CompCytogen 14(3): 313–318 (2020)
doi: 10.3897/CompCytogen.v14i3.54526
http://compcytogen.pensoft.net

SHORT COMMUNICATION

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

Notable homologous variation in chromosomal races of the common shrew

Nina Bulatova[1]

1 *A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow 119071, Russia*

Corresponding author: Nina Bulatova (ninbul@mail.ru)

Academic editor: V.G. Kuznetsova | Received 20 May 2020 | Accepted 10 June 2020 | Published 14 July 2020

http://zoobank.org/B8062F58-FD83-4244-9681-B9DF866072DB

Citation: Bulatova N (2020) Notable homologous variation in chromosomal races of the common shrew. Comparative Cytogenetics 14(3): 313–318. https://doi.org/10.3897/CompCytogen.v14i3.54526

Abstract

This paper is a review of the rare phenomenon of chromosome intraspecies variation manifested in monobrachial homology series in the comprehensively investigated karyotype of the common shrew *Sorex araneus* Linnaeus, 1758 (Eulipotyphla, Mammalia). The detailed dataset on the account of this mammalian species was drawn from the recently published monograph by Searle et al. (2019) "Shrews, Chromosomes and Speciation". The parallels to the law of homologous series in variation by Nikolai Vavilov are discussed.

Keywords

Chromosome rearrangements (CRs), mammal karyotype, N.I. Vavilov heritage, Robertsonian fusions, *Sorex araneus*

Genetics started in the XXth century with rediscovery of G. Mendel's hereditary laws, T.H. Morgan's chromosome theory of heredity and prior evolutionary generalizations of W. Bateson, the author of the term "genetics". Advances of the first two decades in the emerging field of plant genetics have been promptly consolidated into the law of homologous series in variation by Nikolai Vavilov, who was considered himself to be a student of William Bateson. A concise first presentation of the law idea (Vavilov 1920) in Russian was soon published in English in the Journal of Genetics edited by W. Bateson and R.C. Punnett (Vavilov 1922). Since and till now, homology problems remain in focus of different scientific disciplines exploring homologous variation,

from molecular genetics to paleontology and bioinformatics (i.e., Korochkin 1985; Rozhnov 2006; Suslov et al. 2018). For decades, cytogenetic analysis was developing towards the correct assessment of chromosome homology, and today the use of methods of differential staining and, in particular, of chromosome painting makes possible the interspecies comparison on generic and higher taxonomic levels (Ferguson-Smith and Trifonov 2007). This paper aims to review features of homologous chromosomes variation in a mammalian species with one of the best investigated karyotypes, the common shrew *Sorex araneus* Linnaeus, 1758 (Searle et al. 2019). Taking into account the upcoming date of the 100th anniversary of Vavilov's law, it could be a challenge to examine the variety of chromosomal races of *S. araneus* as the phenomenon of series of homologous variation.

The record of chromosomal variation within the common shrew was recently reviewed in essential details in the monograph "Shrews, Chromosomes and Speciation" which summarized more than the 30-year period of joined multidisciplinary studies of *S. araneus* chromosomal races in Eurasia initiated by the International *Sorex araneus* Cytogenetics Committee, ISACC (Searle et al. 2019). Chapter 5 of this book presents the list of chromosomal races discovered over the whole vast species range of *S. araneus*. As generally, geographic names of 76 chromosomal races were listed in an alphabetical order, accompanied with the diagnostic chromosomal formulas (Bulatova et al. 2019). G-band nomenclature was used for the chromosome identification (Searle et al. 1991) and chromosomal races were defined prioritizing the karyotypic and geographic separation adhering to the ISACC rules (Hausser et al. 1994).

The chromosomes of *S. araneus* are composed of 21 chromosomal arms that can be fused in a variety of combinations in different populations forming an astonishing array of chromosomal races. According to the nomenclature, 21 arms are designated by Latin letters (*a* to *s*) in correspondence with the arrangement in decreasing size from largest to smallest.

In the karyotype of *S. araneus*, chromosomes in pairs appear in either the bi-armed (metacentric) or one-armed (acrocentric) form. Among autosomes, three pairs are always bi-armed and demonstrate stable combination of chromosome arms (*af*, *bc* and *tu*). One other pair is always composed of arms *j* and *l*, but can display individual or population Robertsonian polymorphism appearing in acrocentric or/and metacentric forms (*j*, *l* / *jl*) (Ford and Hamerton 1970).

Sex chromosomes of *S. araneus* have complex origin because of the ancient event of the autosome to sex chromosome translocation: a fusion between the original "true" X (arm *e*) and an autosome (arm *d*). Thus, in females, XX pair is represented by bi-armed (*de*) chromosomes, and in males, by a system of triple sex chromosomes – X(*de*) Y1("true" Y)Y2(*d*).

Ten other chromosome arms (*g* to *r*, except *j*, *l*) are fused in a variety of combinations which show the remarkable intraspecies polytypic variation. Such arm reshuffling creates a variety of chromosomal races: 37 different combinations of chromosomal arms fused into metacentrics were detected in 76 described chromosomal races (see tables 5.2 and 5.3 in: Bulatova et al. 2019). So, here the set of chromosomes/arms in

Table 1. Serial presentation of chromosomal race specific metacentrics (monobrachial homologs) defined in *Sorex araneus*. Asterisks mark the fusions absent* in the list of chromosomal races, and potential** for the race/species karyotypes. A double letter designation is given in the alphabetical order following the standard nomenclature of chromosomes of *S. araneus* (Searle et al. 1991). *o*, *q* – NOR-bearing arms.

Arm	***g***	***h***	***i***	***k***	***m***	***n***	***o***	***p***	***q***	***r***	**
g		*	*gi*	*gk*	*gm*	*	*go*	*gp*	*gq*	*gr*	*gh***
h	*		*hi*	*hk*	*	*hn*	*ho*	*	*hq*	*	*gh***, *hm***
i	*gi*	*hi*		*ik*	*im*	*	*io*	*ip*	*iq*	*	
k	*gk*	*hk*	*ik*		*km*	*	*ko*	*kp*	*kq*	*kr*	
m	*gm*	*	*im*	*km*		*mn*	*mo*	*mp*	*mq*	*mr*	*hm***
n	*	*hn*	*	*	*mn*		*no*	*np*	*nq*	*nr*	
o	*go*	*ho*	*io*	*ko*	*mo*	*no*		*op*	*oq*	*or*	NOR
p	*gp*	*	*ip*	*kp*	*mp*	*np*	*op*		*pq*	*pr*	
q	*gq*	*hq*	*iq*	*kq*	*mq*	*nq*	*oq*	*pq*		*qr*	NOR
r	*gr*	*	*	*kr*	*mr*	*nr*	*or*	*pr*	*qr*		
Total of 9	7	5	7	8	8	6	9	8	9	7	

karyotypes of the *S. araneus* is represented in symbols of the standard nomenclature in Latin letters with variable chromosomes being marked with an asterisk:

***af*, *bc*, *de* (XX)/*d* (Y2), *g**, *h**, *i* *, *j*/*l*, *k**, *m**, *n**, *o**, *p**, *q**, *r**, *s* (Y1), *tu*.**

To analyse the peculiarities of the variable group of chromosome arms, the list of the synoptic table 5.3 from Bulatova et al. (2019) was restructured to follow each chromosome variation in Table 1 here. The acrocentric state and fusion variants were labelled with one and two letters, correspondingly, revealing thus all defined series.

In our analysis, each chromosome series begins with an acrocentric state (for instance, *g*) and accumulates varying fusion combinations with other elements of the variable group (in this case – *gi*, *gk*, *gm*, *go*, *gp*, *gq*, *gr*). That is, from nine possible combinations, two variants of the arm *g* fusions are absent from this series (*gh*, *gn*) – but probably could still be found in nature.

All nine possible fusion variants were realized in two cases, for the arms *o* and *q* (Table 1). Along with aforementioned *g* group (lacking *gh* and *gn*), incomplete series are shown for other arms, namely *h* (-*gh*, *hm*, *hp*, *hr*), *i* (-*in*, *ir*), *k* (-*kn*), *m* (-*hm*), *n* (-*gn*, *in*, *kn*), and *p* (-*hp*), and, correspondingly, for their fusion partners (arms *g*, *h*, *i*, *k*, *m*, *n*, *p*, *r*). It is worth noting that some fusions, for instance *gh* and *hm* absent in the *h* (as well as *g* and *m*) series, were found outside the current list of chromosomal races. These are *hm*, present in an F1 interracial hybrid karyotype due to proposed whole arm reciprocal translocation (WART) (Pavlova et al. 2008), and *gh*, identified in the karyotype of a sibling species, *S. satunini* Ognev, 1922 (Borisov and Orlov 2012). Besides, it seems remarkable that the chromosomes *o* and *q*, most "active" in fusions, are carriers of nucleolus organizing region (NOR), located distally at an acrocentric end (Searle et al. 1991).

Fusions predominate among evolutionary changes of karyotypes in the genus *Sorex* Linnaeus, 1758. Cascades of fusions have happened in the past karyotype evo-

lution of this genus according to refined cytogenetic studies (Biltueva et al. 2011) based on chromosome differential staining (homology of G-banding or R-banding patterns) combined with chromosome painting (chromosome homology revealed via fluorescent *in situ* hybridization). Based on the study of 12 congeneric taxa, the authors proposed an ancestral karyotype of Palearctic *Sorex*, AKPS, consisting of 28 chromosomes (2n = 56). Some of these ancestral elements are homologous to whole *S. araneus* chromosomes, while others to parts of the modern chromosomal arms, indicating that multiple evolutionary fusion events led to the formation of *S. araneus* karyotype. Nine autosomes were completely conserved (*h*, *i*, *m*, *n*, *o*, *p*, *q*, *r* and *tu*), while fusion combinations were defined in nine other chromosomes (*a*, *b* consisting of three parts each: *a*1, *a*2, *a*3 and *b*1, *b*2, *b*3; and those consisting of two parts each – *c*, *d*, *f*, *g*, *j*, *k*, *l*) (Table 2). Subsequent fusions between initially formed acrocentrics shaped two characteristic metacentrics of the *S. araneus* karyotype – *jl* and *bc* (Biltueva et al. 2011). The fusion *jl* is species specific apomorphy, while *bc* is a common fusion for the clade consisting of three sibling species, *S. araneus*, *S. satunini*, *S. antinorii* Bonaparte, 1840 (reviewed by Bulatova et al. 2019). The variants of combination of 10 acrocentrics into 1 to 5 permissible race specific metacentrics are multiplied in series of further fusion combinations of every monobrachial homolog (for instance, *gm* with *hi*, *hk*, *hn* – *hi* with *ko*, *kp*, *kq*, *kr* – *ko* with *nr*, *np*, *nq*; and so on). In such a way, at least 58 distinct karyotypic variants were formed which are attributed to 76 known chromosomal races (note that, sometimes, geographically distant races do not show karyotypic differences).

In this review, numerous series of fusion variations in race specific chromosomes of *Sorex araneus* were considered (Tables 1, 2). It is noteworthy that methodical progress ensures the estimation of chromosome homology within and out of the species limits equally. The data on fully complete and incomplete series are available and allow thinking on and filling in the corresponding gaps. That makes the work in this respect to some extent predictable. It is concluded that homologous series recognition could be in particular demonstrative in cases of interspecies and intraspecies karyotypic variation of the Robertsonian type where it can concur with, for instance, routinely proposed understanding of hybrid origin of metacentric homologs (Bakloushinskaya et al. 2019).

The law of homologous series in chromosome variation may be considered a new notion of comparative cytogenetic studies not only of commemorative (Bulatova 2019) but also of fundamental genetic interests.

Table 2. The chromosomal arms of the common shrew in alphabetical order (top line), without *s* (Y1). Homology to the elements of the *Sorex* ancestral karyotype, AKPS, is indicated in the rows below. Number of ancestral homologous elements that formed present-day arms through past fusions was identified by G-banding and chromosome painting by Biltueva et al. (2011).

a	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	**l**	**m**	**n**	**o**	**p**	**q**	**r**	**tu**
a1	b1	c1	d1	e	f1	g1	h	i	j1	k1	l1	m	n	o	p	q	r	tu
a2	b2	c2	d2		f2	g2			j2	k2	l2							
a3	b3																	

Acknowledgements

The author thanks colleagues and professionals for their kind linguistic and editorial input which has been very helpful in finishing this paper.

References

Bakloushinskaya I, Lyapunova EA, Saidov AS, Romanenko SA, O'Brien PCM, Serdyukova NA, Ferguson-Smith MA, Matveevsky S, Bogdanov AS (2019) Rapid chromosomal evolution in enigmatic mammal with XX in both sexes, the Alay mole vole *Ellobius alaicus* Vorontsov et al. 1969 (Mammalia, Rodentia). Comparative Cytogenetics 13(2): 147–177. https://doi.org/10.3897/CompCytogen.v13i2.34224

Biltueva L, Vorobieva N, Perelman P, Trifonov V, Volobouev V, Panov V, Ilyashenko V, Onischenko S, O'Brien P, Yang F, Ferguson-Smith M, Graphodatsky A (2011) Karyotype evolution of Eulipotyphla (Insectivora): The genome homology of seven *Sorex* species revealed by comparative chromosome painting and banding data. Cytogenetics & Genome Research 135: 51–64. https://doi.org/10.1159/000330577

Borisov YM, Orlov VN (2012) A comparison of the chromosome G-banding pattern in two *Sorex* species, *S. satunini* and *S. araneus* (Mammalia, Insectivora). Comparative Cytogenetics 6: 267–271. https://doi.org/10.3897/compcytogen.v6i3.3019

Bulatova N (2019) Revisiting history. The memory of Prof. Hermann J. Muller (1890–1967) in Moscow revived by Helen Muller. Comparative Cytogenetics 13(2): 193–195. https://doi.org/10.3897/CompCytogen.v13i2.37416

Bulatova NS, Biltueva LS, Pavlova SV, Zhdanova NS, Zima J (2019) Chromosomal differentiation in the common shrew and related species. In: Searle J, Polly P, Zima J (Eds) Shrews, Chromosomes and Speciation (Cambridge Studies in Morphology and Molecules: New Paradigms in Evolutionary Bio). Chapter 5. Cambridge University Press, Cambridge, 134–184. https://doi.org/10.1017/9780511895531

Ferguson-Smith MA, Trifonov V (2007) Mammalian Karyotype Evolution. Nature Reviews Genetics 8: 950–962. https://doi.org/10.1038/nrg2199

Ford CE, Hamerton JL (1970) Chromosome polymorphism in the common shrew, *Sorex araneus*. Symposia of the Zoological Society of London 26: 223–236.

Korochkin LI (1985) Parallelisms in molecular organization of genome and problems of evolution. In: Sozinov AA (Ed.) Molecular Mechanisms of Genetic Processes: Molecular Genetics, Evolution and Molecular-Genetic Grounds of Selection. Nauka, Moscow, 132–146.

Pavlova SV, Kolomiets OL, Bulatova NS, Searle JB (2008) Demonstration of a WART in a hybrid zone of the common shrew (*Sorex araneus* Linnaeus, 1758). Comparative Cytogenetics 2: 115–120.

Rozhnov SV (2006) The law of homologue series of N.I. Vavilov and archaic diversity in paleontological data. In: Rozhnov SV (Ed.) Biosphere Evolution and Biodiversity. Moscow, 134–146.

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. [Republished (2010). Comparative Cytogenetics 4: 87–96. https://doi.org/10.3897/compcytogen.v4i1.28

Searle J, Polly P, Zima J [Eds] (2019) Shrews, Chromosomes and Speciation (Cambridge Studies in Morphology and Molecules: New Paradigms in Evolutionary Bio). Cambridge University Press, Cambridge, 475 pp. https://doi.org/10.1017/9780511895531

Suslov V, Ponomarenko M, Rasskazov D (2018) Homologous series and parallel evolution problem. Proceedings of the XI International Symposium of Bioinformatics of genome regulation and structure/systems biology – BGRS/SB-2018. Novosibirsk, August 21–24, 2018. Novosibirsk, 46 pp. https://doi.org/10.18699/BioGenEvo-2018-39

Vavilov NI (1920) The law of homologous series in hereditary variation. 3-rd All-Russian conference of plant breeding in Saratov, June 4, 1920. Saratov, 16 pp. [In Russian]

Vavilov NI (1922) The law of homologous series in variation. Journal of Genetics 12(1): 47–89. https://doi.org/10.1007/BF02983073

CompCytogen 14(3): 319–328 (2020)
doi: 10.3897/CompCytogen.v14i3.53087
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 characterization, rDNA mapping and quantification of the nuclear DNA content in *Seriolella violacea* Guichenot, 1848 (Perciformes, Centrolophidae)

Cristian Araya-Jaime[1,2], Claudio Palma-Rojas[2], Elisabeth Von Brand[3], Alfonso Silva[4]

1 *Instituto de Investigación Multidisciplinar en Ciencia y Tecnología, Universidad de La Serena, Casilla 554, La Serena, Chile* **2** *Laboratorio de Genética y Citogenética Vegetal, Departamento de Biología, Universidad de La Serena. La Serena, Chile* **3** *Departamento de Biología Marina Facultad de Ciencias del Mar, Universidad Católica del Norte Sede Coquimbo, Casilla 117, Coquimbo, Chile* **4** *Laboratorio Cultivo de Peces, Facultad de Ciencias del Mar, Universidad Católica del Norte Sede Coquimbo, Casilla 117, Coquimbo, Chile*

Corresponding author: Cristian Araya-Jaime (cristian.arayaj3@userena.cl)

Academic editor: Kenji Saitoh | Received 9 April 2020 | Accepted 5 June 2020 | Published 14 July 2020

http://zoobank.org/DE161A00-857B-4DEF-B9BE-71FED35A90F0

Citation: Araya-Jaime C, Palma-Rojas C, Von Brand E, Silva A (2020) Cytogenetic characterization, rDNA mapping and quantification of the nuclear DNA content in *Seriolella violacea* Guichenot, 1848 (Perciformes, Centrolophidae). Comparative Cytogenetics 14(3): 319–328. https://doi.org/10.3897/CompCytogen.v14i3.53087

Abstract

Seriolella violacea Guichenot, 1848 is an important component of the fish fauna of the Chilean coast and is of great economic interest. Cytogenetic information for the family Centrolophidae is lacking and the genomic size of five of the twenty-eight species described for this family are is barely known. This study aimed to describe for the first time the karyotype structure via classical and molecular cytogenetics analysis with the goal of identifying the constitutive heterochromatin distribution, chromosome organization of rDNA sequences and quantification of nuclear DNA content. The karyotype of *S. violacea* is composed of 48 chromosomes, with the presence of conspicuous blocks of heterochromatin on chromosomal pairs one and two. FISH assay with a 5S rDNA probe, revealed the presence of fluorescent markings on the heterochromatic block of pair one. The 18S rDNA sites are located exclusively on pair two, characterizing this pair as the carrier of the NOR. Finally, the genomic size of *S. violacea* was estimated at 0.59 pg of DNA as C-value. This work represents the first effort to document the karyotype structure and physical organization of the rDNA sequences in the *Seriolella* genome, contributing with new information to improve our understanding of chromosomal evolution and genomic organization in marine perciforms.

Keywords
chromosomal statis, CMA_3 staining, genome size, Repetitive DNAs

Introduction

In recent years fish cytogenetics has accumulated data that establish evolutionary trends, phylogenetic relationships among different families, species and populations (Arai 2011). This information is of great importance for the management and conservation of natural stocks (Carvalho-Costa et al. 2008). Currently the karyotypes of only 2% of all global marine fish are known (Galetti et al. 2000; Vega et al. 2002; Arai 2011). These studies have been focusing on just a few families of reef and pelagic fish, such as Gerreidae (Calado et al. 2013), Scombridae (Soares et al. 2013), Gobiidae (Lima-Filho et al. 2012), Labridae (Molina et al. 2012; Paim et al. 2014), Haemulidae (Nirchio et al. 2007; Neto et al. 2011) Carangidae (Chai et al. 2009) and Rachycentridae (Jacobina et al. 2011) preferably distributed in the Atlantic Ocean. According to Jara-Seguel et al. (2011) the marine fish fauna of Chile has been little studied, with known cytogenetic data for only some species of the Atherinidae, Galaxiidae, Kyphosidae, Mugilidae, Ophidae and Paralichthydae families being available.

Seriolella violacea (Guichenot, 1848) is an important component of the fish fauna of the Chilean coast and has great economic value (Ojeda et al. 2000). This species has an epipelagic gregarious behavior, forming schools near the coast; adults are found in areas of the continental shelf, as well as within protected bays, along the entire northern coast of Chile. Due to their rapid growth, adaptability and potential market, they currently represent an important candidate for the start of cultivation programs (Angel and Ojeda 2001; Navarrete et al. 2014).

No cytogenetic information is available for the family Centrolophidae, and the chromosomal constitution of the 28 species described in this family is unknown (Arai 2011). In addition the genomic size of five species (Hardie and Hebert 2004) is barely known. Due to this lack of biological information and the high potential for aquaculture that these species represent, it is essential to carry out a cytogenetic characterization; the karyotype and genome size are two primary genetic characteristics of the species, which are of great importance, when studying taxonomy, phylogenetic relationships, evolution and molecular biology.

Considering the absence of cytogenetic information on the Centrolophidae and the biological and economic importance of these pelagic fish, this study aims to describe for the first time the karyotype structure using classical and molecular cytogenetics analysis and quantification of nuclear DNA content in *Seriolella violacea*.

Material and methods

Six individuals, four males and two females, of *S. violacea* were obtained from the Laboratorio Central de Cultivos Marinos belonging to the Universidad Católica del Norte,

Coquimbo-Chile. Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, following the protocol established by Foresti et al. (1993). Approximately 20 metaphase spreads from different individuals were analyzed to confirm the diploid number and karyotype structure of *S. violacea*. The C-banding was carried out according to Sumner (1972); and the use of GC-specific fluorochrome Chromomycin A_3(CMA_3) following Schweizer (1976). The chromosomes were classified according to Levan et al. (1964).

The 18S rDNA and the 5S rDNA probes were obtained by PCR (Polymerase Chain Reaction) from genomic DNA of *Seriolella violacea* using primers NS1F(5'-GTAGTCATATGCTTGTCTC-3'), and NS8R(5'-TCCGCAGGTTCACCTACGGA-3') (Cioffi et al. 2009) and 5SA (5'- TACGCCCGATCTCGTCCGATC-3') and 5SB (5'-GCTGGTATGGCCGTAGC-3') (Pendás et al. 1994), respectively, and subsequently labeled with biotin-16-dUTP and digoxigenin-11-dUTP.

FISH was performed under high stringency conditions using the method described by Pinkel et al. (1986). Slides were incubated with RNase (50 µg/ml) for 1 h at 37 °C. Then the chromosomal DNA was denatured in 70% formamide/2× SSC for 5 min at 70 °C. For each slide, 30 µl of hybridization solution was denatured for 10 min at 95 °C, dropped on the slides and hybridized overnight at 37 °C in a 2× SSC moist chamber. Probe detection was carried out with Avidin-FITC (Sigma) or anti-digoxigenin-rhodamine (Roche). Chromosomes were counterstained with DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories).

Measurements of nuclear DNA content (C-value) were done by microdensitometry in erythrocytes obtained from adult specimens (2♀ and 2♂), analyzing 200 nuclei per sample, using the software Image Pro-Plus 4.0. (Media Cybernetics). The blood was dispersed on slides, air dried, fixed in methanol-acetic acid (3:1 v/v) at 4 °C for 24 h and stained with the Feulgen reaction (Jara-Seguel et al, 2008). Nuclear optic density (OD) is calculated by the software according to the formula OD = log10(1/T) = – log10T; where T = intensity of transmitted light/intensity of incident light. From this estimation, the computer integrates the values of OD obtained for each one of the pixels and it calculates the integrated optical density (IOD = ΣOD). The IOD values, in arbitrary units, were converted to absolute mass of DNA by comparison with erythrocyte smears of rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792), 2C = 5.5 pg, 2n = 58–60) (Hartley and Horne 1985).

Results

The karyotype of *S. violacea* shows 24 pairs of chromosomes (2n = 48; FN = 48), all acrocentric (Fig. 1A). No morphologically differentiated sex chromosomes were found when metaphase plates from males and females were compared. C-positive blocks of constitutive heterochromatin (HC) were observed in pericentromeric regions of few chromosomes, highlighting the presence of two conspicuous HC blocks, one of them in the pericentromeric region of pair one, while the other was in the telomeric region of pair two (Fig. 1B). In addition, these two conspicuous blocks were positive for chromomycin A_3 staining (Fig. 2A).

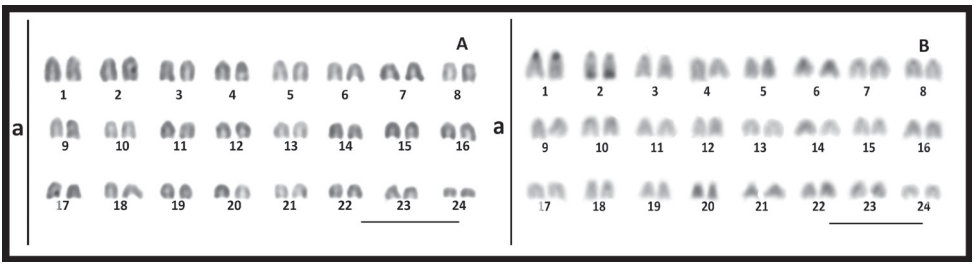


Figure 1. Conventional Giemsa-stained (**A**) and C-banding (**B**) in *Seriolella violacea*. Scale bar: 10 μm.

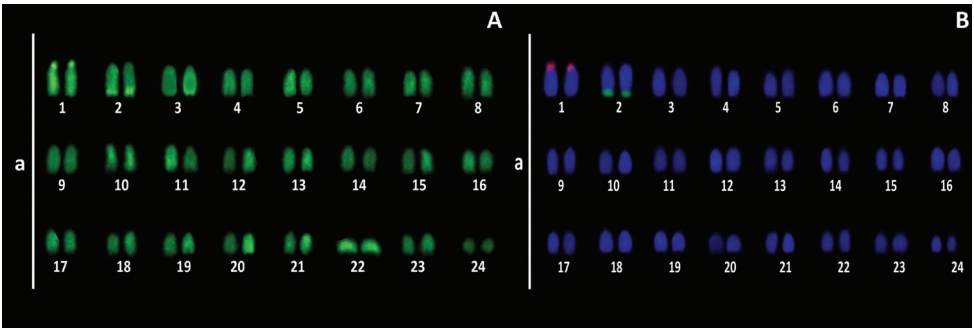


Figure 2. Karyotypes of *Seriolella violacea* after CMA_3 staining (**A**) and dual color FISH with 18S rDNA (green) and 5S rDNA (red) probes (**B**). Scale bar: 10 μm.

Table 1. Known genomic sizes C-Value(pg) for representatives of the Centrolophidae family.

Species	C-Value	Method	Cell Type	St. Species	Reference
Centrolophus niger	0.70	FIA	RBC	BS, GD, OM, RP	Hardie and Hebert 2004
Hyperoglyphe antarctica	0.77	FIA	RBC	BS, GD, OM, RP	Hardie and Hebert 2004
Schedophilus huttoni	0.76	FIA	RBC	BS, GD, OM, RP	Hardie and Hebert 2004
Seriolella punctata	0.78	FIA	RBC	BS, GD, OM, RP	Hardie and Hebert 2004
Tubbia tasmanica	0.76	FIA	RBC	BS, GD, OM, RP	Hardie and Hebert 2004
Seriolella violacea	0.59	FIA	RBC	GD, OM	in this work

FIA: Feulgen Imagen Analysis, **RBC**: Red Bloods Cells, **BS**: *Betta splendens*, **GD**: *Gallus domesticus,* **OM**: *Oncorhynchus mykiss,* **RP**: *Rana pipens.*

Dual FISH detected 18S and 5S rDNA probes on different chromosome pairs (Fig. 2B). Mapping the 5S rDNA probe revealed the presence of fluorescent markings on the heterochromatic block of pair one. The 18S rDNA sites are located exclusively on pair two, in a position coincident to heterocromatics/CMA_3 positive blocks, characterizing pair two as the pair carrying the NOR.

