzygotic isolation. Genetics 154: 1663–1679.

Wójcik J, Ratkiewicz M, Searle JB (2002) Evolution of the common shrew *Sorex araneus*: chromosomal and molecular aspects. In: Gliwicz J (Ed.) Theriology at the turn of a new century, Acta Theriologica 47(1): 139–167.

Wójcik J, Borodin PM, Fedyk S, Fredga K, Hausser J, Mishta A et al. (2003) The list of the chromosome races of the common shrew *Sorex araneus* (updated 2002). Mammalia 67: 169–178. https://doi.org/10.1515/mamm.2003.67.2.169

Yudin BS (1989) [Insectivorous mammals of Siberia]. Nauka, Novosibirsk, 360 pp. [In Russian]

CompCytogen 11(2): 431–433 (2017)
doi: 10.3897/CompCytogen.v11i2.13527
http://compcytogen.pensoft.net

BOOK REVIEW

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Book Review: Kiknadze I., Istomina A., Golygina V., Gunderina L. Karyotypes of Palearctic and Holarctic species of the genus *Chironomus* [Electronic resource] Russian Academy of Sciences, Siberian Branch, Federal Research Center, Institute of Cytology and Genetics. Novosibirsk: Academic Publishing House "GEO", 2016. – 489 p. ISBN 978-5-9908853-2-5.

Valentina Kuznetsova[1]

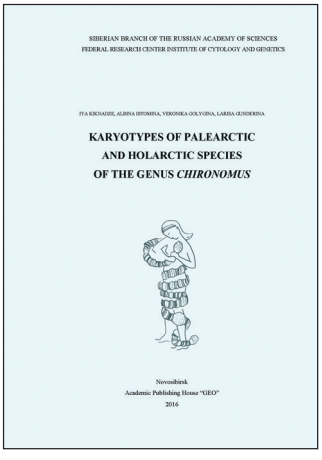
1 *Department of Karyosystematics, Zoological Institute, Russian Academy of Sciences, Universitetskaya nab. 1, 199034, St. Petersburg, Russia*

Corresponding author: *Valentina Kuznetsova* (valentina_kuznetsova@yahoo.com)

Received 3 May 2017 | Accepted 4 May 2017 | Published 19 June 2017

http://zoobank.org/6136E538-58D1-4BFF-8BA7-571045ACA0E2

Citation: Kuznetsova V (2017) Book Review: Kiknadze I., Istomina A., Golygina V., Gunderina L. Karyotypes of Palearctic and Holarctic species of the genus *Chironomus* [Electronic resource] Russian Academy of Sciences, Siberian Branch, Federal Research Center, Institute of Cytology and Genetics. Novosibirsk: Academic Publishing House "GEO", 2016. – 489 p. ISBN 978-5-9908853-2-5. Comparative Cytogenetics 11(2): 431–433. https://doi.org/10.3897/CompCytogen.v11i2.13527



The book "Karyotypes of Palearctic and Holarctic species of the genus *Chironomus*" was published last year in the Russian Federation by Ija Kiknadze, Albina Istomina, Veronika Golygina and Larissa Gunderina (Fig. 1).

Professor I. Kiknadze and her co-authors are noted scientists, who have had a long academic experience in comparative cytogenetics and karyosystematics of the family Chironomidae (Insecta, Diptera, Nematocera), primarily of the species-rich and taxonomically complicated genus *Chironomus* Meigen, 1803, and have made a significant contribution to our understanding of the cytogenetic mechanisms of microevolution in natural systems.

The genus *Chironomus* includes several hundred species distributed almost worldwide (except Antarctica). *Chironomus* larvae are of great concern in aquatic ecosystems and are widely used in ecological and environmental studies of fresh waters. The species identification of *Chironomus* larvae is hence of considerable importance. It is, however, difficult due to the small number of distinct morphological differences between the larvae of many species, especially between those belonging to the sibling species groups. It is generally agreed that the definitive identification of the larvae can be performed using a combination of different lines of evidence including the analysis of traditional morphological characters, molecular markers and structure of salivary-gland polytene chromosomes.