Finally, the nuclear DNA content measured in erythrocytes of *S. violacea* was estimated to be 1.18 ± 0.04 pg (average IOD = 14345 arbitrary units), with a coefficient of variation of 4.2%. Since *S. violacea* is a diploid organism (2n = 48, n = 24), the C-value of 0.59 pg of DNA (Table 1), is equivalent to 578.2 megabase pairs (Mbp).

Discussion

There are no data related to the organization of the repetitive fraction of the genome in the family Centrolophidae. Nevertheless, studies within the marine perciform order, specifically in representatives of the families Ephippidae, Serranidae, Lutjanidae, and Haemulidae have permitted the recognition of a diploid number of 48 chromosomes (completely acrocentric); the non-syntenic state of sequences 5S rDNA and 18S rDNA; and the presence of a single NOR, establishing this pattern as a plesiomorphic characteristic for marine perciforms (Chai et al. 2009; Arai 2011; Neto et al. 2011; Costa et al. 2016; Paim et al. 2017). The repetitive fraction of the genome can be a useful tool for identifying recent genomic changes that have occurred during the evolutionary process, as well as act as potential hotspots for chromosomal rearrangements (Ozouf-Costaz et al. 2004; Valente et al. 2011; Yano et al. 2014). In this sense, *S. violacea* presents exactly the cytogenetic pattern described for marine perciforms, highlighting the association of ribosomal clusters with heterochromatin blocks rich in CG bases in specific chromosome pairs. An association between 18S and 28S rDNA sequences and heterochromatin has been found in other fish, such as salmonids (Pendás et al. 1994; Fujiwara et al. 1998), species of the genera *Epinephelus* Bloch, 1793 (Sola et al. 2000), *Imparfinis* Eigenmann & Norris, 1900 and *Pimelodella* Eigenmann & Eigenmann, 1888 (Gouveia et al. 2013), *Orestias* Valenciennes, 1839 (Araya-Jaime et al. 2017) and sturgeon species (Fontana et al. 2003). This suggests that the repeated HC sequences play an important role and exercise diverse functions in the eukaryotic genome (Grewal and Jia 2007). It has even been postulated that heterochromatin is involved in maintaining the structure of the nucleolus and the integrity of ribosomal DNA repeats (McStay and Grummt 2008). Visualization of a single carrier pair sequence for 18S rDNA is one of the most common features observed in the fish genome, unlike what was observed for the gene 5S ribosomal which may present variations in the chromosomal distribution, apparently through its association with transposable elements, suggesting independent evolutionary pathways for both types of rDNA (Pendás et al. 1994; Martins and Galetti 2001; Cabral-de-Mello et al. 2011; Scacchetti et al. 2012; Sene et al. 2014; Santos et al. 2017; Usso et al. 2019). Teleosts exhibit low levels of compartmentalization in their genomes, which would suggest that the configuration in *S. violacea*, observed for the two types of ribosomal DNA, would represent a relatively simple to organization state (Medrano et al. 1988).

Finally, 0.59 pg of DNA (C-value) measured in erythrocytes of *S. violacea* represents a significantly (20%) lower nuclear DNA content than that of the five species of the Centrolophipadae family analized (Table 1), which on average reach 0.75 pg DNA. Thus, this value represents the smallest genome size known to the family. Currently there are data of nuclear DNA content for 634 species of Perciformes, estimating an average of 0.94 pg of DNA (C-value) for this order of fish, with minimum values of 0.39 pg in *Scienops ocellatus* (Linnaeus, 1766) and maximum of 2.60 in *Lagodon rhomboides* (Linnaeus, 1766) (Hardie and Hebert 2004; Gregory 2020). The evolutionary role genome size plays is the subject of much discussion, but computational biology

has helped to model some patterns. These patterns are clearer when the nuclear DNA content is related to species life history attributes, especially with regards to effective population sizes and their gene flow rates, showing an inverse relationship between population size and the size of the genome (Vinogradov 2004; Labar and Adami 2017; Bobay and Ochman 2018).

Conclusion

In this work, the karyotype of a representative of the Centrolophidae family, *S. violacea*, is described for the first time. Its karyotype is made up of 48 acrocentric chromosomes (2n = 48; FN = 48), simple NOR and ribosomal cistrons (5S-18S rDNA) are not synthetic. Meanwhile, the nuclear DNA content, C-value, was found to be 0.59 pg. It is necessary to perform additional studies physically mapping repetitive DNAs in the other representatives of the genus *Seriolella* Guichenot, 1848, in order to understand the involvement of these sequences in the process of chromosomal evolution that these fish may be experiencing. It is especially necessary to analyze the chromosomal microstructure, given the chromosomal stasis that most marine perciforms present, as this will also expand knowledge of fish fauna which is facing serious conservation issues.

Acknowledgment

This study was funded through a research project from DGIP/ Universidad Católica del Norte 2010. **CAJ:** conceived the study, conducted the experiments, analysed the data and wrote the manuscript. **CPR:** conducted the experiments, analysed the data and manuscript revision. **EVB:** conducted the experiments, analysed the data and manuscript revision. **AS:** analysed the data and manuscript revision. All authors have read and approved the final version.

References

Angel A, Ojeda FP (2001) Structure and trophic organization of subtidal fish assemblages on the northern Chilean coast: The effect of habitat complexity. Marine Ecology Progress Series 217: 81–91. https://doi.org/10.3354/meps217081

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

Araya-Jaime C, Lam N, Pinto IV, Méndez MA, Iturra P (2017) Chromosomal organization of four classes of repetitive DNA sequences in killifish *Orestias ascotanensis* Parenti, 1984 (Cyprinodontiformes, Cyprinodontidae). Comparative Cytogenetics 11: 463–475. https://doi.org/10.3897/compcytogen.v11i3.11729

Bobay LM, Ochman H (2018) Factors driving effective population size and pan-genome evolution in bacteria. BMC Evolutionary Biology 18: 1–153. https://doi.org/10.1186/s12862-018-1272-4

Cabral-de-Mello DC, Cabrero J, López-León MD, Camacho JPM (2011) 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

Calado LL, Bertollo LAC, Costa GWWF, Molina WF (2013) Cytogenetic studies of Atlantic mojarras (Perciformes – Gerreidae): chromosomal mapping of 5S and 18S ribosomal genes using double FISH. Aquaculture Research 44: 829–835. https://doi.org/10.1111/j.1365-2109.2012.03089.x

Carvalho-Costa L, Hatanaka T, Galetti Jr M (2008) Evidence of lack of population substructuring in the Brazilian freshwater fish *Prochilodus costatus*. Genetics and Molecular Biology 31(1): 377–380. https://doi.org/10.1590/S1415-47572008000200036

Chai X, Li X, Lu R, Clarke S (2009) Karyotype analysis of the yellowtail kingfish *Seriola lalandi lalandi* (Perciformes: Carangidae) from South Australia. Aquaculture Research 40: 1735–1741. https://doi.org/10.1111/j.1365-2109.2009.02278.x

Cioffi MB, Martins C, Bertollo LAC (2009) Comparative chromosome mapping of repetitive sequences. Implications for genomic evolution in the fish, *Hoplias malabaricus*. BMC Genetics 10: 1–34. https://doi.org/10.1186/1471-2156-10-34

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: 173–180. https://doi.org/10.1093/jhered/esv136

Fontana F, Lanfredi M, Congiu L, Leis M, Chicca M, Rossi R (2003) Chromosomal mapping of 18S-28S and 5S rRNA genes by two-colour fluorescent in situ hybridization in six sturgeon species. Genome 46: 473–477. https://doi.org/10.1139/g03-007

Foresti F, Oliveira C, de Almeida-Toledo L (1993) A method for chromosome preparations from large fish specimens using in vitro short-term treatment with colchicine. Experientia 49: 810–813. https://doi.org/10.1007/BF01923555

Fujiwara A, Abe S, Yamaha E, Yamazaki F, Yoshida MC (1998) Chromosomal localization and heterochromatin association of ribosomal RNA gene loci and silver-stained nucleolar organizer regions in salmonid fishes. Chromosome Research 6: 463–471. https://doi.org/10.1023/A:1009200428369

Galetti Jr PM, Aguilar CT, Molina WF, Galetti PM, Aguilar CT, Molina WF (2000) An overview of marine fish cytogenetics. Hydrobiologia 420: 55–62. https://doi.org/10.1007/978-94-017-2184-4_6

Gouveia JG, Moraes VPO, Sampaio TR, da Rosa R, Dias AL (2013) Considerations on karyotype evolution in the genera *Imparfinis* Eigenmann and Norris 1900 and *Pimelodella* Eigenmann and Eigenmann 1888 (Siluriformes: Heptapteridae). Reviews in Fish Biology and Fisheries 23: 215–227. https://doi.org/10.1007/s11160-012-9286-2

Gregory TR (2020) Animal Genome Size Database. http://www.genomesize.com [accessed 20, April 2020]

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

Hardie DC, Hebert PD (2004) Genome-size evolution in fishes. Canadian Journal of Fisheries and Aquatic Sciences 61: 1636–1646. https://doi.org/10.1139/f04-106

Hartley SE, Horne MT (1985) Cytogenetic techniques in fish genetics. Journal of Fish Biology 26: 575–582. https://doi.org/10.1111/j.1095-8649.1985.tb04298.x

Jacobina UP, Cioffi MB, Souza LGR, Calado LL, Tavares M, Manzella J, Bertollo LAC, Molina WF (2011) Chromosome mapping of repetitive sequences in *Rachycentron canadum* (Perciformes: Rachycentridae): implications for karyotypic evolution and perspectives for biotechnological uses. Journal of Biomedicine & Biotechnology 2011: 218231. https://doi.org/10.1155/2011/218231

Jara-Seguel P, Lara G, Garcia MP, Valdebenito I (2011) Cytogenetics of Chilean fishes: a commented database. Biocyt 4: 316–326. https://doi.org/10.22201/fesi.20072082.2011.4.75960

Jara-Seguel P, Valdebenito I, Palma-Rojas C, Rebolledo C (2008) Nuclear DNA content in *Galaxias maculatus* (Teleostei: Osmeriformes: Galaxiidae). Latin American Journal of Aquatic Research 36: 87–91. https://doi.org/10.3856/vol36-issue1-fulltext-7

Labar T, Adami C (2017) Genome size and the extinction of small populations. BioRxiv 173690. https://doi.org/10.1101/173690

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

Lima-Filho P, Cioffi M, Bertollo L, Molina WF (2012) Chromosomal and morphological divergences in Atlantic populations of the frillfin goby *Bathygobius soporator* (Gobiidae, Perciformes). Journal of Experimental Marine Biology and Ecology 434: 63–70. https://doi.org/10.1016/j.jembe.2012.08.004

Martins C, Galetti PM (2001) Two 5S rDNA arrays in neotropical fish species: is it a general rule for fishes?. Genetica 111(1–3): 439–446. https://doi.org/10.1023/A:1013799516717

Medrano L, Bernardi G, Couturier J, Dutrillaux B (1988) Chromosome banding and genome compartmentalization in fishes. Chromosoma 96: 178–183. https://doi.org/10.1007/BF00331050

McStay B, Grummt I (2008) The epigenetics of RNA genes: from molecular to chromosome biology. Annual Review of Cell and Developmental Biology 24: 131–157. https://doi.org/10.1146/annurev.cellbio.24.110707.175259

Molina WF, Neto CCM, 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. https://doi.org/10.1016/j.margen.2012.01.001

Navarrete AH, Lagos NA, Ojeda FP (2014) Latitudinal diversity patterns of Chilean coastal fishes: searching for causal processes. Revista Chilena de Historia Natural 87: 1–11. https://doi.org/10.1186/0717-6317-87-2

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

Nirchio M, Gaviria JI, Oliveira C, Ferreira IA, Martins C (2007) Cytogenetic analysis of three species of the genus *Haemulon* (Teleostei: Haemulinae) from Margarita Island, Venezuela. Genetica 131: 135–140. https://doi.org/10.1007/s10709-006-9123-4

Ojeda FP, Labra FA, Muñoz AA (2000) Biogeographic patterns of Chilean littoral fishes. Revista Chilena de Historia Natural. 73(4): 625–641. https://doi.org/10.4067/S0716-078X2000000400007

Ozouf-Costaz C, Brandt J, Körting C, Pisano E, Bonillo C, Coutanceau J-P, Volff JN (2004) Genome dynamics and chromosomal localization of the non-LTR retrotransposons Rex1 and Rex3 in Antarctic fish. Antarctic Science 16: 51–57. https://doi.org/10.1017/S0954102004001816

Paim FG, Aragão da Hora Almeida L, Antunes de Mello Affonso PR, Sobrinho-Scudeler PE, Oliveira C, Diniz D (2017) Chromosomal stasis in distinct families of marine Percomorpharia from South Atlantic. Comparative Cytogenetics 11: 299–307. https://doi.org/10.3897/CompCytogen.11(2).11942

Paim FG, Brandão JHSG, Sampaio I, de Mello Affonso PRA, Diniz D (2014) Genetic identification of bucktooth parrotfish *Sparisoma radians* (Valenciennes, 1840) (Labridae, Scarinae) by chromosomal and molecular markers. Genetics and Molecular Biology 37: 646–651. https://doi.org/10.1590/S1415-47572014005000024

Pendás AM, Moran P, Freije JP, Garcia-Vazquez E (1994) Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenetics and Cell Genetics 67: 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: 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Santos AR, Usso MC, Gouveia JG, Araya-Jaime C, Frantine-Silva W, Giuliano-Caetano L, Foresti F, Dias AL (2017) Chromosomal mapping of repetitive DNA sequences in the genus *Bryconamericus* (Characidae) and DNA barcoding to differentiate populations. Zebrafish 14: 261–271. https://doi.org/10.1089/zeb.2016.1380

Scacchetti PC, Alves JCPP, Utsunomia R, Claro FL, Toledo L, Oliveira C, Foresti F, De Almeida Toledo LF, Oliveira C, Foresti F (2012) Molecular characterization and physical mapping of two classes of 5S rDNA in the genomes of *Gymnotus sylvius* and *G. inaequilabiatus* (Gymnotiformes, Gymnotidae). Cytogenetics and Genome Research 136: 131–137. https://doi.org/10.1159/000335658

Sene VF, Pansonato-Alves JC, Utsunomia R, Oliveira C, Foresti F (2014) Karyotype diversity and patterns of chromosomal evolution in *Eigenmannia* (Teleostei, Gymnotiformes, Sternopygidae). Comparative Cytogenetics 8: 301–311. https://doi.org/10.3897/CompCytogen.v8i4.8396

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58: 307–324. https://doi.org/10.1007/BF00292840

Soares RX, Bertollo LAC, da Costa GWWF, Molina WF (2013) Karyotype stasis in four Atlantic Scombridae fishes: mapping of classic and dual-color FISH markers on chromosomes. Fisheries Science 79: 177–183. https://doi.org/10.1007/s12562-013-0602-0

Sola L, De Innocentiis S, Gornung E, Papalia S, Rossi AR, Marino G, De Marco P, Cataudella S (2000) Cytogenetic analysis of *Epinephelus marginatus* (Pisces: Serranidae), with the

chromosome localization of the 18S and 5S rRNA genes and of the (TTAGGG)(n) telomeric sequence. Marine Biology 137: 47–51. https://doi.org/10.1007/s002270000334

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

Usso MC, Santos AR, Gouveia JG, Frantine-Silva W, Araya-Jaime C, Oliveira MLM, Foresti F, Giuliano-Caetano L, Dias AL (2019) Genetic and chromosomal differentiation of *Rhamdia quelen* (Siluriformes, Heptapteridae) revealed by repetitive molecular markers and DNA barcoding. Zebrafish 16: 87–97. https://doi.org/10.1089/zeb.2018.1576

Valente GT, Mazzuchelli J, Ferreira IA, Poletto AB, Fantinatti BEA, Martins C (2011) Cytogenetic mapping of the retroelements *Rex1*, *Rex3* and *Rex6* among cichlid fish: new insights on the chromosomal distribution of transposable elements. Cytogenetics and Genome Research 133: 34–42. https://doi.org/10.1159/000322888

Vega L, Díaz E, Cross I, Rebordinos L (2002) Caracterizaciones citogenética e isoenzimática del lenguado *Solea senegalensis* Kaup, 1858. Boletín Instituto Español de Oceanografía 18: 245–250.

Vinogradov AE (2004) Genome size and extinction risk in vertebrates. Proceedings of the Royal Society of London. Series B: Biological Sciences 271: 1701–1705. https://doi.org/10.1098/rspb.2004.2776

Yano CF, Bertollo LAC, Molina WF, Liehr T, Cioffi MB (2014) Genomic organization of repetitive DNAs and its implications for male karyotype and the neo-y chromosome differentiation in *Erythrinus erythrinus* (Characiformes, Erythrinidae). Comparative Cytogenetics 8: 139–151. https://doi.org/10.3897/compcytogen.v8i2.7597

CompCytogen 14: 329–338 (2020)
doi: 10.3897/CompCytogen.v14i3.54511
http://compcytogen.pensoft.net

LETTERS TO THE EDITOR

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

Archives: The law of homologous series in variation (N. I. Vavilov)*

Nina Bulatova[1]

1 *A.N. Severtsov Institute of Ecology and Evolution, Moscow, Russia*

Corresponding author: Nina Bulatova (ninbul@mail.ru)

Academic editor: I. Gavrilov-Zimin | Received 20 May 2020 | Accepted 9 June 2020 | Published 14 July 2020

http://zoobank.org/62D36287-5EF9-48A0-9848-3CC6BF421D2B

Citation: Bulatova N (2020) Archives: The law of homologous series in variation (N. I. Vavilov). Comparative Cytogenetics 14(3): 329–338. https://doi.org/10.3897/CompCytogen.v14i3.54511

Keywords
genetics history, homologous variation, memory of Nikolai Vavilov, parallel evolution

Editorial Preface

A century has passed since the days when the law of homologous series in variation was first manifested. This event happened in 1920 in Saratov, in the third post-revolution year, in the frameworks of the III All-Russian Conference on Plant Breeding, then mobilized in view of current needs of agricultural practice, science and education. The report of a 33-year-old professor Nikolai Vavilov, who was accompanied by his students from the Saratov University, caused a sensation. Vavilov's generalization on the phenomenon of the homologous series in variation of cultivated plants was reported on June, 4, 1920 and enthusiastically appreciated by the qualified breeders as a great scientific achievement comparable with the Mendeleev's periodic Law of the chemical elements. On June 21, 1920, a message of the provincial Saratov branch of the Russian Telegraph Agency shared internationally the information on "the greatest discovery of world significance" which was addressed to the State government by the decision of the meeting. Very soon after the initial Russian publication (Vavilov 1920), the paper entitled "The Law of Homologous Series in Variation" was published in the Journal of Ge-

* Originally published in 1922, Journal of Genetics 12 (1): 47–89.

netics, edited by W. Bateson and R.C. Punnett, the elder statesmen of genetics (Vavilov 1922). In 1925, William Bateson, Director of the John Innes Horticultural Institute, with colleagues, visited experimental fields and laboratories of Nikolai Vavilov, Director of the Bureau of Applied Botany and Plant Breeding (future N.I. Vavilov Institute of Plant Breeding) in a Petrograd – Leningrad (now Saint Petersburg) suburb (Fig. 1). The paper took 42 pages of Volume XII (1) (April, 1922, p. 47–89). The substance of this work by Vavilov was recalled in the chapters of N. Timofeeff-Ressovsky (1940) and N. Vavilov (1940) in the monograph "The New Systematics" (Huxley 1940), a synoptic book, preceding the publication on the new synthesis of theory of evolution (Huxley 1942). Since then and till now, genetic nature of homologous variation, the matter of the Vavilov's law, has been in the focus of various disciplines, from agriculture to paleontology, being rejuvenated with the progress of molecular biology. Nowadays, molecular homology can be established universally at various levels, from unique genes to gene orders in chromosomes through genetic, cytogenetic and molecular analyses (Zakharov 1987) up to gene networks studied by bioinformatics (Suslov et al. 2008). It seems rational to meet the 100th anniversary of this significant event of young hereditary science with a digital copy saving the author's idea for future readers and investigators. The text is here reproduced in the Archives format from printed pages of the Introduction (p. 48–53) and the concluding section (p. 86–89) of the original English version (Vavilov 1922). The title page copied on Fig. 2 presents the whole contents of this work. Details of punctuation and citation are generally saved.

N. Bulatova

Introduction [p. 48–53]

Evolution of the study of systematics of plants

The characteristic feature of the history of plant investigation, from Tournefort up to the present, has been the varied conception of systematic units. Further investigation did away with the former conception of species, as introduced by Linné. The history of systematics of plants gives a vivid illustration of attempts to arrange in a convenient and harmonious system all newly discovered morphological and physiological characteristics, the number of which grows rapidly with improved methods of discerning hereditary forms, and with the study of new specimens of the same plants, gathered in different regions. The Linnean species had to be divided into subspecies and varieties (*in sensu bot.*); varieties into races. Genetical studies of the last decades have proved even the divisibility of the minutest morphological and physiological units in systematics (races, Elementararten of de Vries), and established that, although outwardly similar, they can be different genotypically. The same is applicable to the animal world.

Lotsy, in his book Evolution by Means of Hybridization (1916), proposes to introduce a new terminology to distinguish fundamental units in the classification of hereditary forms. He proposes to call the old Linnean species, which, as was shown in the nineteenth century, are of collective nature – "Linneons"; races, varieties, which make up the elementary species of Jordan and de Vries he proposes to define as "Jordanons".

Figure 1. N. I. Vavilov (left below) and Russian geneticists V.A. Dogel, Yu.A. Filipchenko with the visiting European delegation: H. Federley, O. Fogt and W. Bateson (left to right in the second row). 1925, Leningrad (Vavilov 2012).

The term "species", Lotsy would retain (as it seems to us not very successfully) for the modern conception of genetics – the genotype, as a fundamental unit covering similar hereditary groups of individuals.

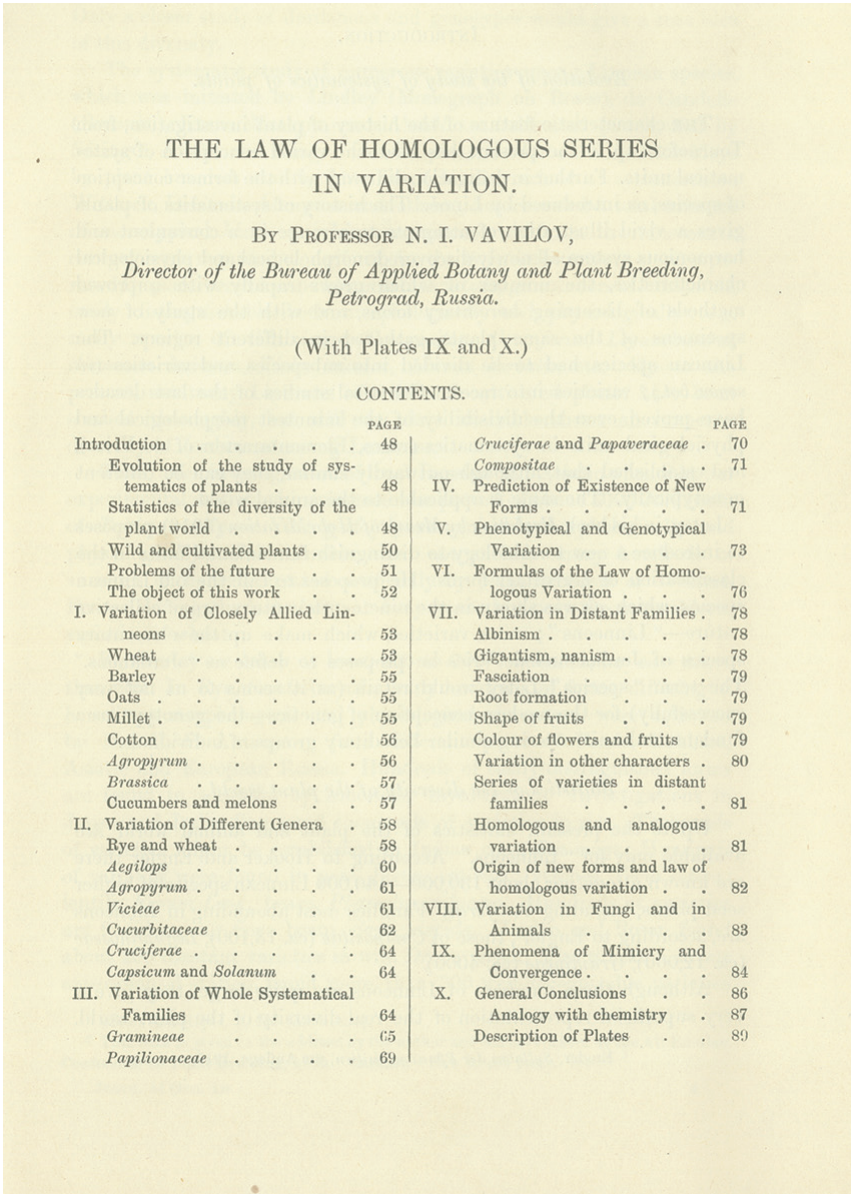
Statistics of the diversity of the plant world

Up to the present, statistics of the plant and animal world are available only for "Linneons". According to Hooker and Engler there are known altogether about 130,000–140,000 Linnean species of higher seed plants, including *Coniferae*. Families most abounding in Linneons are, according to Engler*, those of *Compositae* (ca. 13,100), *Leguminoseae* (ca. 12,000), *Gramineae* (ca. 4,000).

Although these numbers of Linneons are quite large, they give a very superficial representation of the real diversity of the plant world. Only a closer study of Jordanons and genotypes would give a true idea of this diversity.

The systematic study of numerous varieties among Linnean species, which was initiated by Lindley (Monograph on Roses), de Candolle (Brassica), Kraus, Metzger,

* Engler, Syllabus der Pflanzenfamilien, 8te Auflage, 1919.



THE LAW OF HOMOLOGOUS SERIES IN VARIATION.

By Professor N. I. VAVILOV,

Director of the Bureau of Applied Botany and Plant Breeding, Petrograd, Russia.

(With Plates IX and X.)

CONTENTS.

Figure 2. The title page of the Vavilov's paper in the Journal of Genetics, 12(1), 1922.

and Alefeld on cultivated plants, and by Séringe*, Jordan and Naegeli on wild plants, and is continued nowadays by plant breeders and by botanists (Swedish school of systematists: Wittrock, Dalstedt, Almquist and others), has revealed a total absence of monotypical Linneons. Linnean species, which, in the nineteenth century were re-

* Musee helvetique, p. 115 (Aconitum).

garded as uniform, in the twentieth century were separated by plant breeders and systematists into large numbers of Jordanons, easily distinguishable both morphologically and physiologically; e.g. many species of *Gramineae*, *Compositae*, *Cruciferae*, *Leguminoseae*, *Sesamum indicum*, *Viola tricolor*, *Linnea borealis*, etc. Up to the present, not many Linneons of wild and cultivated plants have been studied thoroughly, but still the data available shows an immense diversity of Jordanons among Linneons.

Thus, after investigations of local Russian and Asiatic wheats at our experimental station, the existence was proved of about 3000 Jordanons of *Triticum vulgare* Vill., perfectly recognizable morphologically and physiologically*. This number does not include many hundreds of varieties of hybrids created artificially by plant breeders of Western Europe during the last thirty or forty years, but only the natural local varieties of wheat.

For barley we know at least 600 to 700 Jordanons, for oats more than 600. In Rye, *Secale cereale*, many hundreds of forms, differing in hereditary morphological and physiological characters, were collected by Mrs V. P. Antropova, from different parts of Persia, Bokhara, Asiatic and European Russia. Hundreds of easily distinguished forms are found in sorghum by American investigators. Investigations in Japan and India discovered thousands of varieties in rice. Thousands of varieties might be established in Indian corn, *Zea mays*. Hundreds of varieties were found in peas, *Pisum sativum*; vetches, *Vicia sativa*; lentils, *Ervum Lens*; beans, *Phaseolus vulgaris*. Hundreds of varieties are found among Soya beans, *Soya hispida*. Jordan and Rosen found about 200 constant varieties in wild *Draba verna*. Miss Sinskaja, at our experimental station, found more than 300 well recognizable varieties of *Eruca sativa*, a weed occurring in field of flax in Turkestan and Bokhara. Thousands of forms, perfectly distinguishable, exist among species of *Cucurbita Pepo*, *Cucurbita maxima*, *Citrullus vulgaris* – watermelon, *Cucumis sativus*, and *Cucumis Melo*** . Hundreds of forms are found among wild *Linnea borealis* (Wittrock), *Picea excelsa* (Wittrock), etc.

Wild and cultivated plants

The majority of cultivated and wild Linneons propagated by seeds, are represented by hundreds of well-defined Jordanons. There is no essential difference in this respect between wild and cultivated plants. Wild Linneons, like clover (*Trifolium pratensis*), *Agropyrum cristatum*, *Agropyrum repens*, yellow alfalfa (*Medicago falcata*), *Alopecurus pratensis*, *Brassica elongata*, studied in detail at Russian Experimental Stations by plant breeders (Roudzinski, Lorch, Jegalov, Bogdan), proved to be no less variable than cultivated wheats, barleys, oats, and peas. The monotypic nature of many wild Linnean species is kept only so long as they are studied by a few specimens in the herbarium. The individual study in culture of many samples of the same Linneon inevitably discovers its polymorphic nature.

---

* This data is given in the address by the author and his co-workers at the All-Russian Conference on Plant Breeding, 1920. Saratov. Now in the press.

** These plants were studied at our experimental station by Mr S.M. Boukasov and Mrs S.A. Kartashov.

Still greater diversity is observable in plants multiplying vegetatively or apogamically, like roses, potatoes, apples, *Hieracium* (Naegeli), and *Dahlia*.

We do not exactly know if there are really monotypic Linnean species in nature, fairly well specific and separated from other Linnean species and represented by one variety, one Jordanon only. The whole impression is that the more we study our plants and animals, the more variable thay are, the more varieties we find among Linnean species. Several Linnean species of plants and animals, like roses, wheats, Indian corn, rice, squashes, *Drosophila*, seem to be extremely variable, but these have attracted more attention than others. We easily notice sharp differences in colour, size, and shape of several organs and are rather inattentive to others.

The differences of Jordanons within the limits of the same Linneon, in the shape and colour of their flowers, form and size of leaves, fruits and other organs, are very often no less marked than the differences between Linneons themselves. For instance, some varieties of *Cucurbita Pepo* are characterized by fruit the size of hen's eggs; other varieties, growing under the same conditions, bear fruit three and four feet in diameter. Some varieties of *Sesamum indicum* have opposite leaves and fruits, others have alternate. Some varieties of wheat and rye have simple leaves, without differentiation into vaginae and plates, having no "ligula", or "auriculae"; others have the usual complicated leaves, with "ligula", and "auriculae".

Plants self-fertilized, as wheat, barley, peas, soya, etc., and cross-fertilized, as rye, maize, beet, ale alike polymorphous. The seeming uniformity of several cross-fertilized wild and cultivated plants is only apparent when they are not studied carefully. The difference consists only in the homozygotic nature of many characters in cross-fertilized plants, and in the homozygotic nature of self-fertilized plants. Some recessive characters may be hidden in cross-fertilized plants by the dominance of other characters, but by artificial self-fertilization of these plants, and by inbreeding, it is possible to re-establish them. From what we know at present from the study of Indian corn (Emerson, Collins, and others), of rye, beetroot, *Drosophila*, man himself, cross-fertilized organisms are not less variable than self-fertilized.

The above mentioned numbers of Jordanons are in reality still greater, because, up to the present time, African and Asiatic varieties of even the most important cultivated plants, like wheat, oats, barley, peas, lentils, *Cruciferae*, are almost unknown.

Problems of the future

There is a real need for the study and systematizing of these Jordanons, especially in cultivated plants and domesticated animals, for the benefit of geneticists, as well as systematists and agriculturists. Only the closest study of Jordanons and genotypes will give a real re-presentation of what a Linneon is. To construct the general genetic schemes, it is necessary to know the composition of Linnean species. Before creating new varieties by crossing we ought to know what exists in nature. Even for cereals, *Leguminoseae*, and other most important plants, we have no adequate knowledge of even easily recognizable botanical varieties. Regions of ancient culture in Asia, Africa, and America still preserve numbers of varieties unknown to systematists and plant breeders.

In 1880, Alphonse de Candolle wrote in his remarkable book *La Phytographie*: "Un jour la science traitera les elements de l'espece comme les elements des genres, comme ceux de la famille et tous ces groupes seront coordonnes, les uns au-dessus des autres d'une maniere parfaitement uniforme" (p. 80). This day has arrived, but the task is not very simple. The closest study of some Linneons of cereals, *Leguminoseae*, *Cruciferae*, *Compositae*, and *Cucurbitaceae*, persuades one of the immensity of this work. The diversity of plants and animals is too great to admit of giving a complete list of existing forms. There comes the necessity to establish some principles and schemes of classification.

The near future promises to differentiate the Linneons still more, and to multiply the number of Jordanons and species in Lotsy's sense. Artificial hybridization threatens considerably to enlarge the external diversity of forms.

It may be expedient to define even at the present time the multi-formity in Linneons, not by the number of described and possible compositions, but by the number and list of *varietal characters* through which Jordanons differ from each other, not forgetting that separate characters can be dependent on several hereditary factors or genes, involving complicated genotypical formulae. The complete genotypical compositions of Linneons is a problem for the future.

The multitudinous chaos of innumerable forms obliges investigators to look for some way of simplification. The process of differentiation will go on inevitably, adding to the records of existing forms, and giving a true conception of Linneons. But parallel to differentiation it is natural to search for ways of integration of our knowledge of Jordanons and Linneons themselves. If some 130,000 Linneons are difficult to manage for investigation, the work with tens and hundreds of millions of Jordanons will be still more complicated.

As formerly, in the study of dead organic and inorganic worlds, so at the present, the problem before the investigator of the animal and plant world is to explore the regularities in polymorphism, and to establish its classes.

The object of this work

Below is an attempt to integrate the phenomena of polymorphism which we define as "The Law of Homologous Series of Variation". These regularities were noted by the author during the study of innumerable varieties of cultivated and wild plants.

The ideas expounded below in some parts are not foreign to biological literature. Separate facts of regular variation were known long ago. Naudin noticed them in his classical study of *Cucurbitaceae*. Darwin*, who was in general rather the adherer of fortuitous variations in all directions in his Origin and Variation, paid attention to regular variation, which, as he states, "occasionally" happens in plants and animals.

M.J. Duval-Jouve collected a great many data on the variation of wild Linnean species of *Gramineae*, *Juncaceae* and *Cyperaceae* in his paper on "Variations paralleles des types congeneres" published in 1865 in *Bull. De la Ste. Botanique de France*, Vol. XII. His conclusions in some part come near to the statements of our study. De Vries

* Darwin, Variation of Animals and Plants, Part 2; "Analogous or Parallel Variation."

notices in his *Mutationstheorie* the existence of series of variation. Eimer* in his study of Orthogenesis approached the same subject from a different point of view. Several palaeontologists (Cope, Oscborn) noticed regular variation in animals. More recently Saccardo** and Zederbauer*** gave extremely instructive instances of regular variation in fungi and *Coniferae*.

The detailed study of variation among many different groups, and the great number of new facts permits us to take this subject anew and bring all known facts into the form of a general law to which all organisms are submitted.

X. General conclusions [p. 86–89]

Parallelism in varietal polymorphism, and the existence of regularity in differentiation of greater groups as Linneons, genera, and families, is a great help in the study of varieties in self- and cross-fertilized plants and animals. Instead of searching for unknown forms, the investigator can definitely look for, and foresee, forms lacking in a system, by noticing the similarities with the nearest known Linneons and genera. In this respect a biologist places himself in the position of a chemist, who classifies substances according to their place in a system, and creates them through synthesis.

The investigation of polymorphism and the description of new forms become full of scientific meaning and interest. New forms have to fill vacancies in a system. The collections of immense numbers of butterflies and beetles in our museums and herbariums will play a more worthy role in the immediate future than ever before. For a systematist is not a man who knows all the curiosities of nature, but one who grasps the order and sense of it all.

The existing systems of Linneons and varieties ought to be fundamentally changed, and constructed according to a general plan. Instead of occasional characters, which usually determine species and varieties, it would be more rational to follow a general system. The greatest problem of systematists is to build up a general well sustained monotypical system, where similarity and homological series of variation would be considered as the fundamental basis, instead of an indefinite tangle of names impossible to remember. This may seem rather revolutionary for systematists, and it must be done very carefully, in consideration of existing orders. It would be easier to arrange in general systems of minutest systematical units, varieties and races which are as yet almost untouched by systematists. We have tried this for cultivated plants, and have found it expedient. Instead of remembering endless forms, usually named after occasional places of origin or in honour of persons, we have the possibility of studying a system and introducing into it individual additions, where it may be necessary to do

* G.H.T. Eimer, Die Entstellung der Arten auf Grund von erworbener Eigenschaften nach den Gesetzen organischen Wachsens, Vols. I-III. 1888–1901, Jena.

** P.A. Saccardo, "I Prevedibilli Funghi Futuri secondo la Legge d'Analogia". Degli Atti dei R. Instituto Veneti de Scienze, Lettere ed Arti, Tome VIII. Ser. 7.

*** E. Zederbauer, "Variationsrichtungen der Nadelholzer". Sitzberichte d. Akademie d. Wissenschaften, Wien, Math. Nat. Klasse, 116, Abt. 1. 1907.

so, for single Linneons and genera. We realize well the size and difficulty of the whole problem. Without a differential work, and without studying in detail, the integral work will be groundless. To integrate it is necessary to differentiate. We know that perhaps a century will pass before botanists and zoologists will create, through collective work, an organized world system; but this way is historically necessary and inevitable.

Analogy with chemistry

The above-mentioned analogy of the present day position of the biologist and chemist is deeper than it might seem at first. We have spoken conventionally about characters, colours, hairiness, beardedness, etc. Chemistry says little about the exterior of its substances; it considers the chemical nature of its compounds and their formulas. Numerous chemical substances are required to a harmonious system of combinations of a few elements. The biologist is still far behind. During the last decades, however, genetics has advanced greatly and is rapidly overtaking chemistry – at least the old chemistry of complicated organic compounds. Genetics is creating a laconic language of signs for hereditary factors, determining external characters. The biologist has learned to analyze organisms, and to get a hold on methods for the synthesis of new forms.

The regularities in polymorphism of plants, established by a minute examination of variation in different genera and families which we have examined, can be compared to homologous series of organic chemistry, e.g. carbohydrogen (CH_4, C_2H_4, C_2H_2, …). Its series of compounds differing from each other, are still characterized by many common properties in reactions, by definite cycles of compounds, by definite reactions of exchange and adhesion. Every single hydrocarbon gives a series of compounds similar to that of other hydrocarbon.

In general, genera (*G*1, *G*2, *G*3, …) and Linneons (*L*1, *L*2, *L*3, …) of plants and animals display, in just the same manner, their homologous series of varieties, corresponding to different homologous series of hydrocarbons.

*G*1*L*1 (*a*+*b*+*c*…) _ _ _ _ *G*2*L*1 (*a*+*b*+*c*…)
*G*1*L*2 (*a*+*b*+*c*…) _ _ _ _ *G*2*L*2 (*a*+*b*+*c*…)
*G*1*L*3 (*a*+*b*+*c*…) _ _ _ _ *G*2*L*3 (*a*+*b*+*c*…)
*L*1*a*1, *L*1*a*2, *L*1*a*3, …
*L*2*a*1, *L*2*a*2, *L*2*a*3, …
*L*3*a*1, *L*3*a*2, *L*3*a*3, …

Where *a*1, *a*2, *a*3, … are different characters which distinguish different varieties. The series of forms are strikingly analogous to homologous series of organic chemistry.

Besides their chemical structure, different forms of organized nature are characterized by physical structure, and perhaps it would be better to trace also the analogy of homologous series of plants and animals, with systems and classes of crystallography with definite chemical structure (Crystallo-Chemistry of Fedoroff).

We leave the question, in detail, of these analogies, which is already discussed in literature (Johannsen, Lohmann, Tischler). Further investigations will establish more

precisely the law of homologous variation in plants and animals, and it may be posiible to bring the same series into mathematical expression. The variation in form might be reduced to some geometrical scheme.

The problem of the origin of species cannot be separated from the problem of variation. A great many forms are undoubtedly only different combinations of the same genes, some primary types. The study of variation will give us the possibility of establishing these primary types, the fundamental series of variation of organisms.

The idea of the homologous series in variation in its essence is only a development of the general idea of Goethe's "Metamorphosis of plants", the idea of the unity in variety of C. Dresser*.

In conclusion, we take the liberty of expressing our strong conviction that the most rational and expedient method of studying the diversity of plants and animals open to breeders of both, even for practical purposes, is through the establishment of parallelism and homologous series of variation.

References

Huxley J (1940) The New Systematics. Oxford, London, 583 pp.

Huxley J (1942) Evolution: The Modern Synthesis. London, 770 pp.

Suslov VV, Omeljanchuk NA, Ponomarenko MP, Kolchanov NA (2008) The law of homologous series of N.I. Vavilov and gene networks. Proceedings of the International conference "N.I. Vavilov scientific heritage – a foundation for national and world agriculture", Moscow, November 27–28, 2007. Moscow, 46–75. http://evolbiol.ru/document/988

Timofeeff-Ressovsky NW (1940) Mutations and geographical variation. In: Huxley J (Ed.) The new Systematics. Oxford, London, 73–136.

Vavilov NI (1920) The law of homologous series in hereditary variation. 3-rd All-Russian conference of plant breeding in Saratov, June 4, 1920. Saratov, 16 pp. [In Russian].

Vavilov NI (1922) The law of homologous series in variation. Journal of Genetics 12(1): 47–89. https://doi.org/10.1007/BF02983073

Vavilov NI (1940) The new systematics of cultivated plants. In: Huxley J (Ed.) The new Systematics. Oxford, London, 549–566.

Vavilov NI (2012) Etudes on Genetics History. Moscow, 160 pp.

Zakharov IA (1987) N.I. Vavilov's law of genetic homology in modern genetics. Genetika 11: 1937–1948. [In Russian]

* Christofer Dresser, Unity in Variety. London, 1860. Recently there appeared several works devoted to the general uniformity of phenomena of life, history, psychology. See f.i. K. Marbe, Die Gleichformigkeit in der Welt. Bd. I and II. Munchen 1916–1919. P. Kammerer, Das Gesetz der Serie, 1919.

CompCytogen 14(3): 339–352 (2020)
doi: 10.3897/CompCytogen.v14i3.53367
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Chromosomal and reproductive features of some Oriental and Australasian scale insects (Homoptera, Coccinea)

Ilya A. Gavrilov-Zimin[1]

1 *Zoological Institute, Russian Academy of Sciences, Universitetskaya nab. 1, St. Petersburg, 199034, Russia*

Corresponding author: Ilya A. Gavrilov-Zimin (coccids@gmail.com)

Academic editor: V. G. Kuznetsova | Received 17 April 2020 | Accepted 5 July 2020 | Published 20 July 2020

http://zoobank.org/D2A1B8A1-B2EE-4251-8F36-66B295BA1B73

Citation: Gavrilov-Zimin IA (2020) Chromosomal and reproductive features of some Oriental and Australasian scale insects (Homoptera, Coccinea). Comparative Cytogenetics 14(3): 339–352. https://doi.org/10.3897/CompCytogen.v14i3.53367

Abstract

Fourteen species of scale insects from the families Margarodidae s.l., Pseudococcidae, Eriococcidae, and Coccidae were investigated for the first time in respect to karyotypes, genetic systems, modes of reproduction and general anatomy of the female reproductive system. One of the studied species, *Steatococcus samaraius* Morrison, 1927, showed hermaphroditic reproduction of the female-like specimens, the other species demonstrated bisexual reproduction with a peculiar "Lecanoid" heterochromatinization of the paternal set of chromosomes in male embryos or thelytocous parthenogenesis. *Antonina parazonata* Williams, 2004 and *Saccharolecanium krugeri* (Zehntner, 1897) are recorded here for the first time from Thailand, *Antonina vietnamensis* Williams, 2004 and *Geococcus satellitum* Williams, 2004 – for the first time from Laos.

Keywords

scale insects, giant scales, mealybugs, soft scales, felt scales, chromosome number, karyotype, genetic system, reproductive system

Introduction

The present paper continues a series of the author's publications on the cytogenetics and reproductive biology of scale insects from different regions of the world (Gavrilov 2004, 2007, Gavrilov and Kuznetsova 2007, Gavrilov and Trapeznikova 2007, 2008, 2010, Gavrilov-Zimin 2011, 2012, 2016, 2017, 2018a, b, Gavrilov-Zimin et al. 2015).

Here, 14 previously unstudied species from 13 genera of the families Margarodidae s.l., Pseudococcidae, Eriococcidae, and Coccidae are considered in respect of their karyotypes, genetic systems, modes of reproduction, and general anatomy of the female reproductive system. Unusual aberrant genetic systems of scale insects have been reviewed several times previously (e.g., Hughes-Schrader 1948, Nur 1980, Gavrilov 2007, Gavrilov-Zimin et al. 2015) and will not be discussed here. General evolutionary aspects of scale insect reproductive biology and ontogenesis were analyzed in a special monograph (Gavrilov-Zimin 2018a), that can also be used by readers for the clarifying of the terminology and the higher-level taxa system, explored below.

General anatomic types of the female reproductive system in the scale insects were previously reviewed by De Marzo et al. (1990) basing on a few, mainly European species. However, subsequent studies (for example, Gavrilov and Trapeznikova 2007, Gavrilov-Zimin 2012, 2018a), including the present work, support the view of the mentioned authors (l.c.) that the main types of the reproductive system are characteristic of the higher taxa of scale insects (families, subfamilies, tribes).

Material and methods

Material was collected by the author in different years in Thailand, Laos, Malaysia and Indonesia (Sulawesi, Bali, New Guinea). The detailed collecting data are provided below for each species. All numbers with the letter "K" mean unique collecting numbers for both acetoethanol material and Canada balsam slides. All material is deposited at the Zoological Institute, Russian Academy of Sciences (ZIN RAS), St. Petersburg, Russia.

Both the method for the preparation of permanent morphological slides mounted with Canada balsam and the method of squashing the embryonic cells in lactoacetic orcein for chromosome studies were reported, for example, by Danzig and Gavrilov-Zimin (2014).

All figures and photos, excluding the colour ones, were prepared by the author. The colour photos were prepared by the author with a kind help of D.A. Gapon.

Results and discussion

Family Margarodidae s.l.

***Steatococcus samaraius* Morrison, 1927**
Figs 1a, 2

Material. K 922, Indonesia, Sulawesi, vicinity of Kendari, on twigs of undetermined bush, 10.XI.2011, I.A. Gavrilov-Zimin. K 1071, Malaysia, Borneo, Damai Peninsula, on inflorescences of palm tree (probably *Areca catechu* Linnaeus, 1753), 14.I.2013, I.A. Gavrilov-Zimin.

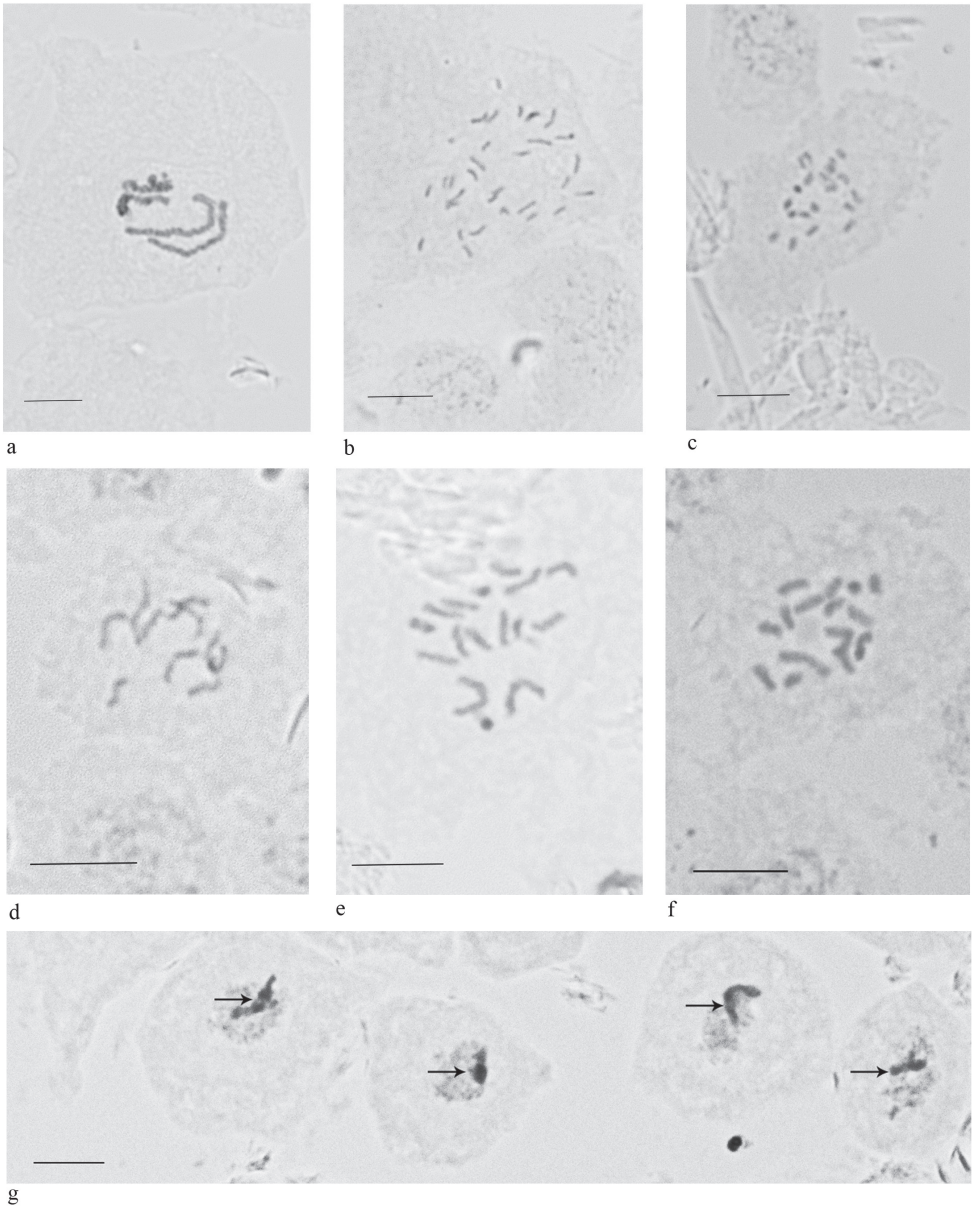


Figure 1. Embryonic cells and chromosomes of the studied species (Margarodidae, Pseudococcidae, Eriococcidae). **a** *Steatococcus samaraius* (2n = 4) **b** *Antonina parazonata* (2n = 30) **c** *A. vietnamensis* (2n = 20) **d** *Mollicoccus guadalcanalanus* (2n = 10) **e** *Acanthococcus* prope *onukii* (2n = 16) **f, g** *Gossypariella siamensis* (2n = 16). **g** Shows interphase cells of male embryos with a Lecanoid heterochromatinization of the paternal set of chromosomes (arrowed in each cell). Scale bars: 10 μm.

New data. 2n = 4; hermaphroditism: the studied female-like ultimolarvae contain sperm bundles in the ovo-testicles. Early stages of embryogenesis (before anatrepsis) occur inside of ovary; then the eggs are laid in the marsupium, where the embryogen-

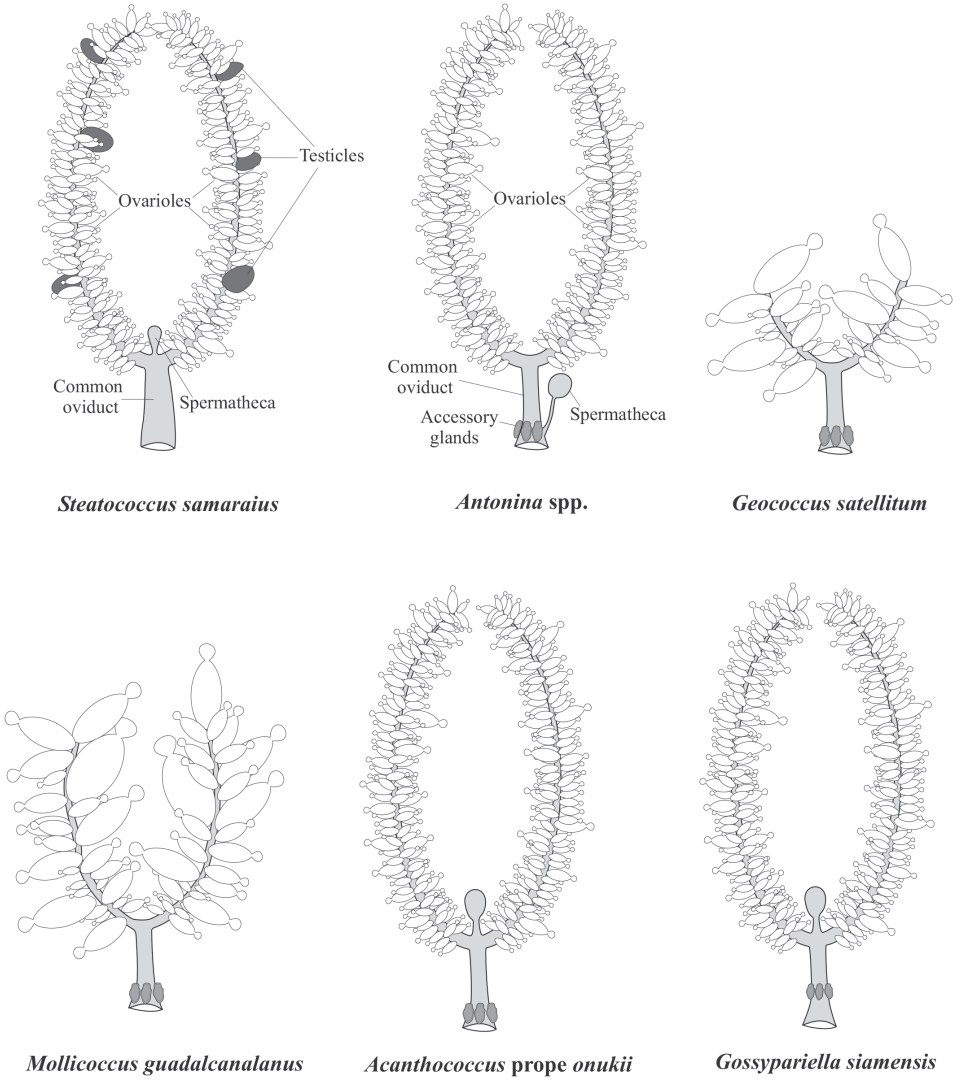


Figure 2. General anatomy of the female (or female-like hermaphrodite in *Steatococcus samaraius*) reproductive system in the studied species (Margarodidae, Pseudococcidae, Eriococcidae).

esis ends. Hermaphroditic reproductive system is generally similar to the usual female system in bisexual species of scale insects, but contains testicular parts, located between numerous ovarioles (Fig. 2).