The book is a tribute to the enormous amount of cytogenetic research on the genus *Chironomus* that has been done by the authors during the decades. The book begins with the table of contents, list of abbreviations and acronyms used to denote the transcriptionally active regions of chromosomes, geographic ranges of chromosome banding sequences, banding frequency occurrence and some other terms, and the list of the studied *Chironomus* sibling species groups (the *aberratus*, the *obtusidens*, the *piger*, the *plumosus* and the *riihimakiensis* groups).

The main body of the book is separated in the two chapters. Chapter I provides the reader with exhaustive and up-to-date information on karyotype structure, polytene chromosome banding sequences and inversion polymorphism in *Chironomus*. Numerous visual displays such as drawings, diagrams, photos, tables, and schemes help the understanding of these complex data.

Michael White, in his notable book "Modes of speciation" (1978) states that "over 90 percent of all speciation events are accompanied by karyotypic changes and in the majority of these cases the structural chromosomal rearrangements have played a primary role in initiating divergence" (p. 324). The book of Kiknadze with co-authors demonstrates well that *Chironomus* species, given the ease of analyzing their giant polytene chromosomes, are the unique subjects for studying the microevolution processes, i.e. evolution on a small-scale. The reader is introduced to the authors' findings making it apparent that most cases of chromosomal polymorphism in *Chironomus* are represented by homo- and heterozygous paracentric inversions. Besides, rare heterozygotes for pericentric inversions, reciprocal translocations and size variation of homologous bands including the centromeric bands (band thickness) are known. Polymorphism for B chromosomes is shown to occur as well.

Chapter II covers the original authors' data and addresses the karyotypes (more precisely, the chromosomal polymorphisms) of sixty three *Chironomus* species referred to the five groups of sibling species and originated from different regions including Russia (European part, Ural, West and East Siberia, Altai, Tuva, the Far East), Kazakhstan, West Europe (Germany, Belgium, the Netherlands, Bulgaria), USA, Canada, China, and Japan. The chapter is particularly noteworthy because of the detailed and fully illustrated presentation of chromosomal polymorphisms in each species and each population under study. What I liked most in this chapter were the wonderful pictures of polytene chromosomes. The polymorphisms are considered and discussed in the

context of evolutionary divergence of species and populations and are used as a basis for reconstruction of ancestral chromosome architecture and further chromosome evolution in the genus *Chironomus*. The banding sequence pattern in *Ch. piger* Strenzke, 1959 suggested by Keyl (1962) as a standard one is applied for chromosome mapping in the species under study.

The book is completed with an extensive reference list, which comprises over 250 items and makes this an indispensable work for every student in *Chironomus* cytogenetics and systematics.

I expect this book to be very well accepted by entomologists, cytogeneticists, evolutionary biologists and advanced students doing research on chironomid cytology, ecology and systematics. The ability to accurately identify *Chironomus* species at the larva stage should facilitate taxonomic, ecological and environmental studies of the group in different geographical regions. Proulx et al. (2013) are still of the opinion (and this is my opinion as well) that only a few of the taxonomists worldwide display the necessary expertise to identify *Chironomus* species using chromosome structure, which is a major drawback for non-cytological experts wishing to do it. In this context, my only criticism of the book by Kiknadze with co-authors from a student's perspective is the conspicuous absence of a glossary. Such a glossary would help the students in defining numerous and highly specific terms that appear throughout the text.

Despite this criticism, the book is excellent, and I would recommend it to everyone who is interested in the genus *Chironomus* cytogenetics and systematics.

The production of the book was carried out with the financial support of the budgetary project of the Russian Academy of Sciences (№ 0324-2015-0003).

References

Keyl H-G (1962) Chromosomenevolution bei *Chironomus*. II. Chromosomenumbauten und phylogenetische Beziehungen der Arten. Chromosoma. 13: 464–514. https://doi.org/10.1007/BF00327342

Proulx I, Martin J, Carew M, Hare L (2013) Using various lines of evidence to identify *Chironomus* species (Diptera: Chironomidae) in eastern Canadian lakes. Zootaxa 3741(4): 401–458. https://doi.org/10.11646/zootaxa.3741.4.1

White MJD (1978) Modes of speciation. W. H. Freeman Co., San Francisco, 454 pp.