Comments. Hermaphroditism is an exceptionally rare phenomenon in Insecta (see, for example, Royer 1975). Up to now hermaphroditic species are known for sure only in the scale insect tribe Iceryini (Margarodidae: Monophlebinae) (Hughes-Schrader 1948, Gavrilov 2007, Gavrilov-Zimin 2018a). Previously, the presence of ovo-testicles in female-like diploid insects has been shown for *Icerya bimaculata* De Lotto, 1959 (Hughes-Shrader 1963), *I. multicicatrices* (Kondo & Unruh, 2009)

(Gavrilov-Zimin 2018a: 27, 190) and *I. purchasi* Maskell, 1879 (Schrader & Hughes-Schrader 1926, Royer 1975). Closely related genus *Steatococcus* Ferris, 1921 (18 species), which differs from *Icerya* Signoret, 1876 (45 species) by the presence of peculiar marsupium, was previously almost unstudied in respect of cytogenetics and reproductive biology, excluding the only American species, *S. tuberculatus* Morrison, 1941. This species was investigated by Hughes-Schrader & Ris (1941) who found that it had 2n = 4 and reproduced bisexually with the appearance of haploid males via facultative parthenogenesis. Here, another species of the genus, *S. samaraius*, a widely distributed Oriental and Australasian pest, was studied and the same chromosome number, 2n = 4, was discovered. However, males were totally absent in any populations of *S. samaraius*, inspected by me in the different countries of the Oriental region and seem to have never been reported in the literature. The preparation of the mature females and larvae expectedly revealed a hermaphroditic condition of the reproductive system of *S. samaraius.* Such a combination of hermaphroditism and haplo-diploidy in closely related species of one genus and even in different populations of the species (as is the case of *Icerya purchasi*; Schrader & Hughes-Schrader 1926) is a peculiar feature of the tribe Iceryini (Hughes-Shrader 1963, Gavrilov-Zimin 2018a). Some authors (Unruh and Gullan 2007) do not consider *Steatococcus* as a separate genus and place its species either in the genus *Icerya* or in another related genus *Crypticerya* Cockerell, 1895. However, such approach leads to the total overlapping of the generic diagnostic characters and to the practical impossibility of assigning newly described species to a certain genus (see Gavrilov-Zimin 2018a: 174, 184, Gavrilov-Zimin and Stekolshikov 2018).

Family Pseudococcidae

***Antonina parazonata* Williams, 2004**
Figs 1b, 2

Material. K 1533, Thailand, Pai, the road to Mae Yen Luang waterfalls, on twigs of bamboo, 13.XI.2019, I.A. Gavrilov-Zimin.

New data. 2n = 30; bisexual reproduction with a Lecanoid heterochromatinization of paternal chromosomes in male embryos; complete ovoviviparity. Female reproductive system is similar to that of other studied mealybugs, i.e. with numerous ovarioles located on the paired oviducts, accessory glands attached to the proximal part of the common oviduct, and a spermatheca located at the same place as accessory glands (Fig. 2)

Comments. Special study of cytogenetics and reproductive biology of the genus *Antonina* Signoret, 1875 and other "legless mealybugs" was done recently (Gavrilov-Zimin 2016). Nine species from 3 genera of legless mealybugs were considered in that paper based on original and literature data and a significant variation of chromosome number was shown: 2n = 10, 12, 16, 20, 22+ Bs, 24, 24 + Bs, and 30. *Antonina parazonata*, studied here showed 2n = 30 as a species from the related monotypic genus *Komodesia* Gavrilov, 2016, namely, *Komodesia circuliplurima* Gavrilov, 2016. For the genus *Antonina*, such a high chromosome number was revealed for the first time.

A. parazonata was previously known from the type localities in India only. It is the first record of this species for Thailand.

***Antonina vietnamensis* Williams, 2004**
Figs 1c, 2

Material. K 1380, Laos, Pak Beng, on twigs of bamboo, 13.VI.2017, I.A. Gavrilov-Zimin.

New data. 2n=20; bisexual reproduction with a Lecanoid heterochromatinization in male embryos; complete ovoviviparity. Female reproductive system is the same type as in *A. parazonata* (Fig. 2).

Comments. *Antonina vietnamensis* has the same chromosome number as a closely related Oriental species of the genus, *A. diversiglandulosa* Gavrilov, 2016.

A. vietnamensis was previously known from the type localities in Vietnam only. It is the first record of the species for Laos.

***Geococcus satellitum* Williams, 2004**
Fig. 2

Material. K 1382, Laos, Pak Beng, on roots of dicotyledonous herb, 13.VI.2017, I.A. Gavrilov-Zimin.

New data. All studied embryos from 3 available females were unsuitable for chromosomal studies due to numerous yolk inclusions. Eggs are laid in loose ovisac at the stage of anatrepsis suggesting incomplete ovoviviparity. Female reproductive system is characterized by an extremely small number of ovarioles and the absence of a spermatheca (Fig. 2).

Comments. Up to now, the genus *Geococcus* Green, 1902 (14 species) has not been studied in terms of cytogenetics and reproductive biology. This is the case with most other related genera of tribe Rhizoecini (or group of the genus *Rhizoecus* Künckel d'Herculais, 1878). Diploid chromosome numbers, 8, 10, and 12, are known only for 5 species of *Rhizoecus* (Danzig & Gavrilov-Zimin, 2015: 428–429); all these species are characterized by a Lecanoid genetic system and bisexual reproduction.

G. satellitum was previously known from the type localities in China and Thailand only. This is the first record of this species for Laos.

***Mollicoccus guadalcanalanus* Williams, 1960**
Figs 1d, 2

Material. K 917, Indonesia, New Guinea, Manokwari, forest near the airport, on leaves of undetermined dicotyledonous herb, 8.XI. 2011, I.A. Gavrilov-Zimin.

New data. 2n = 10; bisexual reproduction with a Lecanoid heterochromatinization in male embryos; eggs are laid in loose ovisac at stage of anatrepsis suggesting incomplete ovoviviparity. Female reproductive system is similar in general details to that of *Geococcus satellitum* (Fig. 2).

Comments. These are the first cytogenetic and reproductive data for monotypic Australasian genus *Mollicoccus* Williams, 1960. The diploid number 10 is considered a modal chromosome number for the family Pseudococcidae as a whole (Nur 1980, Gavrilov 2007, Gavrilov-Zimin et al. 2015).

Family Eriococcidae

Acanthococcus prope *onukii* (Kuwana, 1902)

Figs 1e, 2

Material. K 1513, Thailand, Chiang Mai, slope of Doi Suthep Mt. near the University, on leaves of bamboo, 8.XI.2019, I.A. Gavrilov-Zimin.

New data. 2n = 16; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Eggs are laid in dense wax ovisac at the stage of anatrepsis, i.e. incomplete ovoviviparity is characteristic of the species. Female reproductive system consists of a spermatheca attached at the junction of the oviducts and accessory glands attached at the base of a common oviduct (Fig. 2).

Comments. Only two species of the large genus *Acanthococcus* Signoret, 1875 have been previously studied cytogenetically, i.e. European *A. agropyri* (Borchsenius, 1949) and *A. insignis* (Newstead, 1891), both with 2n = 16 (Gavrilov 2004, 2007). [Nota bene! The studied specimens differ from a common *Acanthococcus onukii* (=*Anophococcus onukii*) in the conical setae with blunt apices. The generic name *Anophococcus* Balachowsky, 1954 is considered here as a synonym of *Acanthococcus* (synonymized by Danzig 1980: 205)].

Gossypariella siamensis (Takahashi, 1942)

Figs 1f–g, 2, 5a

Material. K 1521, Thailand, Chiang Mai, city street near the University, on branches and twigs of an undetermined dicotyledonous tree, probably *Ficus* sp., 9.XI.2019, I.A. Gavrilov-Zimin.

New data. 2n = 16; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Complete ovoviviparity. Female reproductive system is similar with that in the previous species, but accessory glands are located in the middle part of the common oviduct (Fig. 2).

Comments. The genus *Gossypariella* Borchsenius, 1960 includes 4 species distributed in the Oriental region. *G. siamensis* is the first species of the genus studied cytogenetically.

Family Coccidae

***Coccus viridis* (Green, 1889)**
Figs 3a, 4

Material. K 939, Indonesia, Bali, mountain forest above Lake Buyan, about 1200 m altitude, on leaves of an undetermined tree, 13.XI. 2011, I.A. Gavrilov-Zimin.

New data. 2n = 18; there is no heterochromatinization (and thus no Lecanoid system) in all 50 studied embryos from 3 females, no sperm in spermathecae and no males in the studied population; so, the thelytocous reproduction is characteristic of this species. Complete ovoviviparity. Female reproductive system is of the usual for the soft scales type (Fig. 4).

Comments. The type species of the genus, *Coccus hesperidum* Linnaeus, 1758, shows 2n =14 and different variants of parthenogenesis (Thomsen 1927, 1929, Nur 1979), whereas two other studied species, *C. longulus* (Douglas, 1887) and *Coccus* sp., were reported by Moharana (1990) as having 2n=18, but without any comments on genetic system and reproductive peculiarities . All other (more than 110) species of the genus *Coccus* Linnaeus, 1758, are still unstudied cytogenetically.

***Discochiton expansum* (Green, 1896)**
Figs 3b, 4

Material. K 1121, Thailand, Malay Peninsula, Khao Lak, forest above the city, on leaves of an undetermined bush, 8.XI. 2013, I.A. Gavrilov-Zimin.

New data. 2n = 18; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Complete ovoviviparity. Female reproductive system has the usual structure, but accessory glands are poorly visible (Fig. 4).

Comments. The recently erected genus *Discochiton* Hodgson & Williams, 2018 comprises 21 species, and *D. expansum* is the first species of the genus studied cytogenetically.

***Drepanococcus chiton* (Green, 1909)**
Fig. 4

Material. K 864, Indonesia, New Guinea, vicinity of Jayapura, Entrop, on stem of a dicotyledonous herb, 30.X. 2011, I.A. Gavrilov-Zimin.

New data. There were no embryonic cells suitable for chromosomal analysis in the available material. The reproduction is bisexual with a Lecanoid heterochromatinization in male embryos. All studied females contained embryos at early stages of embryogenesis (up to anatrepsis). Female reproductive system has the usual structure (Fig. 4).

Comments. The only other species of the genus, *D. cajani* (Maskell, 1891), was previously studied cytogenetically by Moharana (1990), who reported 2n = 18 with no other comments on the species.

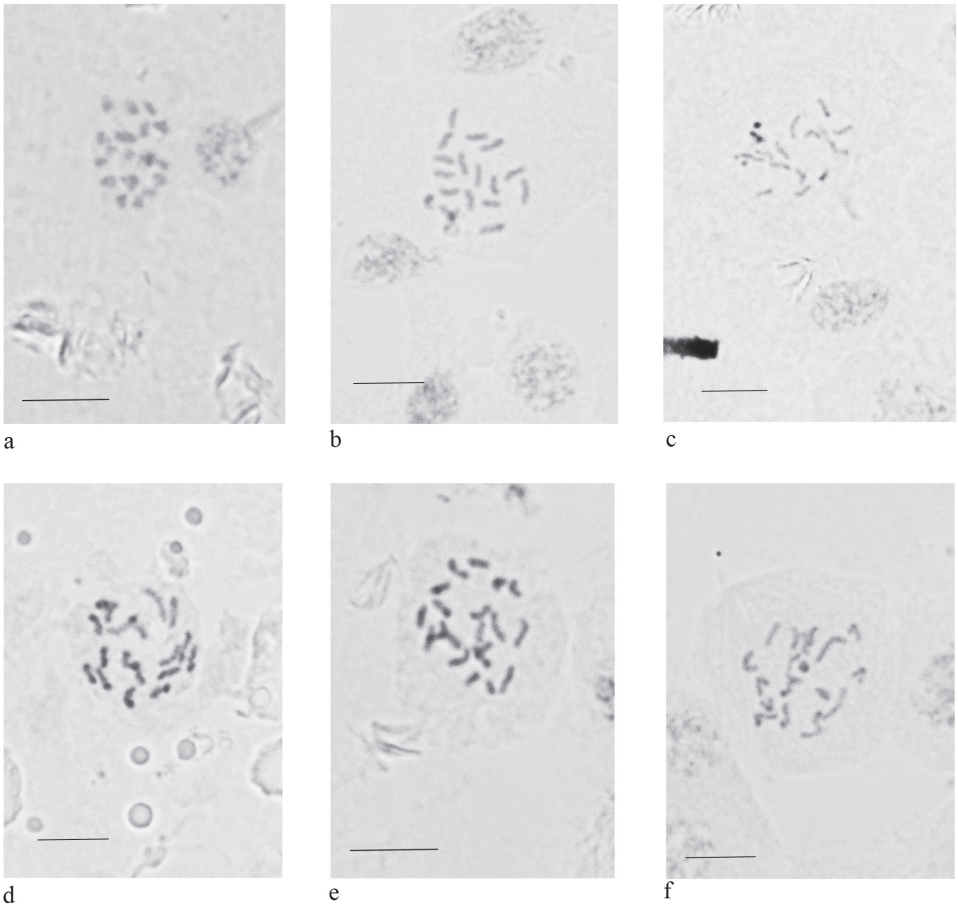


Figure 3. Embryonic cells and chromosomes of the studied species (Coccidae). **a** *Coccus viridis* (2n = 18) **b** *Discochiton expansum* (2n = 18) **c** *Luzulaspis australis* (2n = 18) **d** *Megalocryptes buteae* (2n = 18) **e** *Megapulvinaria maxima* (2n = 20) **f** *Saccharolecanium krugeri* (2n = 18). Scale bars: 10 μm.

Luzulaspis australis (Maskell, 1894)

Figs 3c, 4

Material. K 861, Indonesia, New Guinea, vicinity of Jayapura, Entrop, under leaf sheathes of a Poaceae grass, 30.X. 2011, I.A. Gavrilov-Zimin.

New data. 2n = 18; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. The eggs are laid in a long wax ovisac at the stage of late anatrepsis; i.e. incomplete ovoviviparity is characteristic of the species. Female reproductive system has the usual structure (Fig. 4).

Comments. The genus *Luzulaspis* Cockerell, 1902 comprises about 25 species, but only one of them, European *L. dactylis* Green, 1928, has been thus far studied cytogenetically (Gavrilov 2004). This species was found to have 2n = 18 and a bisexual reproduction with a Lecanoid heterochromatinization as well as presently studied Australasian *L. australis*.

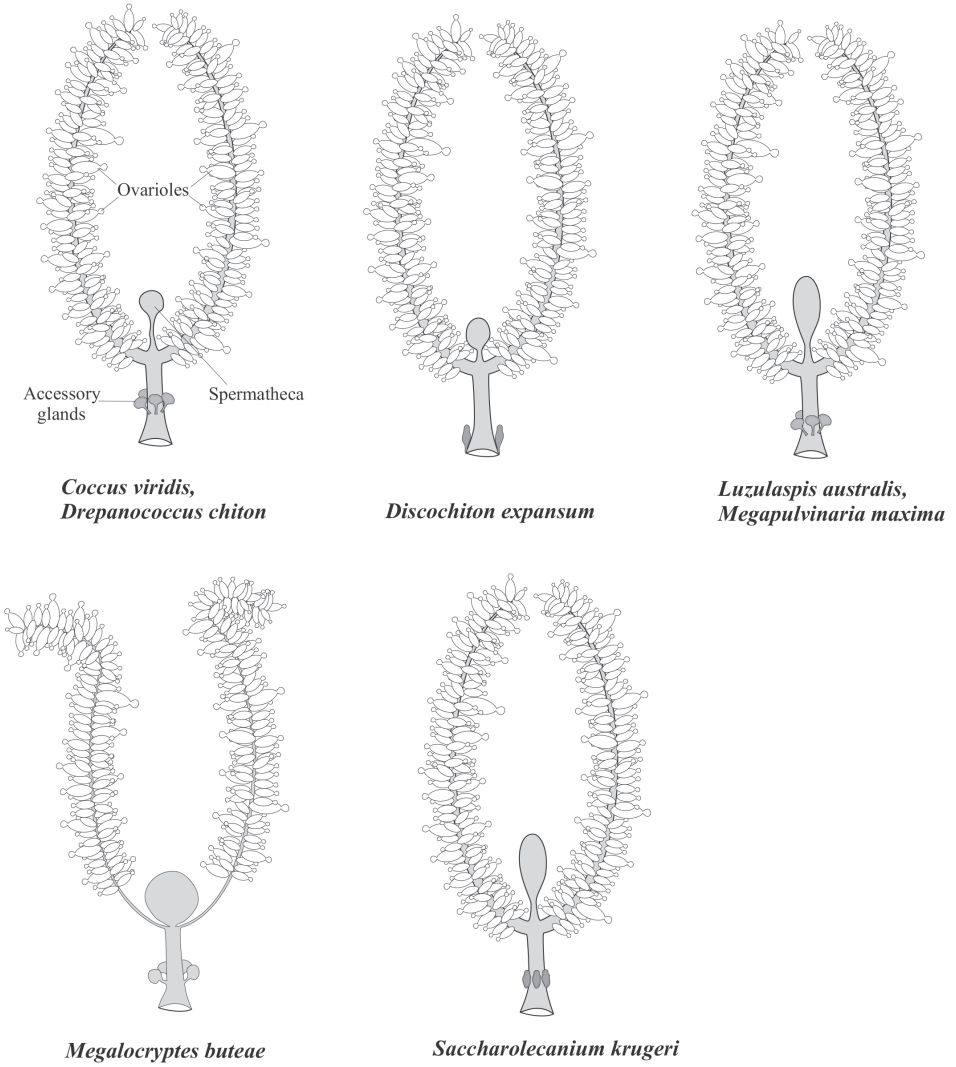


Figure 4. General anatomy of the female reproductive system in the studied species (Coccidae).

Megalocryptes buteae Takahashi, 1942

Figs 3d, 4, 5c

Material. K 1536, Thailand, Pai, on twigs of an undetermined dicotyledonous tree, 13.XI.2019, I.A. Gavrilov-Zimin.

New data. 2n = 18; there is no heterochromatinization in all 72 studied embryos from 3 females, no sperm in spermathecae and no males in the population suggesting thus thelytokous reproduction. Female reproductive system is distinguished by unusually long and thin lateral oviducts (Fig. 4). Cleavage divisions in the egg start just prior to oviposition.

Comments. These are the first cytogenetic and reproductive data for the small Oriental genus *Megalocryptes* Takahashi, 1942 which comprises two species only.

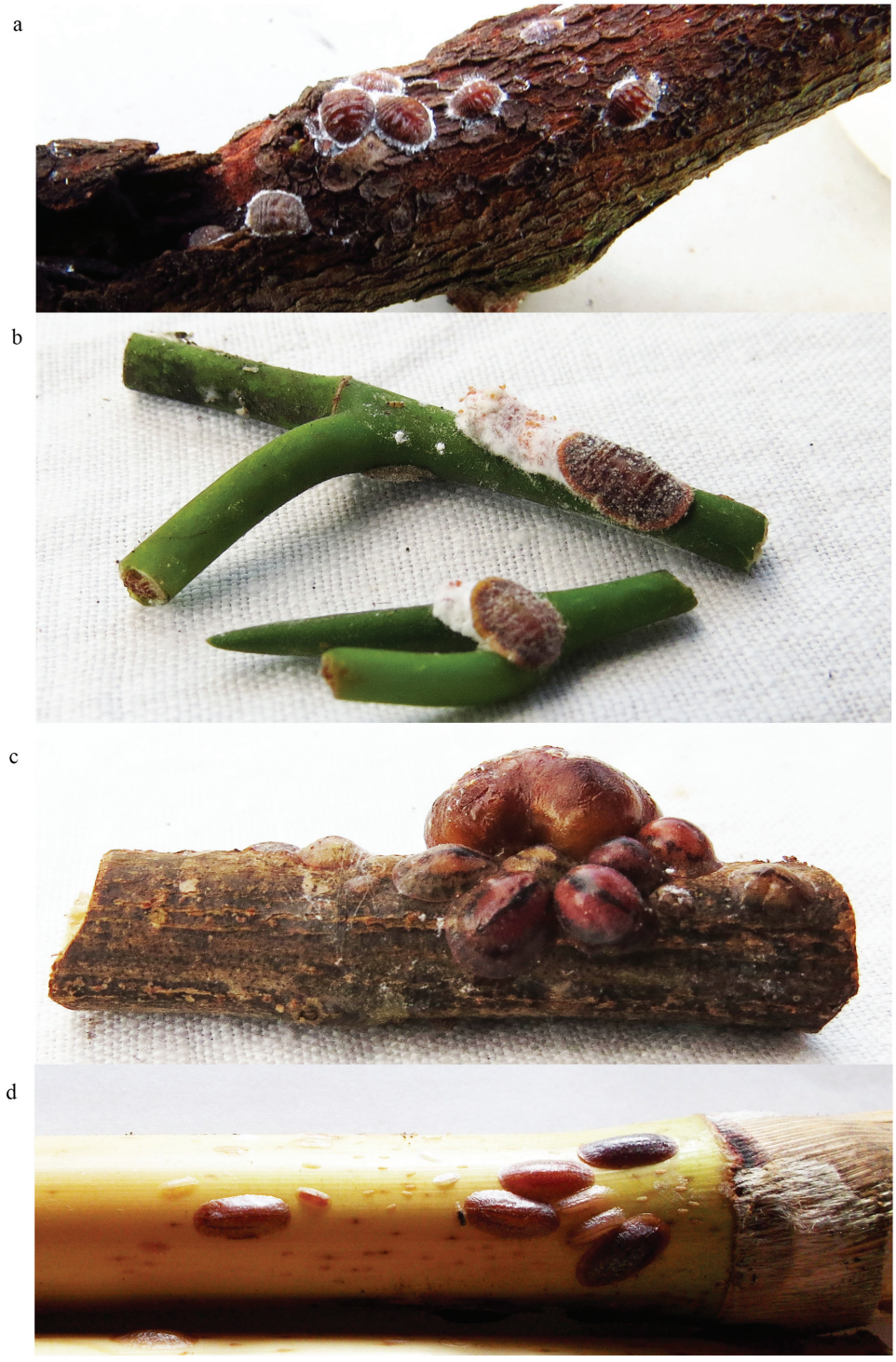


Figure 5. Females of some species on twigs of host plants. **a** *Gossypariella siamensis* **b** *Megapulvinaria maxima* **c** *Megalocryptes buteae* (with a colony of *Kerria* sp. at the background) **d** *Saccharolecanium krugeri*.

***Megapulvinaria maxima* (Green, 1904)**
Figs 3e, 4, 5b

Material. K 1531, Thailand, Pai, on leaves and twigs of an undetermined dicotyledonous tree, 13.XI.2019, I.A. Gavrilov-Zimin.

New data. 2n = 20; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Incomplete ovoviviparity: embryogenesis (until the late anatrepsis) partially occurs inside of the mother's body. Female reproductive system has the usual structure (Fig. 4).

Comments. Different European members of the tribe Pulvinariini have been previously studied cytogenetically (Gavrilov 2007, Gavrilov and Trapeznikova 2008). Four Oriental species from the genera *Chloropulvinaria* Borchsenius, 1952, *Pseudopulvinaria* Atkinson, 1889 and *Pulvinaria* Targioni Tozzetti, 1866 were studied by Moharana (1990), who reported chromosome numbers with no comments or details. *M. maxima* is the first species of the genus *Megapulvinaria* Yang, 1982 studied in terms of chromosome number; the kartotype 2n = 20 is found for the first time in the tribe Pulvinariini in general.

***Saccharolecanium krugeri* (Zehntner, 1897)**
Figs 3f, 4, 5d

Material. K 1368, Thailand, vicinity of Chiang Rai, forest above the Mae Fah Luang University, under the leaf sheathes of ?*Saccharum* sp., 8.VI.2017, I.A. Gavrilov-Zimin.

New data. 2n = 18; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Complete ovoviviparity. Female reproductive system has the usual structure (Fig. 4).

Comments. These are the first cytogenetic and reproductive data for the small Oriental genus *Saccharolecanium* Williams, 1980, which comprises two species only. *S. krugeri* is noted here for the first time for the territory of Thailand.

Acknowledgements

Financial support for this study was provided by the research grant no. 19-54-18002 from the Russian Foundation for Basic Research. The part of the material was prepared for the collection of the Zoological Institute, Russian Academy of Sciences according to the state projects nos. AAAA-A19-119020790106-0 and AAAA-A19-119020690101-6. Expedition expenses of different years were partly covered by Percy Sladen Memorial Fund. The author thanks D.A. Gapon for his help with the photographing of some studied insects in the nature.

References

Danzig EM (1980) Koktsydy Dal'nego Vostoka SSSR (Homoptera, Coccinea) s analizom filogenii koktsyd mirovoy fauny. Leningrad, 367 pp. [In Russian] [English Edition: Danzig EM (1986) Coccids of the Far-Eastern USSR (Homoptera: Coccinea). Phylogenetic analysis of coccids in the world fauna. New Delhi, 450 pp.]

Danzig EM, Gavrilov-Zimin IA (2014) Palaearctic mealybugs (Homoptera: Coccinea: Pseudococcidae). Part 1. Subfamily Phenacoccinae. St. Petersburg, 678 pp. (Fauna of Russia and neighbouring countries. New series, № 148. Insecta: Hemiptera: Arthroidignatha).

Danzig EM, Gavrilov-Zimin IA (2015) Palaearctic mealybugs (Homoptera: Coccinea: Pseudococcidae). Part 2. Subfamily Pseudococcidae. St. Petersburg, 619 pp. (Fauna of Russia and neighbouring countries. New series, № 149. Insecta: Hemiptera: Arthroidignatha). https://doi.org/10.31610/zsr/2015.24.2.236

De Marzo L, Romano V, Tranfaglia A (1990) Types of the reproductive system in some scale insects (Homoptera: Coccoidea). Proceedings of the VI International Symposium of Scale Insect Studies. Krakow, August 6–12, 1990, 2. Krakow, 41–46.

Gavrilov IA (2004) Taxonomic and cytogenetic studies of scale insects (Homoptera: Coccinea) of European Russia. Proceedings of the Zoological Institute RAS 300: 77–82.

Gavrilov IA (2007) A catalogue of chromosome numbers and genetic systems of scale insects (Homoptera: Coccinea) of the world. Israel Journal of Entomology 37: 1–45.

Gavrilov-Zimin IA (2011) New cytogenetic data for some Palaearctic species of scale insects (Homoptera: Coccinea) with karyosystematic notes. Comparative Cytogenetics 5(5): 375–390. https://doi.org/10.3897/compcytogen.v5i5.2116

Gavrilov-Zimin IA (2012) A contribution to the taxonomy, cytogenetics and reproductive biology of the genus *Aclerda* Signoret (Homoptera, Coccinea, Aclerdidae). Comparative Cytogenetics 6(4): 389–395. https://doi.org/10.3897/compcytogen.v6i4.4320

Gavrilov-Zimin IA (2016) Cytogenetic and taxonomic studies of some legless mealybugs (Homoptera: Coccinea: Pseudococcidae). Comparative Cytogenetics 10(4): 587–601. https://doi.org/10.3897/compcytogen.v10i4.10503

Gavrilov-Zimin IA (2017) Contribution to the cytogenetics of Kuwaniini scale insects (Homoptera, Coccinea, Margarodidae s.l.). Comparative Cytogenetics 11(4): 659–663. https://doi.org/10.3897/CompCytogen.v11i4.20168

Gavrilov-Zimin IA (2018a) Ontogenesis, morphology and higher classification of archaecococcids (Homoptera: Coccinea: Orthezioidea). Zoosystematica Rossica (Supplementum 2). 260 pp. https://doi.org/10.31610/zsr/2018.supl.2.1

Gavrilov-Zimin IA (2018) First illustration of chromosomes and genetic system of Lecanodiaspidinae (Homoptera: Coccinea: Asterolecaniidae s.l.). Comparative Cytogenetics 12(3): 439–443. https://doi.org/10.3897/CompCytogen.v12i3.29648

Gavrilov IA, Kuznetsova VG (2007) On some terms in scale insects cytogenetics and reproductive biology (Homoptera: Coccinea). Comparative Cytogenetics 1(2): 169–174.

Gavrilov-Zimin IA, Stekolshikov AV (2018) A new species of the genus *Steatococcus* Ferris, 1921 (Homoptera: Coccinea: Margarodidae) with some additions to fauna of Republic of Mali. Entomological Review 98(7): 865–867. https://doi.org/10.1134/S0013873818070060

Gavrilov-Zimin IA, Stekolshikov AV, Gautam DC (2015) General trends of chromosomal evolution in Aphidococca (Insecta, Homoptera, Aphidinea + Coccinea). Comparative Cytogenetics 9(3): 335–422. https://doi.org/10.3897/CompCytogen.v9i3.4930

Gavrilov IA, Trapeznikova IV (2007) Karyotypes and reproductive biology of some mealybugs (Homoptera: Coccinea: Pseudococcidae). Comparative Cytogenetics 1(2): 139–148.

Gavrilov IA, Trapeznikova IV (2008) Cytogenetic studies of European Pulvinariini (Homoptera: Coccinea). Comparative Cytogenetics 2(2): 123–131.

Gavrilov IA, Trapeznikova IV (2010) Karyotypes of six previously unstudied European mealybugs (Homoptera: Pseudococcidae). Comparative Cytogenetics 4(2): 203–205. https://doi.org/10.3897/compcytogen.v4i2.44

Hughes-Schrader S (1948) Cytology of coccids (Coccoidea-Homoptera). Advances in Genetics 2: 127–203. https://doi.org/10.1016/S0065-2660(08)60468-X

Hughes-Schrader S (1963) Hermaphroditism in an African coccid, with notes on other Margarodids (Coccoidea-Homoptera). Journal of Morphology 113: 173–184. https://doi.org/10.1002/jmor.1051130205

Hughes-Schrader S, Ris H (1941) The diffuse spindle attachment of coccids, verified by the mitotic behavior of induced chromosome fragments. Journal of Experimental Zoology 87: 429–456. https://doi.org/10.1002/jez.1400870306

Moharana S (1990) Cytotaxonomy of coccids (Homoptera: Coccoidea). Proceedings of the VI International Symposium of Scale Insect Studies. Krakow, August 6–12, 1990, 2. Krakow, 47–54.

Nur U (1979) Gonoid thelytoky in soft scale insects (Coccidae: Homoptera). Chromosoma (Berlin) 72: 89–104. https://doi.org/10.1007/BF00286431

Nur U (1980) Evolution of unusual chromosome systems in scale insects (Coccoidea: Homoptera). In: Blackman RL, Hewitt GM, Ashburner M (Eds) Insect Cytogenetics. London, 97–117.

Royer M (1975) Hermaphroditism in Insects. Studies on *Icerya purchasi*. In: Reinboth R (Ed.) Intersexuality in the Animal Kingdom. Springer, Berlin, Heidelberg, 135–145. https://doi.org/10.1007/978-3-642-66069-6_14

Schrader F, Hughes-Schrader S (1926) Haploidy in *Icerya purchasi*. Zeitschrift für Wissenschaftliche Zoologie 128: 182–200.

Thomsen M (1927) Studien über die partenogenese bei einigen Cocciden und Aleyrodiden. Zeitschrift für Zellforschung und Mikroskopische Anatomie 5(1/2): 1–116. https://doi.org/10.1007/BF00398903

Thomsen M (1929) Sex-determination in *Lecanium*. In: Jordan K, Horn W (Eds) Fourth International Congress of Entomology, Ithaca, August 1928, 2, Ithaca, 18–24.

Unruh CM, Gullan PJ (2007) Molecular data reveal convergent reproductive strategies in iceryine scale insects (Hemiptera: Coccoidea: Monophlebidae), allowing the re-interpretation of morphology and a revised generic classification. Systematic Entomology 33: 8–50. https://doi.org/10.1111/j.1365-3113.2007.00404.x

CompCytogen 14(3): 353–367 (2020)
doi: 10.3897/CompCytogen.v14i3.53533
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 highly rearranged karyotype of the hangingfly *Bittacus sinicus* (Mecoptera, Bittacidae): the lowest chromosome number in the order

Ying Miao[1], Bao-Zhen Hua[2]

1 *College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China* **2** *College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China*

Corresponding author: Bao-Zhen Hua (huabzh@nwafu.edu.cn)

Academic editor: V. Lukhtanov | Received 22 April 2020 | Accepted 4 July 2020 | Published 30 July 2020

http://zoobank.org/885B5AE8-3F19-42C1-86C5-904DDD26CF55

Citation: Miao Y, Hua B-Z (2020) The highly rearranged karyotype of the hangingfly *Bittacus sinicus* (Mecoptera, Bittacidae): the lowest chromosome number in the order. Comparative Cytogenetics 14(3): 353–367. https://doi.org/10.3897/CompCytogen.v14i3.53533

Abstract

Cytogenetic features of the hangingfly *Bittacus sinicus* Issiki, 1931 were investigated for the first time using C-banding and DAPI (4',6-diamidino-2-phenylindole) staining. The karyotype analyses show that the male *B. sinicus* possesses the lowest chromosome number ($2n$ = 15) ever observed in Mecoptera, and an almost symmetric karyotype with M_{CA} (Mean Centromeric Asymmetry) of 12.55 and CV_{CL} (Coefficient of Variation of Chromosome Length) of 19.78. The chromosomes are either metacentric or submetacentric with their sizes decreasing gradually. Both the C-banding and DAPI$^+$ patterns detect intermediate heterochromatin on the pachytene bivalents of *B. sinicus*, definitely different from the heterochromatic segment at one bivalent terminal of other bittacids studied previously. The male meiosis of *B. sinicus* is chiasmate with two chiasmata in metacentric bivalents and one in the submetacentric bivalent. The sex determination mechanism is X0(♂), which is likely plesiomorphic in Bittacidae. Two alternative scenarios of karyotype origin and evolution in *Bittacus* Latreille, 1805 are discussed.

Keywords

C-banding technique, chromosome rearrangement, cytogenetics, DAPI, evolution, Holometabola, meiosis

Introduction

Bittacidae is the second largest family of Mecoptera, and currently consists of over 200 species in 18 genera in the world (Zhang et al. 2020). The adults of Bittacidae comprise an exclusive group that possesses three pairs of elongated raptorial legs with a single claw at pretarsus and adopts a predacious feeding strategy (Bornemissza 1966; Byers and Thornhill 1983; Penny 2006; Tan and Hua 2008; Ma et al. 2014). They are commonly known as hangingflies because between flights they are unable to stand on a surface but hang themselves from the edges of leaves or twigs using the prehensile foretarsi (Thornhill 1977; Tan and Hua 2008). *Bittacus* Latreille, 1805 is the largest and most widespread genus of Bittacidae, and comprises more than 2/3 species of the family recorded from all zoogeographical regions (Penny and Byers 1979). Owing to considerable morphological variations (Lambkin 1988; Chen et al. 2013) and complicated distribution patterns (Penny 1975; Li and Ren 2009), the evolutionary relationship within this genus remains largely unknown to date.

Chromosomes of eukaryotic organisms may carry crucial information related to the species diversification and evolution (Gokhman and Kuznetsova 2006; Noor et al. 2007; Faria and Navarro 2010). The variations of chromosome number reflect the result of complicated chromosomal rearrangements and may help reveal the evolutionary relationships of sibling species (White 1974; Lukhtanov et al. 2005; Kandul et al. 2007; Faria and Navarro 2010). The chromosomal morphology may provide substantial information related to structural rearrangements, which may contribute to the increased level of divergence among taxa (Rieseberg and Burke 2001; Navarro and Barton 2003; Butlin 2005). Such studies have been well documented in many insect groups, including aquatic bugs (Stoianova et al. 2020), psyllids (Nokkala et al. 2019), bush crickets (Kociński et al. 2018), beetles (Dutrillaux and Dutrillaux 2019), butterflies (Dincă et al. 2011), warrior wasps (Menezes et al. 2019), and ants (Pereira et al. 2018). In Bittacidae, however, the cytogenetic information is poorly documented, with only six species reported to date (Matthey 1950; Atchley and Jackson 1970; Miao and Hua 2017, 2019).

According to the limited cytogenetic data available, the chromosome number varies extensively in Bittacidae (Matthey 1950; Atchley and Jackson 1970; Miao and Hua 2017, 2019). It is $2n$ = 25 in *B. italicus* (Müller, 1766), $2n$ = 27 in *B. flavidus* Huang et Hua, 2005, $2n$ = 29 in *B. pilicornis* Westwood, 1846, $2n$ = 31 in *B. stigmaterus* Say, 1823, $2n$ = 35 in *B. planus* Cheng, 1949, and $2n$ = 41 in *Terrobittacus implicatus* (Huang et Hua in Cai et al., 2006). Each species examined has a distinctive karyotype, which represents an important diagnostic feature in Bittacidae and provides useful information on the evolutionary relationship of Mecoptera (Miao and Hua 2017, 2019).

In this paper, we present for the first time information on the karyotype and male meiosis of the hangingfly *Bittacus sinicus* Issiki, 1931, attempting to enrich our knowledge of the chromosome evolution of *Bittacus* and to contribute to the cytogenetic data for a better understanding of the evolutionary history of Bittacidae.

Materials and methods

Adult collecting

Adults of *B. sinicus* (Fig. 1A) were collected from Shimian County (29°03'00"N, 102°21'00"E, elev. 1800–1890 m), Sichuan Province in China from July to August in 2016 and Paomashan (30°02'36"N, 101°57'33"E, elev. 2600 m), Sichuan Province in China in late July 2018, respectively.

Insect rearing

Live adults were reared in screen-wired cages (40 × 60 × 60 cm) containing twigs and leaves of plants and moist absorbent cotton (Miao and Hua 2019). Eggs, larvae and pupae were incubated and reared in plastic containers with humid humus. Live flies and frozen pupae of *Musca domestica* Linnaeus, 1758 (Diptera, Muscidae) were provided as food for the adults and larvae, respectively. Temperature was kept at 16 ± 2 °C for larvae, 21 ± 2 °C for pupae, and 23 ± 2 °C for adults. Relative humidity was maintained at 75 % ± 10 % (Miao and Hua 2017).

Cytogenetic analyses

Chromosome spreads were prepared using the testes of larvae and pupae following Imai et al. (1988). The mitotic metaphase and early stages of meiosis were obtained from males of the third and fourth (last) instar larvae, and the male meiosis I/II mainly from young pupae. Totally 66 larvae (46 from Shimian County and 20 from Paomashan) and 12 pupae (nine from Shimian County and three from Paomashan) of *B. sinicus* were used for chromosome preparations.

C-banding was obtained using the same technique as in Miao and Hua (2019). The fluorochrome DAPI (4',6-diamidino-2-phenylindole) staining was performed to characterize the $DAPI^+$ heterochromatin (the shiny blue regions rich in AT bases) on chromosomes, following Rebagliati et al. (2003).

Photographs were taken with a Nikon DS-Fil digital camera mounted on a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). The fluorescence signals were observed with a UV filter (330–385 nm).

Statistical analyses

Five spermatogonial cells with well-spread chromosomes at mitotic metaphase were used to statistically analyze the chromosomes of *B. sinicus* following the procedures of Miao and Hua (2017). The captured images were quantified using the NIS-Element

D 3.22 software (Nikon, Tokyo, Japan). The chromosomal morphology was determined based on the arm ratio where chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or telocentric (t) (Levan et al. 1964). The following features of chromosomes were measured: absolute chromosome length (*AL*), long arm length (*L*), short arm length (*S*), arm ratio ($r = L/S$), centromeric index ($i = S \times 100/AL$), and relative chromosome length (*RL*) of each chromosome ($RL = AL \times 100/\sum AL$). The evaluated data are presented as mean ± SD.

The karyotype asymmetry is represented by two components, the heterogeneous degree of chromosome lengths (interchromosomal asymmetry) and the prevalence of telo-/subtelocentric chromosomes (intrachromosomal asymmetry) (Astuti et al. 2017). Two separate parameters were assessed, i.e. Coefficient of Variation of Chromosome Length (CV_{CL}) (Paszko 2006) and Mean Centromeric Asymmetry (M_{CA}) (Peruzzi and Eroğlu 2013).

Results

Karyology

The males of *B. sinicus* possess $2n = 15$ (Fundamental Number *FN* = 30), with the karyotype formula of 13 m + 2 sm (Fig. 1B, C).

The *AL* ranges from 7.47 ± 0.26 to 3.72 ± 0.05 μm, and the *RL* from 8.43 ± 0.29 to 4.20 ± 0.05. Autosomal bivalents decrease gradually in size, and the sex chromosome (X) is the smallest of the set. The total length of all chromosomes is 88.65 μm (Table 1).

Table 1. Morphometric analyses of the chromosomes of *Bittacus sinicus* based on five spermatogonial cells from a male larva.

Pair No.	*AL* ± SD (μm)	*RL* ± SD	*L* ± SD (μm)	*S* ± SD (μm)	(*L* – *S*)/(*L* + *S*)	*i*	*r*	Type
1	3.98 ± 0.06	4.49 ± 0.07	2.62 ± 0.05	1.36 ± 0.18	0.32	34.11	1.93	sm
	4.29 ± 0.02	4.83 ± 0.03	2.75 ± 0.03	1.53 ± 0.02	0.29	35.74	1.80	sm
2	4.97 ± 0.24	5.61 ± 0.27	2.67 ± 0.10	2.30 ± 0.10	0.07	46.27	1.16	m
	5.38 ± 0.04	6.07 ± 0.05	3.18 ± 0.22	2.20 ± 0.15	0.18	40.84	1.45	m
3	6.00 ± 0.17	6.77 ± 0.19	3.45 ± 0.05	2.55 ± 0.12	0.15	42.55	1.35	m
	6.12 ± 0.08	6.90 ± 0.09	3.35 ± 0.03	2.76 ± 0.06	0.10	45.19	1.21	m
4	6.45 ± 0.08	7.27 ± 0.09	3.48 ± 0.05	2.97 ± 0.12	0.08	46.00	1.17	m
	6.50 ± 0.21	7.33 ± 0.24	3.68 ± 0.22	2.83 ± 0.13	0.13	43.45	1.30	m
5	6.59 ± 0.15	7.44 ± 0.17	3.49 ± 0.13	3.10 ± 0.29	0.06	47.08	1.12	m
	6.60 ± 0.15	7.44 ± 0.17	3.49 ± 0.11	3.11 ± 0.20	0.06	47.16	1.12	m
6	6.92 ± 0.64	7.80 ± 0.72	3.93 ± 0.09	2.99 ± 0.12	0.14	43.18	1.32	m
	6.62 ± 0.61	7.46 ± 0.69	3.56 ± 0.26	3.05 ± 0.17	0.08	46.14	1.17	m
7	7.04 ± 0.11	7.94 ± 0.12	3.92 ± 0.09	3.12 ± 0.01	0.11	44.31	1.26	m
	7.47 ± 0.26	8.43 ± 0.29	3.97 ± 0.26	3.50 ± 0.25	0.06	46.90	1.13	m
8 (X)	3.72 ± 0.05	4.20 ± 0.05	1.98 ± 0.13	1.75 ± 0.09	0.06	46.94	1.13	m

Notes: *AL*, absolute chromosome length (actual length of chromosomes); *RL*, relative chromosome length (*RL* = *AL*/total length of the chromosome complement); SD = standard deviation; *L*, long arm length; *S*, short arm length; *i*, centromeric index ($i = s \times 100/AL$); *r*, arm ratio ($r = L/S$); m, metacentric; sm, submetacentric.

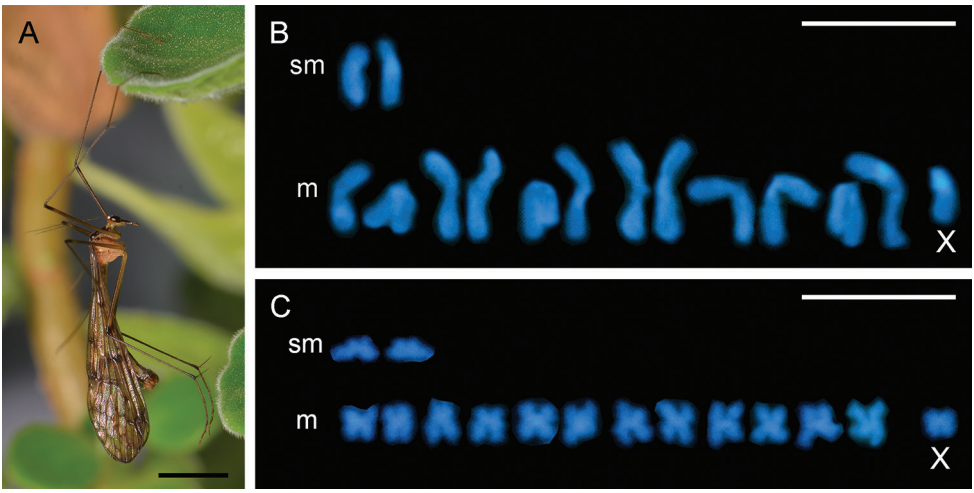


Figure 1. Karyotypes of *Bittacus sinicus* with DAPI staining **A** habitus of male adult **B** spermatogonial metaphase **C** meiotic anaphase I. Abbreviations: m, metacentric; sm, submetacentric; X, sex chromosome. Scale bars: 5 mm (**A**); 10 μm (**B, C**).

The M_{CA} is calculated as 12.55 and the CV_{CL} is 19.78. The relatively low degrees of both intrachromosomal and interchromosomal asymmetries indicate that the karyotype of *B. sinicus* is almost symmetric.

Banding patterns

Conspicuous heterochromatin was observed on the meiotic bivalents of *B. sinicus* after C-banding and DAPI staining (Fig. 2). Both treatments reveal that the autosomal bivalents exhibit intermediate heterochromatin. The sex chromosome is heteropycnotic and totally heterochromatic at the early pachytene (Fig. 2A, C), but becomes isopycnic with two heterochromatic dots later (Fig. 2B, D).

Chiasmate male meiosis

The synaptic attraction between the homologues terminates from the pachytene to diplotene. The early diplotene appears to be the diffuse stage, which can be interpreted as uncondensed bivalents connected by chiasmata (Fig. 3A). During this stage, the intermediate region of the bivalents is heavily stained and arranged dispersedly, while the remaining bivalents are weakly stained and are often overlooked consequently. The chromosomes move apart in repulsion and are held together only at exchange points, which appear as visible chiasmata in the diplotene stage (Fig. 3B). Metacentric bivalents exhibit two terminal chiasmata and look like large rings, whereas the submetacentric one usually

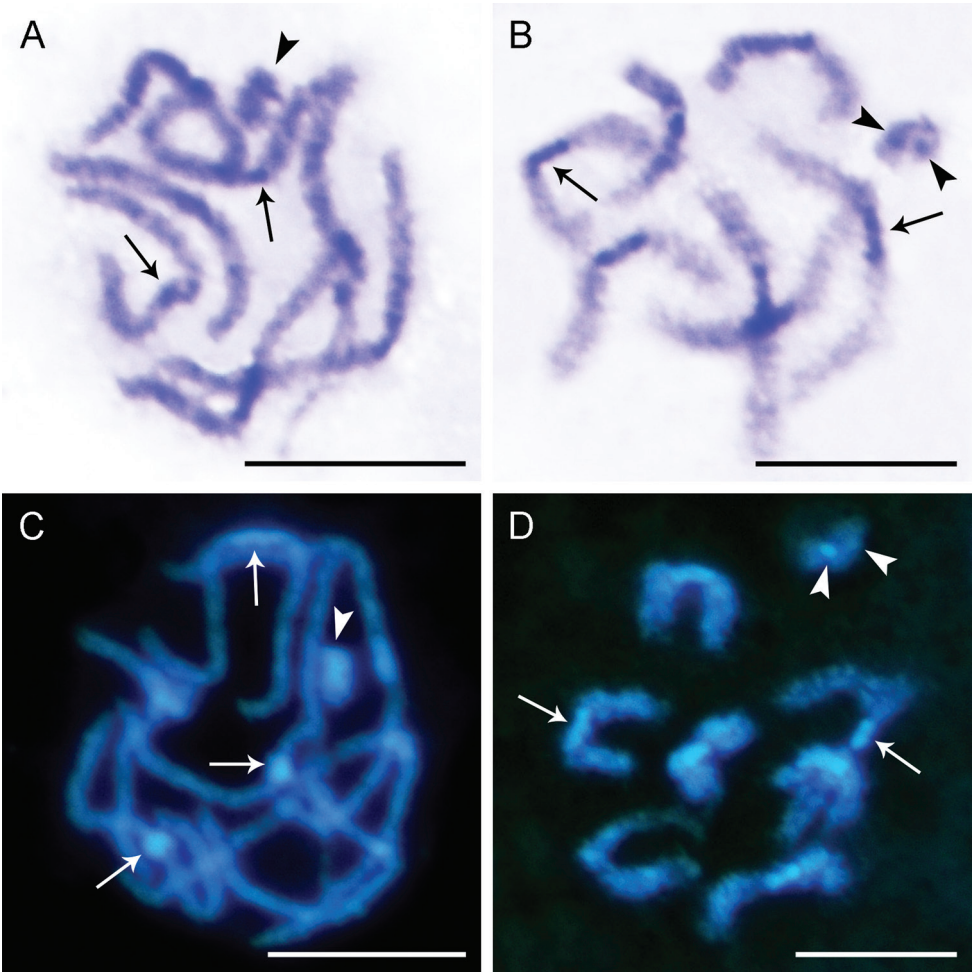


Figure 2. Pachytene bivalents of *Bittacus sinicus*, stained with C-banding (**A, B**) and DAPI (**C, D**) **A, C** early pachytene, showing the intermediate heterochromatin on bivalents and the heteropycnotic sex chromosome (arrowhead) **B, D** late pachytene, showing the sex chromosome with a dot-shaped heterochromatic block (arrowheads). Arrows point to the intermediate heterochromatin. Scale bars: 10 μm.

contains only one terminal chiasma at the long-arm side as a long rod-shape. Chiasmata can be clearly visible after some condensation of the chromosomes at diakinesis (Fig. 3C). In *B. sinicus* the mean chiasma count per cell was 13.2 (50 cells, ranging from 13 to 14).

Bivalents assemble at the equatorial plate in metaphase I (Fig. 3D) and become oriented with their centromeres poleward (Fig. 3E). In *B. sinicus* the rod-shaped bivalent is bound by one chiasma at one arm end (asterisk in Fig. 3F), whereas the ring-shaped bivalents have both arms bound by chiasmata. The autosomal bivalents separate into dyads, whereas the X univalent moves undividedly to one pole (Fig. 3G–I), indicating that *B. sinicus* has the initial-/prereductional meiosis. Each dyad consists of two divergent chromatids associated only in the regions proximal to the centromere (Fig. 3G,

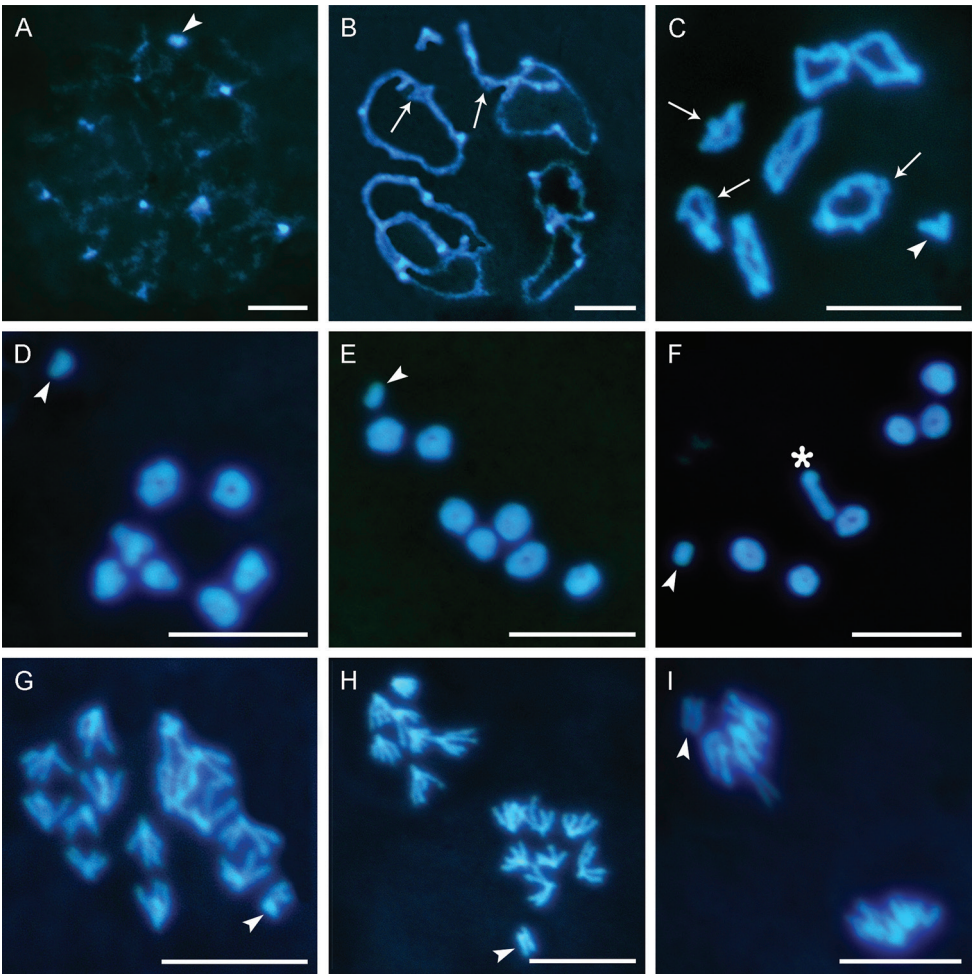


Figure 3. Meiosis I of *Bittacus sinicus* **A** diffuse diplotene with the condensed sex chromosome and decondensed bivalents **B** diplotene, showing the bivalents are held together only at exchange points (arrows) **C** diakinesis, showing the evident chiasmata (arrows) **D** bivalents assembling at the equatorial plate in metaphase I (polar view) **E, F** metaphase I in side view, showing the ring-shaped bivalents with two chiasmata and rod-shaped bivalent with one terminal chiasma (asterisk) **G** anaphase disjunction, showing the divided bivalents and the undivided sex chromosome **H** anaphase I, showing the chromosome number of *B. sinicus* is $2n = 15$ **I** telophase I. Arrowheads show the sex chromosome. Scale bars: 10 μm.

H). Both submetacentric and metacentric dyads are four armed with a double V-shape in anaphase I. The dyads reach the opposite poles and fuse into an indistinguishable mass of chromatin in telophase I (Fig. 3I).

Meiosis II takes place immediately after the first meiotic division. The movement of the X univalent toward only one pole at anaphase I leads to the formation of two classes of nuclei (Fig. 4A, B). The sister chromatids of each dyad are widely splayed, but are held together at the centromere in prometaphase II (Fig. 4C). The centromeric

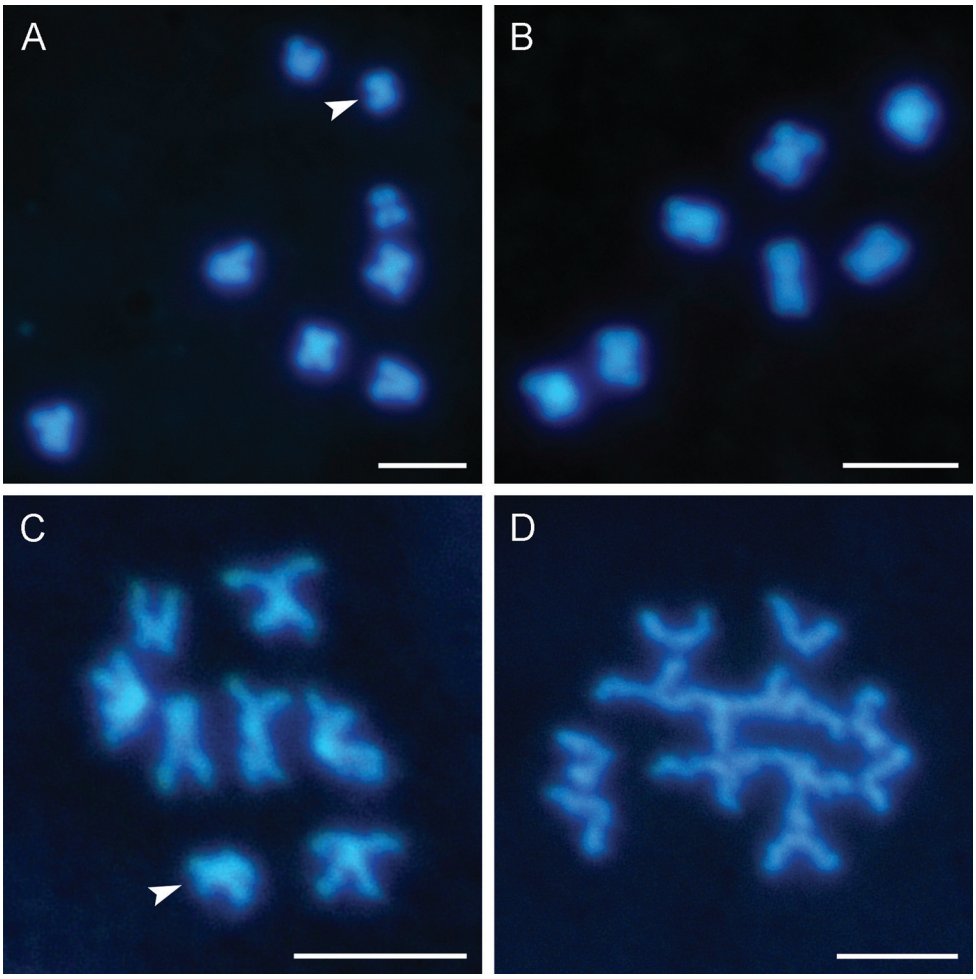


Figure 4. Meiosis II of *Bittacus sinicus* **A, B** the secondary spermatocytes: **A** with $n = 8$ **B** with $n = 7$ **C** prometaphase II, showing the striking repulsion between the sister chromatids of each dyad chromosome **D** anaphase II, showing the separation of sister chromatids. Arrowheads show the sex chromosome. Scale bars: 5 μm.

cohesion between the two sister chromatids is removed in anaphase II, and the sister chromatids are pulled apart by microtubules attached to the kinetochore (Fig. 4D).

Sex chromosome system

The diploid somatic chromosome number ($2n$) is reduced to the haploid gametic chromosome number (n) during the first meiosis. Both the autosomes and the sex chromosome exhibit pre-reductional type of meiosis. The haploid chromosome numbers are different between the two daughter nuclei with $n = 7 + X$ (Fig. 4A) and $n = 7$ (Fig. 4B), indicating an X0 sex system of the male *B. sinicus*.

Discussion

The present study is the first attempt to investigate the karyotype and male meiosis of *B. sinicus*. As in other bittacids studied previously, *B. sinicus* has the chiasmate meiosis and the X0(♂) sex determination mechanism, which are likely the plesiomorphies in Bittacidae (Matthey 1950; Atchley and Jackson 1970; Miao and Hua 2017, 2019).

Bittacus sinicus has the lowest chromosome number $2n = 15$ ever observed in Mecoptera. Previously, $2n = 17$ chromosomes recorded for *Nannochorista dipteroides* Tillyard, 1917 (Nannochoristidae) was considered the lowest number reported for this order (Bush 1966). Despite limited chromosome data available, the chromosome number exhibits considerable variations among the families of Mecoptera, from $2n = 15$ to 41 in Bittacidae, $2n = 19$ to 31 in Boreidae (Cooper 1951, 1974), $2n = 17$ to 27 in Nannochoristidae (Bush 1966), and $2n = 35$ to 47 in Panorpidae (Naville and Beaumont 1934; Ullerich 1961; Atchley and Jackson 1970; Xu et al. 2013; Miao et al. 2017, 2019).

In Bittacidae, each species examined has a distinctive karyotype, and the two genera (*Bittacus* and *Terrobittacus* Tan et Hua, 2009) investigated are distinguishable cytogenetically. *Bittacus* has relatively low chromosome numbers and symmetric karyotypes, while *Terrobittacus* has a higher chromosome number and less symmetric karyotype (Miao and Hua 2017), suggesting that the chromosomal changes may have participated in the lineage differentiation of Bittacidae.

Interestingly, the sex chromosome is the smallest element in the karyotype of *B. sinicus*, but is larger than the majority of autosomes in other bittacids studied (Miao and Hua 2017, 2019). Therefore, we speculate that autosome-autosome fusions may contribute to the karyotype formation in *B. sinicus*. Similar rearrangements are also suggested for some recently differentiated species of the scorpionflies Panorpidae (Miao et al. 2019). A notable example is *Neopanorpa lipingensis* Cai et Hua, 2009, which has a distinct chromosome number of $2n = 33$, not $2n = 41$ found in most members of *Neopanorpa* van der Weele, 1909, indicating that fusion events occurred at least eight times among the autosomes.

The C-banding pattern of *B. sinicus* is represented by intermediate blocks on pachytene bivalents and is definitely different from the heterochromatic segment at one bivalent terminal in other bittacids (Atchley and Jackson 1970; Miao and Hua 2017, 2019), implying that inversions may participate in the changes of chromosome morphology.

Conspicuous bands are detectable on pachytene bivalents using the DAPI staining. In general, the terminal DAPI$^+$ (AT-rich) heterochromatin at one side of a bivalent is the most frequent pattern, which has been observed in the majority of Panorpidae and Bittacidae investigated (Miao and Hua 2017, 2019; Miao et al. 2019). In *B. sinicus*, however, the DAPI$^+$ bands are present in the intermediate regions of all bivalents (Fig. 2C, D). Bivalents with intermediate DAPI$^+$ heterochromatin were also found in the species of *Neopanorpa* and were considered as important evidence for the evolutionary reduction of chromosome number in Panorpidae (Miao et al. 2019).

Two alternative hypotheses (fission and fusion) can explain the karyotype formation in the genus *Bittacus*. The fission hypothesis assumes that the cytogenetic features of *B. sinicus* are primitive with a low chromosome number, relatively large autosomes and reduced heterochromatin. The karyotype changes of *Bittacus* (Miao and Hua

2017, 2019) are similar to those of ants and wasps, in which the centric fissions tend to increase the chromosome number and accumulate chromatin (mainly heterochromatin) (Imai et al. 1986, 1994, 2001).

Alternatively, the fusion hypothesis may also explain the karyotype variations found in *Bittacus*. The karyotype of *B. sinicus* is considered the derived condition and is shaped by Robertsonian translocations of acrocentric chromosomes and/or reciprocal translocations between meta-/submetacentric and acrocentric ones, which are generated by pericentric inversions. During the translocation events, small centromeric chromosomes (in addition to the final fused chromosomes) may be produced and lost within a few cell cycles. Such scenarios may explain the elimination of centromeres and heterochromatin toward the *B. sinicus* karyotype, and has been suggested for many monocentric organisms, such as the plant *Arabidopsis thaliana* (Linnaeus, 1758) (Lysak et al. 2006), the flatworm *Aspidogaster limacoides* Diesing, 1834 (Bombarová et al. 2015), the pangolin *Manis javanica* (Desmarest, 1822) (Nie et al. 2009), the mouse *Akodon* Meyen, 1833 (Ventura et al. 2009), the grasshopper *Ronderosia* Cigliano, 1997 (Orthoptera, Acrididae) (Castillo et al. 2019), the beetle *Dichotomius* Hope, 1838 (Coleoptera, Scarabaeidae) (Cabral-de-Mello et al. 2011), and the ants Myrmicinae (Cardoso et al. 2014). Based on the phylogeny of the Chinese Bittacidae (YM, unpublished data), we speculate that the cytogenetic features observed in *B. sinicus* may be derived conditions, including the low number of chromosomes, relatively large sizes of autosomes and the intermediate distribution of heterochromatin.

Chromosome rearrangements are proposed as an important driving force of diversification since they lead to speciation via formation of reproductive incompatibility or recombination suppression (Navarro and Barton 2003; Ayala and Coluzzi 2005; Butlin 2005; Kandul et al. 2007; Brown and O'Neill 2010; Kirkpatrick 2010; Mills and Cook 2014). According to the models of chromosomal speciation, there is an increasing level of divergence near rearrangement breakpoints, which tend to accumulate alleles involved in the reproductive isolation (Coghlan et al. 2005; Faria and Navarro 2010). In *Bittacus*, the cytogenetic data available indicate that the chromosomal evolution involves progressive changes in chromosome number and karyotype structure. However, it remains unclear whether these chromosomal rearrangements are an integral component and driving force of the speciation process or they are established later, after speciation is completed. Further investigations of additional species, combined with molecular phylogeny and fluorescent in situ hybridization (telomere and 18S rDNA probes), are needed to shed more light on this issue.

Acknowledgements

We are grateful to Lu Liu and Ning Li for assistance in specimen collection. We also thank Qiong-Hua Gao and Wei Du for species identification. We express our special thanks to Lorenzo Peruzzi and Rodolpho Menezes for their valuable comments and suggestions in the revision of the manuscript. This study was funded by the National Natural Science Foundation of China (grant number 31672341) and the China Postdoctoral Science Foundation (grant number 2019M663830).

References

Astuti G, Roma-Marzio F, Peruzzi L (2017) Traditional cytotaxonomic studies: can they still provide a solid basis in plant systematics? Flora Mediterranea 27: 91–98.

Atchley WR, Jackson RC (1970) Cytological observations on spermatogenesis in four species of Mecoptera. Canadian Journal of Genetics and Cytology 12(2): 264–272. https://doi.org/10.1139/g70-039

Ayala FJ, Coluzzi M (2005) Chromosome speciation: humans, *Drosophila*, and mosquitoes. Proceedings of the National Academy of Sciences of the United States of America 102(suppl. material 1): 6535–6542. https://doi.org/10.1073/pnas.0501847102

Bombarová M, Špakulová M, Kello M, Nguyen P, Bazsalovicsová E, Králová-Hromadová I (2015) Cytogenetics of *Aspidogaster limacoides* (Trematoda, Aspidogastrea): karyotype, spermatocyte division, and genome size. Parasitology Research 114(4): 1473–1483. https://doi.org/10.1007/s00436-015-4330-5

Bornemissza GF (1966) Observations on the hunting and mating behaviours of two species of scorpion flies (Bittacidae: Mecoptera). Australian Journal of Zoology 14(3): 371–382. https://doi.org/10.1071/ZO9660371

Brown JD, O'Neill RJ (2010) Chromosomes, conflict, and epigenetics: chromosomal speciation revisited. Annual Review of Genomics and Human Genetics 11: 291–316. https://doi.org/10.1146/annurev-genom-082509-141554

Bush GL (1966) The comparative cytology of the Choristidae and Nannochoristidae (Mecoptera). American Philosophical Society Yearbook 1966: 326–328

Butlin RK (2005) Recombination and speciation. Molecular Ecology 14(9): 2621–2635. https://doi.org/10.1111/j.1365-294X.2005.02617.x

Byers GW, Thornhill R (1983) Biology of the Mecoptera. Annual Review of Entomology 28: 203–228. https://doi.org/10.1146/annurev.en.28.010183.001223

Cabral-de-Mello DC, Moura RC, Martins C (2011) Cytogenetic mapping of rRNAs and histone H3 genes in 14 species of *Dichotomius* (Coleoptera, Scarabaeidae, Scarabaeinae) beetles. Cytogenetic and Genome Research 134: 127–135. https://doi.org/10.1159/000326803

Cardoso DC, das Graças Pompolo S, Cristiano MP, Tavares MG (2014) The role of fusion in ant chromosome evolution: insights from cytogenetic analysis using a molecular phylogenetic approach in the genus *Mycetophylax*. PLoS ONE 9(1): e87473. https://doi.org/10.1371/journal.pone.0087473

Castillo ERD, Martí DA, Maronna MM, Scattolini MC, Cabral-de-Mello DC, Cigliano MM (2019) Chromosome evolution and phylogeny in *Ronderosia* (Orthoptera, Acrididae, Melanoplinae): clues of survivors to the challenge of sympatry? Systematic Entomology 44(1): 61–74. https://doi.org/10.1111/syen.12317

Chen J, Tan J-L, Hua B-Z (2013) Review of the Chinese *Bittacus* (Mecoptera: Bittacidae) with descriptions of three new species. Journal of Natural History 47(21–22): 1463–1480. https://doi.org/10.1080/00222933.2012.763065

Coghlan A, Eichler EE, Oliver SG, Paterson AH, Stein L (2005) Chromosome evolution in eukaryotes: a multi-kingdom perspective. Trends in Genetics 21(12): 673–682. https://doi.org/10.1016/j.tig.2005.09.009

Cooper KW (1951) Compound sex chromosomes with anaphasic precocity in the male mecopteran, *Boreus brumalis* Fitch. Journal of Morphology 89(1): 37–57. https://doi.org/10.1002/jmor.1050890104

Cooper KW (1974) Sexual biology, chromosomes, development, life histories and parasites of *Boreus*, especially of *B. notoperates*. A southern California *Boreus*. II. (Mecoptera: Boreidae). Psyche 81: 84–120. https://doi.org/10.1155/1974/48245

Dincă V, Lukhtanov VA, Talavera G, Vila R (2011) Unexpected layers of cryptic diversity in wood white *Leptidea* butterflies. Nature Communications 2: 324. https://doi.org/10.1038/ncomms1329

Dutrillaux A-M, Dutrillaux B (2019) Different behaviour of C-banded peri-centromeric heterochromatin between sex chromosomes and autosomes in polyphagan beetles. Comparative Cytogenetics 13(2): 179–192. https://doi.org/10.3897/CompCytogen.v13i2.34746

Faria R, Navarro A (2010) Chromosomal speciation revisited: rearranging theory with pieces of evidence. Trends in Ecology & Evolution 25(11): 660–669. https://doi.org/10.1016/j.tree.2010.07.008

Gokhman VE, Kuznetsova VG (2006) Comparative insect karyology: current state and applications. Entomological Review 86(3): 352–368. https://doi.org/10.1134/S0013873806030110

Imai HT, Maruyama T, Gojobori T, Inoue Y, Crozier RH (1986) Theoretical bases for karyotype evolution. 1. The minimum-interaction hypothesis. The American Naturalist 128(6): 900–920. https://doi.org/10.1086/284612

Imai HT, Satta Y, Takahata N (2001) Integrative study on chromosome evolution of mammals, ants and wasps based on the minimum interaction theory. Journal of Theoretical Biology 210(4): 475–497. https://doi.org/10.1006/jtbi.2001.2327

Imai HT, Taylor RW, Crosland MW, Crozier RH (1988) Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. The Japanese Journal of Genetics 63(2): 159–185. https://doi.org/10.1266/jjg.63.159

Imai HT, Taylor RW, Crozier RH (1994) Experimental bases for the minimum interaction theory. I. Chromosome evolution in ants of the *Myrmecia pilosula* species complex (Hymenoptera: Formicidae: Myrmeciinae). The Japanese Journal of Genetics 69(2): 137–182. https://doi.org/10.1266/jjg.69.137

Kandul NP, Lukhtanov VA, Pierce NE (2007) Karyotypic diversity and speciation in *Agrodiaetus* butterflies. Evolution 61(3): 546–559. https://doi.org/10.1111/j.1558-5646.2007.00046.x

Kirkpatrick M (2010) How and why chromosome inversions evolve. PLoS Biology 8(9): e1000501. https://doi.org/10.1371/journal.pbio.1000501

Kociński M, Grzywacz B, Chobanov D, Warchałowska-Śliwa E (2018) New insights into the karyotype evolution of the genus *Gampsocleis* (Orthoptera, Tettigoniinae, Gampsocleidini). Comparative Cytogenetics 12(4): 529–538. https://doi.org/10.3897/CompCytogen.v12i4.29574

Lambkin KJ (1988) An Australian species of the genus *Bittacus* Latreille (Mecoptera: Bittacidae). Memoirs of the Queensland Museum 25(2): 439–444. https://biodiversitylibrary.org/page/43242356

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52(2): 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Li Y-L, Ren D (2009) History and development of researches on Bittacidae (Insecta: Mecoptera). Acta Geoscientica Sinica 30(4): 554–560.

Lukhtanov VA, Kandul NP, Plotkin JB, Dantchenko AV, Haig D, Pierce NE (2005) Reinforcement of pre-zygotic isolation and karyotype evolution in *Agrodiaetus* butterflies. Nature 436(7049): 385–389. https://doi.org/10.1038/nature03704

Lysak MA, Berr A, Pecinka A, Schmidt R, McBreen K, Schubert I (2006) Mechanisms of chromosome number reduction in *Arabidopsis thaliana* and related Brassicaceae species. Proceedings of the National Academy of Sciences of the United States of America 103(13): 5224–5229. https://doi.org/10.1073/pnas.0510791103

Ma N, Huang J, Hua B-Z (2014) Fine structure and functional morphology of the mouthparts of *Bittacus planus* and *Terrobittacus implicatus* (Insecta: Mecoptera: Bittacidae). Zoologischer Anzeiger 253(6): 441–448. https://doi.org/10.1016/j.jcz.2014.05.001

Matthey R (1950) La formule chromosomique et le type de digamétie chez *Bittacus italicus* Müll. (Mecoptera). Archiv der Julius-Klaus-Stiftung für Vererbungs Forschung 25: 605–611

Menezes RST, Gazoni T, Costa MA (2019) Cytogenetics of warrior wasps (Vespidae: *Synoeca*) reveals intense evolutionary dynamics of ribosomal DNA clusters and an unprecedented number of microchromosomes in Hymenoptera. Biological Journal of the Linnean Society 126(4): 925–935. https://doi.org/10.1093/biolinnean/bly210

Miao Y, Hua B-Z (2017) Cytogenetic comparison between *Terrobittacus implicatus* and *Bittacus planus* (Mecoptera: Bittacidae) with some phylogenetic implications. Arthropod Systematics & Phylogeny 75(2): 175–183. http://www.senckenberg.de/files/content/forschung/publikationen/arthropodsystematics/asp_75_2/01_asp_75_2_miao_175-183.pdf

Miao Y, Hua B-Z (2019) Chromosomal characteristics of the hangingfly *Bittacus flavidus* Huang & Hua (Mecoptera: Bittacidae) and their phylogenetic implications. Acta Entomologica Sinica 62(6): 732–742. https://doi.org/10.1007/s00709-019-01415-w

Miao Y, Ma N, Hua B-Z (2017) Cytotaxonomy and molecular phylogeny of the genus *Cerapanorpa* Gao, Ma & Hua, 2016 (Mecoptera: Panorpidae). Scientific Reports 7: 4493. https://doi.org/10.1038/s41598-017-04926-9

Miao Y, Wang J-S, Hua B-Z (2019) Molecular phylogeny of the scorpionflies Panorpidae (Insecta: Mecoptera) and chromosomal evolution. Cladistics 35: 385–400. https://doi.org/10.1111/cla.12357

Mills PJ, Cook LG (2014) Rapid chromosomal evolution in a morphologically cryptic radiation. Molecular Phylogenetics and Evolution 77: 126–135. https://doi.org/10.1016/j.ympev.2014.03.015

Navarro A, Barton NH (2003) Accumulating postzygotic isolation genes in parapatry: a new twist on chromosomal speciation. Evolution 57(3): 447–459. https://doi.org/10.1111/j.0014-3820.2003.tb01537.x

Naville A, Beaumont J (1934) Les chromosomes des Panorpes. Bulletin Biologique de la France et de la Belgique 68: 98–107

Nie W-H, Wang J-H, Su W-T, Wang Y-X, Yang F-T (2009) Chromosomal rearrangements underlying karyotype differences between Chinese pangolin (*Manis pentadactyla*) and Malayan pangolin (*Manis javanica*) revealed by chromosome painting. Chromosome Research 17(3): 321–329. https://doi.org/10.1007/s10577-009-9027-0

Nokkala C, Kuznetsova VG, Rinne V, Nokkala S (2019) Description of two new species of the genus *Cacopsylla* Ossiannilsson, 1970 (Hemiptera, Psylloidea) from northern Fennoscandia

recognized by morphology, cytogenetic characters and *COI* barcode sequence. Comparative Cytogenetics 13(4): 367–382. https://doi.org/10.3897/CompCytogen.v13i4.47395

Noor MAF, Garfield DA, Schaeffer SW, Machado CA (2007) Divergence between the *Drosophila pseudoobscura* and *D. persimilis* genome sequences in relation to chromosomal inversions. Genetics 177(3): 1417–1428. https://doi.org/10.1534/genetics.107.070672

Paszko B (2006) A critical review and a new proposal of karyotype asymmetry indices. Plant Systematics and Evolution 258(1): 39–48. https://doi.org/10.1007/s00606-005-0389-2

Penny ND (1975) Evolution of the extant Mecoptera. Journal of the Kansas Entomological Society 48(3): 331–350

Penny ND (2006) A review of our knowledge of California Mecoptera. Proceedings of the California Academy of Sciences 57(9): 365–372

Penny ND, Byers GW (1979) A check-list of the Mecoptera of the world. Acta Amazonica 9(2): 365–388. https://doi.org/10.1590/1809-43921979092365

Pereira TTP, Reis ACCC, Cardoso DC, Cristiano MP (2018) Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger, 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera). Comparative Cytogenetics 12(1): 13–26. https://doi.org/10.3897/CompCytogen.v12i1.21799

Peruzzi L, Eroğlu HE (2013) Karyotype asymmetry: again, how to measure and what to measure? Comparative Cytogenetics 7(1): 1–9. https://doi.org/10.3897/compcytogen.v7i1.4431

Rebagliati PJ, Papeschi AG, Mola LM (2003) Meiosis and fluorescent banding in *Edessa meditabunda* and *E. rufomarginata* (Heteroptera: Pentatomidae: Edessinae). European Journal of Entomology 100(1): 11–18. https://doi.org/10.14411/eje.2003.002

Rieseberg LH, Burke JM (2001) A genic view of species integration. Journal of Evolutionary Biology 14(6): 883–886. https://doi.org/10.1046/j.1420-9101.2001.00339.x

Stebbins GL (1971) Chromosomal Evolution in Higher Plants. Edward Arnold, London, 216 pp.

Stoianova D, Simov N, Vu MQ, Nguyen DM, Grozeva S (2020) New data on karyotype, spermatogenesis and ovarian trophocyte ploidy in three aquatic bug species of the families Naucoridae, Notonectidae, and Belostomatidae (Nepomorpha, Heteroptera). Comparative Cytogenetics 14(1): 139–156. https://doi.org/10.3897/CompCytogen.v14i1.48709

Tan J-L, Hua B-Z (2008) Structure of raptorial legs in *Bittacus* (Mecoptera : Bittacidae). Acta Entomologica Sinica 51(7): 745–752. http://www.insect.org.cn/EN/Y2008/V51/I7/745

Thornhill R (1977) The comparative predatory and sexual behavior of hangingflies (Mecoptera: Bittacidae). Occasional Papers of the Museum of Zoology University of Michigan 677: 1–43. http://deepblue.lib.umich.edu/bitstream/2027.42/57113/1/OP677.pdf

Ullerich FH (1961) Achiasmatische spermatogenese bei der skorpionsfliege *Panorpa* (Mecoptera). Chromosoma 12(1): 215–232. https://doi.org/10.1007/BF00328920

Ventura K, O'Brien PCM, Yonenaga-Yassuda Y, Ferguson-Smith MA (2009) Chromosome homologies of the highly rearranged karyotypes of four Akodon species (Rodentia, Cricetidae) resolved by reciprocal chromosome painting: the evolution of the lowest diploid number in rodents. Chromosome Research 17(8): 1063–1078. https://doi.org/10.1007/s10577-009-9083-5

White MJD (1974) Genetic Mechanisms of Speciation in Insects. Springer, Dordrecht, 170 pp. https://doi.org/10.1007/978-94-010-2248-4

Xu B, Li Y-K, Hua B-Z (2013) A chromosomal investigation of four species of Chinese Panorpidae (Insecta, Mecoptera). Comparative Cytogenetics 7(3): 229–239. https://doi.org/10.3897/compcytogen.v7i3.5500

Zhang Y-N, Du W, Hua B-Z (2020) Three new species of the genus *Bittacus* Latreille, 1805 (Mecoptera: Bittacidae), with a key to the species of Bittacidae in South China. Zootaxa 4718(3): 381–390. https://doi.org/10.11646/zootaxa.4718.3.6

CompCytogen 14(3): 369–385 (2020)
doi: 10.3897/CompCytogen.v14i3.52726
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 FISH-mapping of TTAGG telomeric sequences to the chromosomes of leafcutter ants (Formicidae, Myrmicinae): is the insect canonical sequence conserved?

Carini Picardi Morais de Castro[1,2], Danon Clemes Cardoso[1], Ricardo Micolino[3], Maykon Passos Cristiano[1]

1 *Departamento de Biodiversidade, Evolução e Meio Ambiente, Universidade Federal de Ouro Preto (UFOP), MG, Brazil* **2** *Departament de Genètica, Facultat de Biologia, Universitat de Barcelona (UB), Barcelona, Spain* **3** *Programa de Pós-graduação em Genética, Universidade Federal do Paraná (UFPR), Curitiba, PR, Brazil*

Corresponding author: Danon Clemes Cardoso (danon@ufop.edu.br); Maykon Passos Cristiano (maykon@ufop.edu.br)

Academic editor: Marco Gebiola | Received 30 March 2020 | Accepted 30 May 2020 | Published 14 August 2020

http://zoobank.org/02AEB841-9BD3-4C95-B03F-36B062B6D1B2

Citation: de Castro CPM, Cardoso DC, Micolino R, Cristiano MP (2020) Comparative FISH-mapping of TTAGG telomeric sequences to the chromosomes of leafcutter ants (Formicidae, Myrmicinae): is the insect canonical sequence conserved?. Comparative Cytogenetics 14(3): 369–385. https://doi.org/10.3897/CompCytogen.v14i3.52726

Abstract

Telomeric sequences are conserved across species. The most common sequence reported among insects is $(TTAGG)_n$, but its universal occurrence is not a consensus because other canonical motifs have been reported. In the present study, we used fluorescence *in situ* hybridization (FISH) using telomeric probes with $(TTAGG)_6$ repeats to describe the telomere composition of leafcutter ants. We performed the molecular cytogenetic characterization of six *Acromyrmex* Mayr, 1865 and one *Atta* Fabricius, 1804 species (*Acromyrmex ambiguus* (Emery, 1888), *Ac. crassispinus* (Forel, 1909), *Ac. lundii* (Guérin-Mèneville, 1838), *Ac. nigrosetosus* (Forel, 1908), *Ac. rugosus* (Smith, 1858), *Ac. subterraneus subterraneus* (Forel, 1893), and *Atta sexdens* (Linnaeus, 1758)) and described it using a karyomorphometric approach on their chromosomes. The diploid chromosome number 2n = 38 was found in all *Acromyrmex* species, and the karyotypic formulas were as follows: *Ac. ambiguus* 2K = 14M + 12SM + 8ST + 4A, *Ac. crassispinus* 2K = 12M + 20SM + 4ST + 2A, *Ac. lundii* 2K = 10M + 14SM + 10ST + 4A, *Ac. nigrosetosus* 2K = 12M + 14SM + 10ST + 2A, and *Ac. subterraneus subterraneus* 2K = 14M + 18SM + 4ST + 2A. The exact karyotypic formula was not established for *Ac. rugosus*. FISH analyses revealed the telomeric regions in all the chromosomes of the species studied in the present work were marked by the $(TTAGG)_6$ sequence. These results reinforce the premise that Formicidae presents high homology between their genera for the presence of the canonical sequence $(TTAGG)_n$.

Keywords
evolution, FISH, insects, leafcutter ants, telomere

Introduction

Cytogenetic studies have been performed on more than 750 ant species, most of which describe only the chromosome number and morphology (Lorite and Palomeque 2010; Cardoso et al. 2018a). However, the cytogenetic information available so far represents less than 5% of the known ant species. Formicidae is very diverse with respect to both karyotype and species. The subfamily Myrmicinae comprises more than 400 species with established karyotypes and haploid chromosome counts varying from n = 2 to n = 35 (Cardoso et al. 2018a). Myrmicinae includes the leafcutter ants in the genera *Atta* Fabricius, 1804 to *Acromyrmex* Mayr, 1865 that occur exclusively in the Neotropical region and are extremely important herbivores in the habitats that they occupy. They cut thousands of fresh plant pieces that are transported to nests and this habit is essential for cycling soil nutrients, mainly carbon (Farji-Brener and Ghermandi 2008). In some cases, *Atta* and *Acromyrmex* are considered agricultural pests due to the economic damages caused by their habit of cutting green leaves; therefore, most studies usually focus on their ecology, geographic distribution, and population control (Loeck et al. 2003). However, both genera need a systematic revision and a complete picture of their unclear phylogenetic relationships.

The genus *Atta* includes 17 species (Bolton 2020), of which five have an established karyotype. All species present the diploid chromosome number, 2n = 22, and the karyotype formula, 2K = 18M + 4A, except for *Atta robusta* Borgmeier, 1939, which has the formula 2K = 18M + 2SM + 2ST (reviewed in Cardoso et al. 2018a). The genus *Acromyrmex* has 34 species and 29 subspecies that are currently recognized (Bolton 2020), it has the diploid chromosome number 2n = 38 and its karyotype formula is variable (Barros et al. 2016; reviewed in Cardoso et al. 2018a). The exceptions in the genus are *Acromyrmex ameliae* de Souza, Soares & Della Lucia, 2007, that has 2n = 36 (reviewed in Cardoso et al. 2018a) and *Acromyrmex striatus* (Roger, 1863) which presents 2n = 22 (Cristiano et al. 2013). The only species whose karyotype has been characterized by morphometric analyses so far is *Ac. striatus* (Cristiano et al. 2013). Such chromosomal features are essential for understanding chromosomal variants and the possible genetic barriers among phylogenetic groups (Cardoso et al. 2018b). *Ac. striatus* is a key species within the evolutionary history of leafcutter ants because molecular analyses and its karyotype establishment resulted in reclassification of *Acromyrmex* as paraphyletic. Although *Ac. striatus* shares the characteristics of both *Acromyrmex* and *Atta*, it presents peculiarities such as its karyotype formula 2K = 20M + 2SM, indicating that *Ac. striatus* should be better classified as a genus distinct from its sibling leafcutter ants (Cristiano et al. 2013).

Karyo-evolutionary pathways can be accurately established from molecular analyses by means of fluorescence *in situ* hybridization (FISH), a chromosomal mapping

technique that allows identification of specific genomic regions through hybridization of fluorescent probes to the genetic material (Speicher and Carter 2005). Probe origin may range from single or repetitive sequences to large genomic sequences and probes from telomeric repeating regions are commonly applied in cytogenetic studies (Micolino et al. 2019a, b, 2020; Travenzoli et al. 2019). Telomeres are located at terminal portions of chromosomes, which are enriched with repetitive bases of adenine (A), guanine (G), and thymine (T) and the number of repeated base pairs can be extremely conserved among some taxonomic groups (Blackburn 1991; Zakian 1995). Four different telomeric sequences have been identified in Insecta, but the pentanucleotide region $(TTAGG)_n$ is present in most insects (Okazaki et al. 1993; Sahara et al. 1999). Thus, it is presumed that this motif is derived from a common ancestor and is therefore homologous among the class orders (Vítková et al. 2005). However, many Hymenoptera families do not present the sequence in their chromosomes (Menezes et al. 2017), whereas some families have several species that show telomeric regions marked by the presence of $(TTAGG)_n$ or the vertebrate canonical repetition $(TTAGGG)_n$, as in the case of Apidae (Sahara et al. 1999), Formicidae (Okazaki et al. 1993; Meyne et al. 1995; Lorite et al. 2002; Wurm et al. 2011) and Tenthredinidae, which has two species presenting the insect canonical sequence (Gokhman and Kuznetsova 2018).

The pentanucleotide sequence has apparently evolved from the canonical sequence $(TTAGGG)_n$ and has changed during insect diversification. This is supported by families that show the presence of $(TTAGGG)_n$ and also by genera which present a different telomeric sequence such as $(TCAGG)_n$, which is observed in some Coleoptera families (Kuznetsova et al. 2019). The differences in telomeric sequences within the class Insecta can be explained by biological mechanisms that preserve the telomere integrity. Telomerase is the enzyme responsible for maintaining repetitive sequences on telomeres; however, many alternative telomerase-independent mechanisms also act in telomere conservation. In this manner, the $(TTAGG)_n$ sequence has been lost and recovered several times during the evolution of insects (Kuznetsova et al. 2019).

Other than chromosome number, not much cytogenetic information is available regarding leafcutter ants, and FISH analyses involving telomeric probes are available only for *Ac. striatus* (Pereira et al. 2018). Further, the distribution of canonical repeats and telomerase systems is still an open question among insects (Kuznetsova et al. 2019). Thus, in the present study, we analyzed the homology between the telomeric regions of leafcutter ant species *Ac. ambiguus* (Emery, 1888), *Ac. crassispinus* (Forel, 1909), *Ac. lundii* (Guérin-Mèneville, 1838), *Ac. nigrosetosus* (Forel, 1908), *Ac. rugosus* (Smith, 1858), *Ac. subterraneus subterraneus* (Forel, 1893), and *Atta sexdens* (Linnaeus, 1758) by FISH chromosome mapping using the $(TTAGG)_6$ probe. We aimed to accumulate evolutionary evidence for the presence of an insect canonical telomere motif on the chromosomes of leafcutter ants. We further performed a detailed karyomorphometric analysis to establish karyotypes and classify chromosome, and described two new chromosome counts.

Material and methods

Chromosome preparation and fluorescence *in situ* hybridization (FISH)

The ant colonies were collected from different Brazilian states in 2018. *Acromyrmex ambiguus* was collected from Ilha Comprida – SP (24°44'28"S, 47°32'24"W); the species *Ac. crassispinus* (Ouro Preto – 20°17'15"S, 43°30'29"W), *Ac. rugosus* (Marliéria – 19°43'21"S, 42°43'26"W), *Ac. nigrosetosus* (Ouro Preto – 20°17'15"S, 43°30'29"W), *Ac. subterraneus subterraneus* (Viçosa – 20°48'35.5"S, 42°51'31.07"W), and *At. sexdens* (Marliéria – 19°43'21"S, 42°43'26"W) were collected in Minas Gerais – MG; *Ac. lundii* was collected in Dom Pedrito – RS (30°58'5"S, 54°40'23"W). The nests were kept at the Laboratório de Genética Evolutiva e de Populações of the Universidade Federal de Ouro Preto. The brain ganglia of post-defective larvae were extracted in hypotonic solution of colchicine (0.005%), as described by Imai et al. (1988) with modifications described by Cardoso et al. (2012), to obtain the metaphasic chromosomes.

FISH experiments were performed as described by Micolino et al. (2019a). The $(TTAGG)_6$ motif was directly labeled with Cy3 at the 5' terminal (Sigma, St. Louis, MO, USA). Briefly, slides were submitted to RNA degradation for 1 h in a humid chamber at 37 °C, were washed in 2× SSC, and treated with 0.005% pepsin for 10 min. After washing in 1× PBS, the slides were fixed with 10% formaldehyde for 10 min. Another wash in 1× PBS was performed and then, the slides were dehydrated in an alcohol series. Chromosomal denaturation was promoted by adding 70% formamide at 75 °C for 5 min. Another alcohol dehydration series was performed before adding 2 μL of the $(TTAGG)_6$ probe and 18 μL of HybMix to each slide in the dark. The slides were incubated overnight in a humid chamber at 37 °C. Finally, the slides were washed in 2× SSC solution, 1× SSC, 4× SSC Tween (during 5 min in each solution), and then rapidly in 1× PBS. Dehydration was performed in an alcohol series and DAPI was added as a counterstain. To select 10 metaphases with chromosomal integrity and evident probe marking, the slides were visualized on a Zeiss Axio Imager Z2 fluorescence microscope coupled with an image capture system and the resulting images were further edited using Adobe Photoshop CC Software.

Karyomorphometry

The slides were stained with a 4% Giemsa solution and visualized on a Zeiss Axio Imager Z2 microscope with image capture. For each species, we selected 10 metaphases with chromosomal integrity, evident centromeres and no overlapping. Karyomorphometry and chromosomal classification were performed as described by Cristiano et al. (2017). The chromosomes were measured using Image-Pro Plus (Media Cybernetics, Rockville, MD) and some chromosome characteristics were evaluated. For each chromosome, we measured the total length (TL) end-to-end, short arm (S), and long arm (L) sizes calculated by the distance between the arm end and centromeric region. The

karyotype length (KL) was calculated by summing the total length of all chromosomes. The relative size (RL) of each chromosome was calculated in relation to the total size of all chromosomes (TL × 100 / ΣTL). The ratio (r) between the length of the long arm and short arm (r = L / S) was calculated to classify the chromosomes as described by Levan et al. (1964) with modifications reported by Crozier (1970).

Results

The typical chromosome number of *Acromyrmex* (2n = 38) was found in all species of the genus analyzed in the present work. The karyotype of *Ac. lundii* and *Ac. nigrosetosus* were described for the first time and, that of *Ac. ambiguus* was described for the first time from a Brazilian population. The two largest chromosomal pairs were the first subtelocentric and the first metacentric. The karyotype formula was variable (see below) and in *Ac. crassispinus*, *Ac. lundii*, *Ac. nigrosetosus*, and *Ac. subterraneus subterraneus*, most chromosomes presented an r ratio between 1.67 and 3.00; therefore, these were classified as submetacentric. The chromosomal classification of *Ac. ambiguus* was different from that of other species, as it mainly presents metacentric chromosomes. *Ac. ambiguus* has the karyotype formula 2K = 14M + 12SM + 8ST + 4A (Figure 1, Table 1). *Ac. crassispinus* presented 2K = 12M + 20SM + 4ST + 2A (Figure 2, Table 2) and its chromosomes are larger when compared to other species. *Ac. lundii* has the karyotype formula 2K = 10M + 14SM + 10ST + 4A (Figure 3, Table 3). *Ac. nigrosetosus* presented 2K = 12M + 14SM + 10ST + 2A and its chromosomes seem smaller than those of the other species (Figure 4, Table 4). *Ac. subterraneus subterraneus* has 2K = 14M +

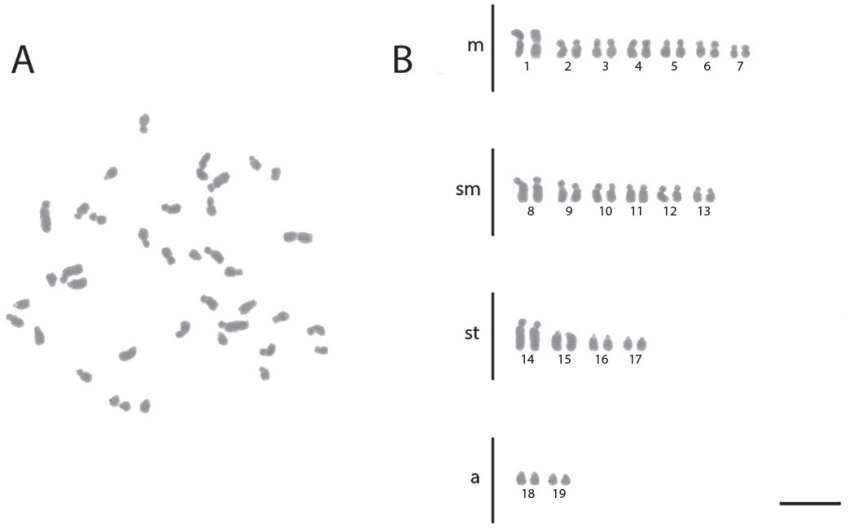


Figure 1. Conventional staining of mitotic cells of *Acromyrmex ambiguus* **A** the metaphase and **B** diploid karyotype with 2n = 38. Scale bar: 5 μm.

Table 1. Karyomorphometric analyses of the chromosomes of *Acromyrmex ambiguus*.

Chromosomes	TL	L	S	RL	r	Classification
1	5.13 ± 1.90	2.79 ± 1.03	2.34 ± 0.89	4.41 ± 0.39	1.20 ± 0.14	Metacentric
1	4.85 ± 1.87	2.60 ± 1.01	2.26 ± 0.89	4.17 ± 0.46	1.15 ± 0.15	Metacentric
2	3.35 ± 1.11	1.96 ± 0.68	1.39 ± 0.43	2.91 ± 0.14	1.40 ± 0.14	Metacentric
2	3.18 ± 0.98	1.87 ± 0.58	1.31 ± 0.41	2.78 ± 0.05	1.43 ± 0.14	Metacentric
3	3.11 ± 0.94	1.83 ± 0.59	1.29 ± 0.36	2.72 ± 0.07	1.41 ± 0.10	Metacentric
3	3.08 ± 0.93	1.80 ± 0.58	1.28 ± 0.36	2.69 ± 0.07	1.40 ± 0.10	Metacentric
4	3.01 ± 0.92	1.76 ± 0.57	1.25 ± 0.36	2.63 ± 0.06	1.40 ± 0.13	Metacentric
4	2.92 ± 0.89	1.77 ± 0.54	1.15 ± 0.35	2.55 ± 0.09	1.54 ± 0.10	Metacentric
5	2.86 ± 0.84	1.71 ± 0.52	1.15 ± 0.33	2.50 ± 0.08	1.48 ± 0.13	Metacentric
5	2.76 ± 0.77	1.62 ± 0.47	1.14 ± 0.32	2.43 ± 0.15	1.43 ± 0.18	Metacentric
6	2.65 ± 0.69	1.61 ± 0.44	1.04 ± 0.25	2.35 ± 0.18	1.54 ± 0.12	Metacentric
6	2.56 ± 0.66	1.47 ± 0.36	1.09 ± 0.33	2.27 ± 0.21	1.39 ± 0.18	Metacentric
7	2.28 ± 0.62	1.37 ± 0.40	0.90 ± 0.23	2.02 ± 0.22	1.51 ± 0.10	Metacentric
7	2.13 ± 0.55	1.22 ± 0.31	0.90 ± 0.26	1.89 ± 0.17	1.37 ± 0.18	Metacentric
8	4.35 ± 1.37	3.17 ± 1.02	1.18 ± 0.36	3.79 ± 0.18	2.68 ± 0.27	Submetacentric
8	4.11 ± 1.27	3.01 ± 0.95	1.10 ± 0.33	3.59 ± 0.16	2.73 ± 0.19	Submetacentric
9	3.35 ± 0.99	2.36 ± 0.77	0.98 ± 0.26	2.94 ± 0.13	2.40 ± 0.41	Submetacentric
9	3.15 ± 0.94	2.21 ± 0.76	0.94 ± 0.21	2.76 ± 0.08	2.33 ± 0.44	Submetacentric
10	3.11 ± 0.91	2.15 ± 0.68	0.95 ± 0.25	2.73 ± 0.09	2.25 ± 0.32	Submetacentric
10	3.07 ± 0.92	2.17 ± 0.73	0.90 ± 0.22	2.69 ± 0.07	2.41 ± 0.44	Submetacentric
11	2.98 ± 0.91	2.08 ± 0.66	0.90 ± 0.26	2.60 ± 0.08	2.33 ± 0.28	Submetacentric
11	2.90 ± 0.86	2.00 ± 0.63	0.90 ± 0.24	2.54 ± 0.07	2.20 ± 0.22	Submetacentric
12	2.70 ± 0.68	1.77 ± 0.58	0.93 ± 0.20	2.40 ± 0.22	2.08 ± 0.28	Submetacentric
12	2.57 ± 0.67	1.76 ± 0.45	0.81 ± 0.23	2.29 ± 0.24	2.21 ± 0.22	Submetacentric
13	2.47 ± 0.66	1.73 ± 0.47	0.75 ± 0.20	2.19 ± 0.23	2.33 ± 0.28	Submetacentric
13	2.19 ± 0.51	1.49 ± 0.39	0.70 ± 0.14	1.96 ± 0.25	2.10 ± 0.26	Submetacentric
14	5.22 ± 1.84	4.15 ± 1.60	1.07 ± 0.30	4.50 ± 0.32	3.86 ± 0.97	Subtelocentric
14	4.76 ± 1.56	3.79 ± 1.33	0.97 ± 0.26	4.14 ± 0.19	3.86 ± 0.66	Subtelocentric
15	3.23 ± 1.24	2.61 ± 1.03	0.62 ± 0.22	2.77 ± 0.29	4.13 ± 0.62	Subtelocentric
15	2.99 ± 1.15	2.35 ± 0.93	0.64 ± 0.25	2.56 ± 0.31	3.68 ± 0.65	Subtelocentric
16	2.69 ± 1.05	2.15 ± 0.88	0.54 ± 0.19	2.29 ± 0.25	3.98 ± 0.60	Subtelocentric
16	2.55 ± 0.96	1.98 ± 0.76	0.57 ± 0.21	2.18 ± 0.20	3.49 ± 0.45	Subtelocentric
17	2.39 ± 0.87	1.91 ± 0.73	0.48 ± 0.17	2.05 ± 0.16	4.00 ± 0.93	Subtelocentric
17	2.21 ± 0.76	1.74 ± 0.58	0.48 ± 0.20	1.91 ± 0.14	3.93 ± 1.11	Subtelocentric
18	2.03 ± 0.48	1.83 ± 0.43	0.20 ± 0.06	1.82 ± 0.22	9.14 ± 1.41	Acrocentric
18	1.95 ± 0.47	1.73 ± 0.41	0.22 ± 0.07	1.74 ± 0.20	8.27 ± 0.99	Acrocentric
19	1.85 ± 0.43	1.66 ± 0.39	0.19 ± 0.04	1.66 ± 0.19	9.02 ± 0.91	Acrocentric
19	1.75 ± 0.42	1.57 ± 0.39	0.18 ± 0.03	1.56 ± 0.19	8.79 ± 1.21	Acrocentric
KL	114.44					

TL: total length; L: long arm length; S: short arm length; RL: relative length; r: arm ratio, KL: karyotype length.

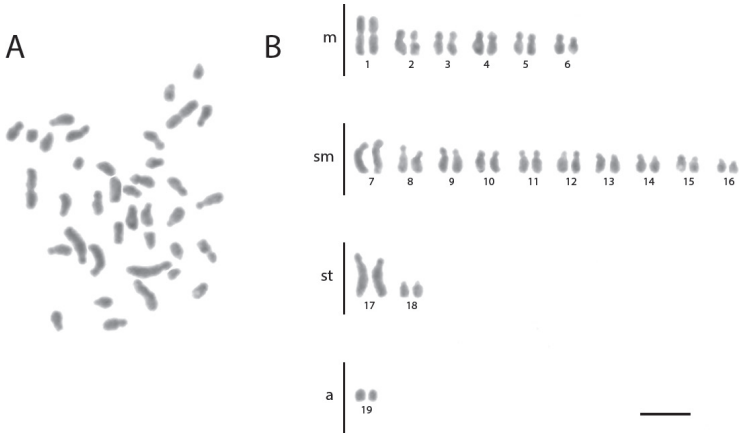


Figure 2. Conventional staining of mitotic cells of *Acromyrmex crassispinus* **A** the metaphase and **B** diploid karyotype with 2n = 38. Scale bar: 5 μm.

Table 2. Karyomorphometric analyses of the chromosomes of *Acromyrmex crassispinus*.

Chromosomes	TL	L	S	RL	r	Classification
1	5.84 ± 0.93	3.10 ± 0.51	2.74 ± 0.47	4.35 ± 0.22	1.14 ± 0.12	Metacentric
1	5.66 ± 0.93	3.04 ± 0.44	2.62 ± 0.50	4.21 ± 0.21	1.17 ± 0.08	Metacentric
2	3.96 ± 0.76	2.36 ± 0.53	1.61 ± 0.28	2.94 ± 0.20	1.47 ± 0.20	Metacentric
2	3.74 ± 0.60	2.24 ± 0.32	1.50 ± 0.31	2.79 ± 0.10	1.51 ± 0.14	Metacentric
3	3.63 ± 0.56	2.10 ± 0.24	1.45 ± 0.20	2.71 ± 0.10	1.46 ± 0.15	Metacentric
3	3.58 ± 0.56	1.98 ± 0.31	1.60 ± 0.29	2.67 ± 0.10	1.25 ± 0.14	Metacentric
4	3.48 ± 0.50	2.04 ± 0.34	1.43 ± 0.19	2.60 ± 0.08	1.43 ± 0.13	Metacentric
4	3.38 ± 0.48	2.01 ± 0.27	1.37 ± 0.23	2.53 ± 0.12	1.49 ± 0.14	Metacentric
5	3.23 ± 0.46	1.94 ± 0.30	1.30 ± 0.18	2.42 ± 0.15	1.50 ± 0.10	Metacentric
5	3.11 ± 0.49	1.85 ± 0.33	1.27 ± 0.18	2.33 ± 0.18	1.45 ± 0.13	Metacentric
6	2.94 ± 0.53	1.63 ± 0.42	1.14 ± 0.27	2.19 ± 0.21	1.43 ± 0.16	Metacentric
6	3.01 ± 1.11	1.86 ± 0.89	1.14 ± 0.31	2.21 ± 0.57	1.60 ± 0.45	Metacentric
7	5.02 ± 0.83	3.57 ± 0.54	1.45 ± 0.37	3.74 ± 0.12	2.53 ± 0.42	Submetacentric
7	4.72 ± 0.86	3.22 ± 0.90	1.50 ± 0.45	3.51 ± 0.24	2.49 ± 0.29	Submetacentric
8	3.99 ± 0.58	2.70 ± 0.44	1.29 ± 0.23	2.98 ± 0.12	2.14 ± 0.39	Submetacentric
8	3.85 ± 0.59	2.66 ± 0.40	1.20 ± 0.23	2.87 ± 0.08	2.25 ± 0.34	Submetacentric
9	3.78 ± 0.57	2.65 ± 0.37	1.13 ± 0.24	2.82 ± 0.08	2.39 ± 0.34	Submetacentric
9	3.70 ± 0.60	2.56 ± 0.50	1.14 ± 0.19	2.75 ± 0.08	2.29 ± 0.45	Submetacentric
10	3.64 ± 0.57	2.51 ± 0.45	1.13 ± 0.20	2.71 ± 0.07	2.25 ± 0.41	Submetacentric
10	3.56 ± 0.52	2.43 ± 0.35	1.12 ± 0.22	2.65 ± 0.05	2.20 ± 0.33	Submetacentric
11	3.48 ± 0.48	2.41 ± 0.36	1.07 ± 0.17	2.60 ± 0.08	2.27 ± 0.34	Submetacentric
11	3.39 ± 0.50	2.32 ± 0.40	1.07 ± 0.18	2.53 ± 0.07	2.19 ± 0.42	Submetacentric
12	3.34 ± 0.48	2.31 ± 0.39	1.02 ± 0.14	2.49 ± 0.09	2.27 ± 0.33	Submetacentric
12	3.25 ± 0.46	2.21 ± 0.39	1.05 ± 0.12	2.43 ± 0.11	2.10 ± 0.31	Submetacentric
13	3.15 ± 0.48	2.14 ± 0.39	1.01 ± 0.13	2.35 ± 0.12	2.13 ± 0.27	Submetacentric
13	2.98 ± 0.50	2.07 ± 0.40	0.92 ± 0.15	2.22 ± 0.12	2.27 ± 0.39	Submetacentric
14	2.84 ± 0.43	1.90 ± 0.35	0.93 ± 0.13	2.11 ± 0.06	2.06 ± 0.34	Submetacentric
14	2.77 ± 0.43	1.91 ± 0.33	0.86 ± 0.13	2.07 ± 0.07	2.23 ± 0.28	Submetacentric
15	2.71 ± 0.43	1.88 ± 0.26	0.83 ± 0.22	2.02 ± 0.09	2.34 ± 0.44	Submetacentric
15	2.67 ± 0.43	1.79 ± 0.30	0.87 ± 0.16	1.99 ± 0.08	2.08 ± 0.31	Submetacentric
16	2.55 ± 0.43	1.75 ± 0.32	0.80 ± 0.16	1.90 ± 0.11	2.24 ± 0.43	Submetacentric
16	2.48 ± 0.45	1.68 ± 0.32	0.80 ± 0.17	1.84 ± 0.18	2.14 ± 0.37	Submetacentric
17	6.43 ± 1.18	5.09 ± 0.95	1.34 ± 0.28	4.77 ± 0.20	3.83 ± 0.48	Subtelocentric
17	5.99 ± 0.93	4.67 ± 0.74	1.31 ± 0.20	4.46 ± 0.15	3.58 ± 0.29	Subtelocentric
18	2.34 ± 0.44	1.83 ± 0.35	0.51 ± 0.10	1.75 ± 0.18	3.65 ± 0.62	Subtelocentric
18	2.09 ± 0.43	1.68 ± 0.37	0.41 ± 0.09	1.55 ± 0.15	4.13 ± 0.76	Subtelocentric
19	2.03 ± 0.37	1.82 ± 0.32	0.21 ± 0.06	1.51 ± 0.13	9.02 ± 1.69	Acrocentric
19	1.91 ± 0.26	1.70 ± 0.23	0.20 ± 0.05	1.43 ± 0.10	8.69 ± 1.68	Acrocentric
KL	134.22					

TL: total length; L: long arm length; S: short arm length; RL: relative length; r: arm ratio, KL: karyotype length.

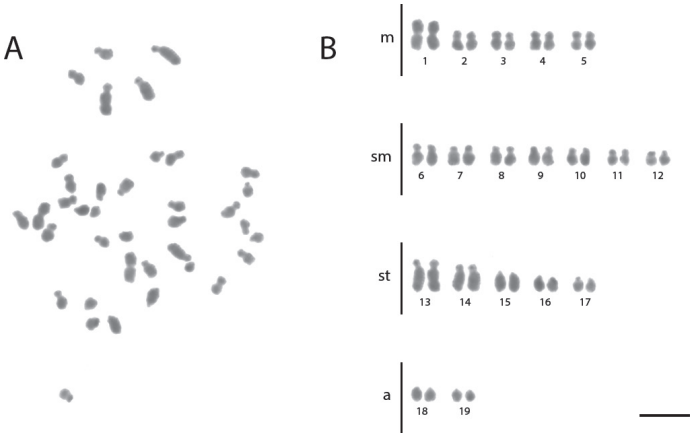


Figure 3. Conventional staining of mitotic cells of *Acromyrmex lundii* **A** the metaphase and **B** diploid karyotype with 2n = 38. Scale bar: 5 μm.

Table 3. Karyomorphometric analyses of the chromosomes of *Acromyrmex lundii*.

Chromosomes	TL	L	S	RL	r	Classification
1	5.00 ± 1.17	2.77 ± 0.70	2.23 ± 0.49	4.42 ± 0.27	1.24 ± 0.11	Metacentric
1	4.67 ± 1.10	2.62 ± 0.68	2.05 ± 0.44	4.14 ± 0.28	1.27 ± 0.13	Metacentric
2	3.16 ± 0.63	1.81 ± 0.35	1.35 ± 0.30	2.82 ± 0.08	1.35 ± 0.11	Metacentric
2	3.06 ± 0.66	1.79 ± 0.36	1.27 ± 0.32	2.72 ± 0.10	1.43 ± 0.16	Metacentric
3	2.91 ± 0.61	1.70 ± 0.38	1.21 ± 0.28	2.59 ± 0.09	1.42 ± 0.21	Metacentric
3	2.92 ± 0.62	1.67 ± 0.32	1.25 ± 0.32	2.60 ± 0.09	1.37 ± 0.18	Metacentric
4	2.81 ± 0.53	1.67 ± 0.32	1.14 ± 0.22	2.51 ± 0.08	1.47 ± 0.11	Metacentric
4	2.75 ± 0.51	1.63 ± 0.30	1.13 ± 0.21	2.46 ± 0.08	1.45 ± 0.09	Metacentric
5	2.61 ± 0.43	1.53 ± 0.28	1.07 ± 0.19	2.34 ± 0.15	1.44 ± 0.18	Metacentric
5	2.42 ± 0.45	1.43 ± 0.30	0.99 ± 0.16	2.17 ± 0.20	1.44 ± 0.13	Metacentric
6	3.38 ± 0.65	2.30 ± 0.44	1.09 ± 0.22	3.02 ± 0.15	2.12 ± 0.22	Submetacentric
6	3.24 ± 0.60	2.17 ± 0.42	1.08 ± 0.20	2.89 ± 0.11	2.02 ± 0.21	Submetacentric
7	3.19 ± 0.64	2.19 ± 0.43	1.00 ± 0.22	2.84 ± 0.12	2.20 ± 0.19	Submetacentric
7	3.12 ± 0.62	2.19 ± 0.48	0.93 ± 0.17	2.78 ± 0.13	2.36 ± 0.32	Submetacentric
8	3.02 ± 0.55	2.07 ± 0.39	0.95 ± 0.21	2.70 ± 0.13	2.21 ± 0.36	Submetacentric
8	2.96 ± 0.53	2.00 ± 0.39	0.96 ± 0.19	2.65 ± 0.12	2.12 ± 0.40	Submetacentric
9	2.88 ± 0.48	1.95 ± 0.35	0.94 ± 0.17	2.58 ± 0.14	2.10 ± 0.30	Submetacentric
9	2.80 ± 0.46	1.89 ± 0.32	0.91 ± 0.18	2.51 ± 0.15	2.12 ± 0.36	Submetacentric
10	2.70 ± 0.50	1.80 ± 0.36	0.90 ± 0.17	2.41 ± 0.12	2.02 ± 0.28	Submetacentric
10	2.57 ± 0.48	1.76 ± 0.32	0.82 ± 0.19	2.30 ± 0.13	2.19 ± 0.33	Submetacentric
11	2.40 ± 0.44	1.62 ± 0.32	0.78 ± 0.14	2.14 ± 0.11	2.07 ± 0.26	Submetacentric
11	2.28 ± 0.38	1.58 ± 0.34	0.70 ± 0.07	2.05 ± 0.12	2.26 ± 0.44	Submetacentric
12	2.18 ± 0.32	1.47 ± 0.21	0.71 ± 0.14	1.96 ± 0.14	2.10 ± 0.26	Submetacentric
12	2.06 ± 0.34	1.40 ± 0.26	0.67 ± 0.10	1.85 ± 0.13	2.12 ± 0.34	Submetacentric
13	5.01 ± 1.21	3.87 ± 0.99	1.14 ± 0.24	4.43 ± 0.24	3.38 ± 0.39	Subtelocentric
13	4.87 ± 1.13	3.85 ± 1.03	1.02 ± 0.12	4.31 ± 0.22	3.74 ± 0.66	Subtelocentric
14	4.24 ± 0.99	3.24 ± 0.79	1.00 ± 0.20	3.75 ± 0.17	3.23 ± 0.15	Subtelocentric
14	4.03 ± 1.00	3.08 ± 0.76	0.95 ± 0.24	3.56 ± 0.21	3.24 ± 0.17	Subtelocentric
15	3.22 ± 0.69	2.56 ± 0.51	0.66 ± 0.20	2.86 ± 0.19	4.00 ± 0.68	Subtelocentric
15	3.00 ± 0.68	2.33 ± 0.45	0.66 ± 0.25	2.66 ± 0.24	3.78 ± 0.97	Subtelocentric
16	2.65 ± 0.66	2.09 ± 0.49	0.56 ± 0.18	2.35 ± 0.26	3.89 ± 0.74	Subtelocentric
16	2.38 ± 0.51	1.85 ± 0.40	0.53 ± 0.12	2.12 ± 0.16	3.53 ± 0.26	Subtelocentric
17	2.27 ± 0.47	1.75 ± 0.34	0.53 ± 0.14	2.03 ± 0.14	3.40 ± 0.39	Subtelocentric
17	2.09 ± 0.33	1.67 ± 0.30	0.42 ± 0.08	1.88 ± 0.14	4.07 ± 0.88	Subtelocentric
18	2.06 ± 0.37	1.83 ± 0.33	0.23 ± 0.04	1.85 ± 0.12	7.92 ± 0.58	Acrocentric
18	1.93 ± 0.32	1.70 ± 0.29	0.23 ± 0.03	1.73 ± 0.14	7.58 ± 1.05	Acrocentric
19	1.75 ± 0.29	1.56 ± 0.25	0.18 ± 0.05	1.57 ± 0.11	8.76 ± 1.38	Acrocentric
19	1.64 ± 0.29	1.48 ± 0.26	0.16 ± 0.04	1.47 ± 0.07	9.09 ± 0.89	Acrocentric
KL	112.23					

TL: total length; L: long arm length; S: short arm length; RL: relative length; r: arm ratio, KL: karyotype length.

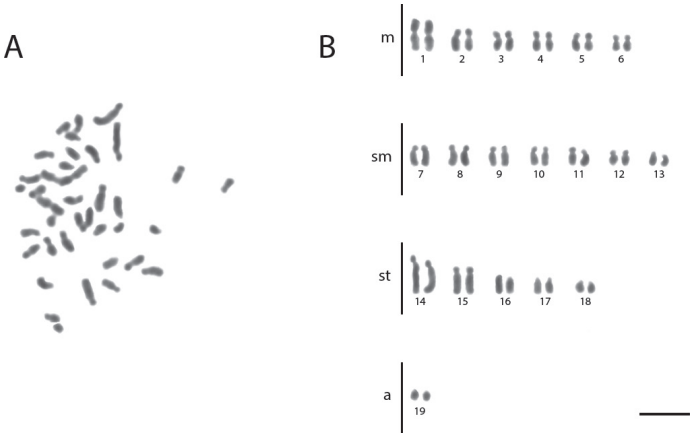


Figure 4. Conventional staining of mitotic cells of *Acromyrmex nigrosetosus* **A** the metaphase and **B** diploid karyotype with 2n = 38. Scale bar: 5 μm.

Table 4. Karyomorphometric analyses of the chromosomes of *Acromyrmex nigrosetosus*.

Chromosomes	TL	L	S	RL	r	Classification
1	4.40 ± 1.10	2.40 ± 0.55	2.00 ± 0.57	4.34 ± 0.34	1.22 ± 0.11	Metacentric
1	4.17 ± 1.00	2.24 ± 0.57	1.93 ± 0.44	4.12 ± 0.18	1.16 ± 0.08	Metacentric
2	2.92 ± 0.61	1.75 ± 0.33	1.18 ± 0.29	2.90 ± 0.18	1.51 ± 0.12	Metacentric
2	2.79 ± 0.58	1.68 ± 0.34	1.12 ± 0.24	2.77 ± 0.12	1.51 ± 0.10	Metacentric
3	2.71 ± 0.54	1.57 ± 0.38	1.14 ± 0.20	2.70 ± 0.10	1.38 ± 0.21	Metacentric
3	2.65 ± 0.53	1.61 ± 0.33	1.06 ± 0.22	2.64 ± 0.09	1.52 ± 0.14	Metacentric
4	2.59 ± 0.53	1.54 ± 0.34	1.04 ± 0.21	2.57 ± 0.07	1.48 ± 0.13	Metacentric
4	2.53 ± 0.55	1.48 ± 0.34	1.05 ± 0.22	2.50 ± 0.09	1.42 ± 0.15	Metacentric
5	2.44 ± 0.55	1.48 ± 0.35	0.96 ± 0.20	2.42 ± 0.12	1.54 ± 0.11	Metacentric
5	2.37 ± 0.55	1.42 ± 0.32	0.96 ± 0.24	2.35 ± 0.13	1.48 ± 0.09	Metacentric
6	2.24 ± 0.56	1.33 ± 0.35	0.90 ± 0.22	2.21 ± 0.19	1.48 ± 0.12	Metacentric
6	2.06 ± 0.39	1.24 ± 0.25	0.82 ± 0.14	2.06 ± 0.14	1.52 ± 0.14	Metacentric
7	2.99 ± 0.55	2.11 ± 0.41	0.88 ± 0.17	2.98 ± 0.18	2.42 ± 0.27	Submetacentric
7	2.88 ± 0.56	2.00 ± 0.42	0.88 ± 0.18	2.87 ± 0.14	2.29 ± 0.33	Submetacentric
8	2.77 ± 0.56	1.90 ± 0.41	0.87 ± 0.18	2.76 ± 0.09	2.21 ± 0.26	Submetacentric
8	2.71 ± 0.51	1.87 ± 0.32	0.84 ± 0.22	2.70 ± 0.10	2.29 ± 0.40	Submetacentric
9	2.69 ± 0.52	1.87 ± 0.43	0.81 ± 0.11	2.67 ± 0.09	2.30 ± 0.36	Submetacentric
9	2.61 ± 0.46	1.84 ± 0.34	0.77 ± 0.15	2.60 ± 0.13	2.42 ± 0.27	Submetacentric
10	2.58 ± 0.46	1.79 ± 0.33	0.79 ± 0.16	2.57 ± 0.12	2.29 ± 0.35	Submetacentric
10	2.52 ± 0.45	1.72 ± 0.35	0.81 ± 0.14	2.51 ± 0.14	2.14 ± 0.33	Submetacentric
11	2.43 ± 0.47	1.67 ± 0.35	0.76 ± 0.14	2.41 ± 0.15	2.20 ± 0.29	Submetacentric
11	2.33 ± 0.46	1.59 ± 0.33	0.74 ± 0.15	2.31 ± 0.13	2.15 ± 0.25	Submetacentric
12	2.24 ± 0.42	1.52 ± 0.28	0.71 ± 0.17	2.23 ± 0.11	2.20 ± 0.39	Submetacentric
12	2.16 ± 0.42	1.45 ± 0.32	0.70 ± 0.15	2.14 ± 0.08	2.10 ± 0.39	Submetacentric
13	2.04 ± 0.40	1.39 ± 0.29	0.65 ± 0.14	2.03 ± 0.20	2.19 ± 0.41	Submetacentric
13	1.91 ± 0.35	1.31 ± 0.25	0.60 ± 0.13	1.91 ± 0.20	2.25 ± 0.42	Submetacentric
14	4.69 ± 1.10	3.73 ± 0.91	0.97 ± 0.20	4.64 ± 0.20	3.85 ± 0.39	Subtelocentric
14	4.40 ± 0.94	3.50 ± 0.84	0.90 ± 0.14	4.36 ± 0.12	3.89 ± 0.65	Subtelocentric
15	3.72 ± 0.82	2.84 ± 0.64	0.88 ± 0.19	3.68 ± 0.14	3.23 ± 0.16	Subtelocentric
15	3.50 ± 0.84	2.67 ± 0.64	0.83 ± 0.20	3.46 ± 0.18	3.22 ± 0.23	Subtelocentric
16	2.61 ± 0.66	2.05 ± 0.49	0.57 ± 0.19	2.60 ± 0.43	3.76 ± 0.79	Subtelocentric
16	2.35 ± 0.51	1.83 ± 0.40	0.51 ± 0.13	2.34 ± 0.31	3.64 ± 0.52	Subtelocentric
17	2.18 ± 0.51	1.73 ± 0.38	0.45 ± 0.14	2.17 ± 0.27	3.97 ± 0.59	Subtelocentric
17	2.07 ± 0.51	1.63 ± 0.40	0.44 ± 0.12	2.05 ± 0.22	3.73 ± 0.59	Subtelocentric
18	1.84 ± 0.50	1.47 ± 0.41	0.36 ± 0.11	1.81 ± 0.16	4.11 ± 0.54	Subtelocentric
18	1.70 ± 0.44	1.36 ± 0.32	0.34 ± 0.12	1.68 ± 0.12	4.15 ± 0.70	Subtelocentric
19	1.52 ± 0.33	1.36 ± 0.31	0.16 ± 0.03	1.51 ± 0.13	8.45 ± 1.05	Acrocentric
19	1.42 ± 0.29	1.27 ± 0.26	0.16 ± 0.03	1.42 ± 0.12	8.19 ± 0.79	Acrocentric
KL	100.73					

TL: total length; L: long arm length; S: short arm length; RL: relative length; r: arm ratio, KL: karyotype length.

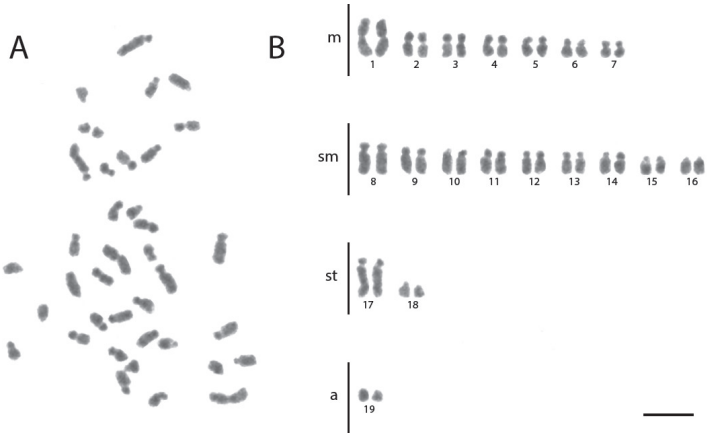


Figure 5. Conventional staining of mitotic cells of *Acromyrmex subterraneus subterraneus* **A** the metaphase and **B** diploid karyotype with 2n = 38. Scale bar: 5 μm.

Table 5. Karyomorphometric analyses of the chromosomes of *Acromyrmex subterraneus subterraneus*.

Chromosomes	TL	L	S	RL	r	Classification
1	5.03 ± 0.96	2.72 ± 0.53	2.31 ± 0.46	4.42 ± 0.37	1.19 ± 0.11	Metacentric
1	4.78 ± 0.94	2.55 ± 0.48	2.23 ± 0.48	4.20 ± 0.38	1.15 ± 0.10	Metacentric
2	3.31 ± 0.64	1.88 ± 0.33	1.43 ± 0.34	2.91 ± 0.22	1.34 ± 0.18	Metacentric
2	3.18 ± 0.48	1.82 ± 0.29	1.37 ± 0.21	2.81 ± 0.11	1.33 ± 0.14	Metacentric
3	3.08 ± 0.45	1.81 ± 0.28	1.28 ± 0.20	2.72 ± 0.10	1.42 ± 0.15	Metacentric
3	3.01 ± 0.44	1.78 ± 0.26	1.23 ± 0.20	2.65 ± 0.09	1.46 ± 0.13	Metacentric
4	2.96 ± 0.46	1.77 ± 0.30	1.19 ± 0.17	2.61 ± 0.08	1.49 ± 0.11	Metacentric
4	2.91 ± 0.45	1.69 ± 0.28	1.22 ± 0.18	2.56 ± 0.09	1.38 ± 0.12	Metacentric
5	2.87 ± 0.45	1.71 ± 0.28	1.16 ± 0.19	2.53 ± 0.10	1.48 ± 0.13	Metacentric
5	2.80 ± 0.42	1.70 ± 0.26	1.10 ± 0.17	2.48 ± 0.11	1.54 ± 0.12	Metacentric
6	2.70 ± 0.42	1.57 ± 0.18	1.12 ± 0.26	2.38 ± 0.13	1.45 ± 0.21	Metacentric
6	2.59 ± 0.42	1.50 ± 0.22	1.09 ± 0.24	2.29 ± 0.18	1.40 ± 0.20	Metacentric
7	2.46 ± 0.38	1.46 ± 0.21	1.00 ± 0.20	2.18 ± 0.17	1.48 ± 0.17	Metacentric
7	2.33 ± 0.39	1.40 ± 0.25	0.93 ± 0.15	2.05 ± 0.15	1.51 ± 0.14	Metacentric
8	4.35 ± 0.99	3.12 ± 0.69	1.22 ± 0.29	3.82 ± 0.47	2.56 ± 0.26	Submetacentric
8	4.05 ± 0.76	2.97 ± 0.59	1.08 ± 0.18	3.56 ± 0.32	2.74 ± 0.23	Submetacentric
9	3.42 ± 0.50	2.35 ± 0.41	1.08 ± 0.17	3.02 ± 0.12	2.20 ± 0.39	Submetacentric
9	3.32 ± 0.53	2.29 ± 0.44	1.03 ± 0.18	2.92 ± 0.14	2.26 ± 0.45	Submetacentric
10	3.23 ± 0.53	2.30 ± 0.41	0.93 ± 0.15	2.84 ± 0.15	2.49 ± 0.34	Submetacentric
10	3.20 ± 0.52	2.19 ± 0.37	1.01 ± 0.19	2.82 ± 0.15	2.19 ± 0.31	Submetacentric
11	3.10 ± 0.45	2.12 ± 0.35	0.98 ± 0.15	2.74 ± 0.09	2.17 ± 0.30	Submetacentric
11	3.04 ± 0.44	2.11 ± 0.33	0.93 ± 0.13	2.68 ± 0.07	2.27 ± 0.17	Submetacentric
12	3.01 ± 0.44	2.10 ± 0.38	0.91 ± 0.11	2.65 ± 0.09	2.31 ± 0.36	Submetacentric
12	2.94 ± 0.41	2.03 ± 0.34	0.91 ± 0.13	2.60 ± 0.10	2.26 ± 0.40	Submetacentric
13	2.77 ± 0.40	1.92 ± 0.35	0.84 ± 0.11	2.45 ± 0.20	2.29 ± 0.41	Submetacentric
13	2.68 ± 0.43	1.85 ± 0.33	0.83 ± 0.10	2.37 ± 0.19	2.21 ± 0.20	Submetacentric
14	2.58 ± 0.38	1.80 ± 0.30	0.77 ± 0.11	2.28 ± 0.16	2.34 ± 0.30	Submetacentric
14	2.48 ± 0.36	1.73 ± 0.24	0.75 ± 0.16	2.20 ± 0.17	2.35 ± 0.39	Submetacentric
15	2.43 ± 0.35	1.61 ± 0.23	0.82 ± 0.16	2.15 ± 0.17	2.00 ± 0.32	Submetacentric
15	2.29 ± 0.32	1.60 ± 0.26	0.68 ± 0.08	2.03 ± 0.14	2.35 ± 0.34	Submetacentric
16	2.23 ± 0.30	1.54 ± 0.21	0.69 ± 0.13	1.98 ± 0.15	2.26 ± 0.35	Submetacentric
16	2.16 ± 0.26	1.44 ± 0.17	0.71 ± 0.11	1.91 ± 0.11	2.05 ± 0.26	Submetacentric
17	4.94 ± 0.77	3.84 ± 0.63	1.1 ± 0.17	4.35 ± 0.20	3.49 ± 0.29	Subtelocentric
17	4.76 ± 0.70	3.73 ± 0.59	1.03 ± 0.15	4.20 ± 0.14	3.64 ± 0.46	Subtelocentric
18	2.12 ± 0.30	1.70 ± 0.29	0.42 ± 0.05	1.87 ± 0.12	4.12 ± 0.88	Subtelocentric
18	1.99 ± 0.27	1.58 ± 0.24	0.41 ± 0.08	1.76 ± 0.13	4.03 ± 1.01	Subtelocentric
19	1.82 ± 0.28	1.63 ± 0.24	0.19 ± 0.04	1.62 ± 0.20	8.84 ± 1.31	Acrocentric
19	1.61 ± 0.25	1.46 ± 0.21	0.16 ± 0.03	1.42 ± 0.14	9.23 ± 1.74	Acrocentric
KL	114.53					

TL: total length; L: long arm length; S: short arm length; RL: relative length; r: arm ratio, KL: karyotype length.

18SM + 4ST + 2A (Figure 5, Table 5). For *Ac. rugosus* and *At. sexdens* only the chromosome number was established, but no detailed karyomorphometry was performed.

FISH analyses revealed that all chromosomes of all *Acromyrmex* species and *Atta sexdens* are positively marked at both arms in the telomeric regions with the presence of the canonical insect sequence $(TTAGG)_6$ and no signals for interstitial telomeric sites were detected (Figures 6A–F, 7). The intensity and size of the probe marking was varied between the chromosomes and metaphases of each species.

Discussion

The insect canonical repeat $(TTAGG)_n$ has been observed in 30 species of ants using different methods (Okazaki et al. 1993; Meyne et al. 1995; Lorite et al. 2002; Wurm

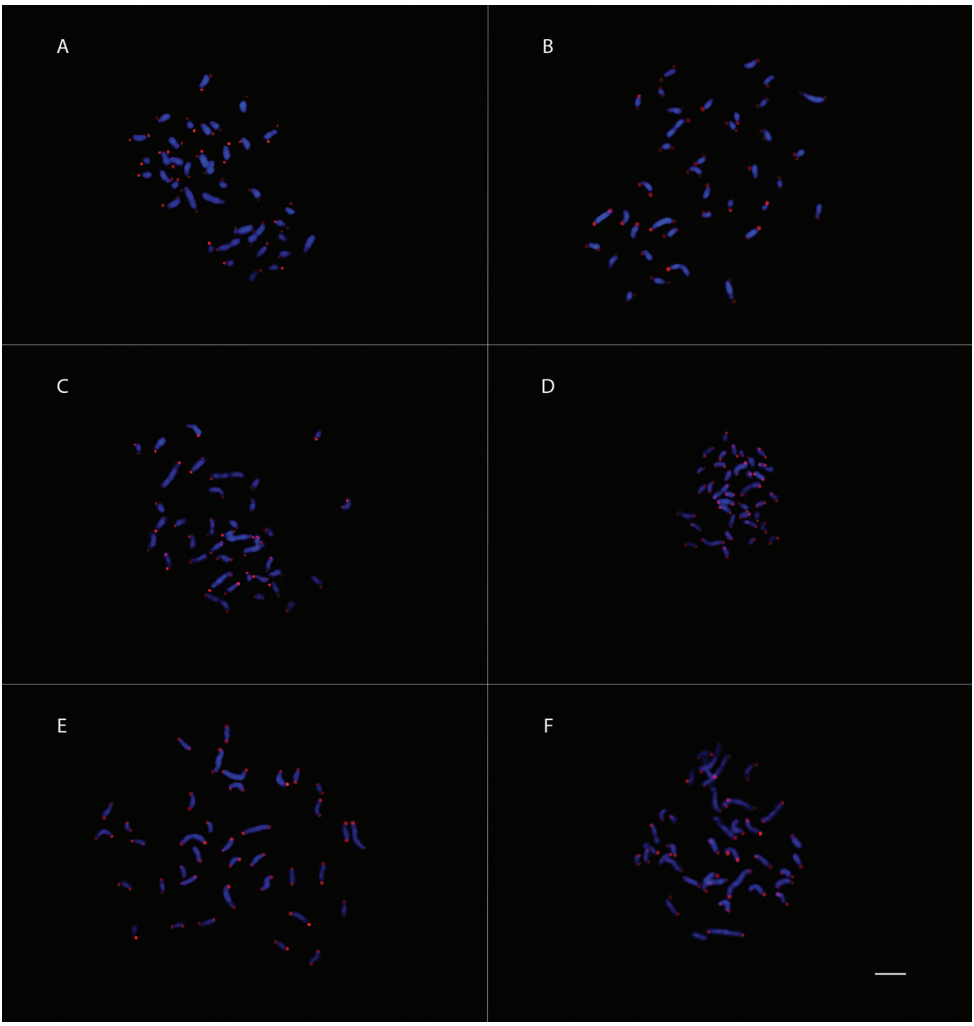


Figure 6. FISH mapping of mitotic metaphase chromosomes using a $(TTAGG)_6$ telomeric probe; DAPI stain in blue and Cy3 in red **A** *Acromyrmex ambiguus* **B** *Acromyrmex crassispinus* **C** *Acromyrmex lundii* **D** *Acromyrmex nigrosetosus* **E** *Acromyrmex rugosus* and **F** *Acromyrmex subterraneus subterraneus*. Scale bar: 5 μm.

et al. 2011; Pereira et al. 2018), but FISH studies were mostly performed with *Myrmecia* species (Meyne et al. 1995). The only analysis involving a leafcutter ant has been performed on *Ac. striatus*, which also presents $(TTAGG)_6$ labeling in the telomeres of both arms of all 22 chromosomes and does not show markings in other chromosomal regions (Pereira et al. 2018). The present study adds information about one species of *Atta* (*At. sexdens*) and six *Acromyrmex* species (*Ac. ambiguus*, *Ac. crassispinus*, *Ac. lundii*, *Ac. nigrosetosus*, *Ac. rugosus*, *Ac. subterraneus subterraneus*). We also describe the chromosome number and structure of *Ac. lundii* and *Ac. nigrosetosus* for the first time. The karyotype description for *Ac. ambiguus* from Brazil revealed the same diploid chromo-

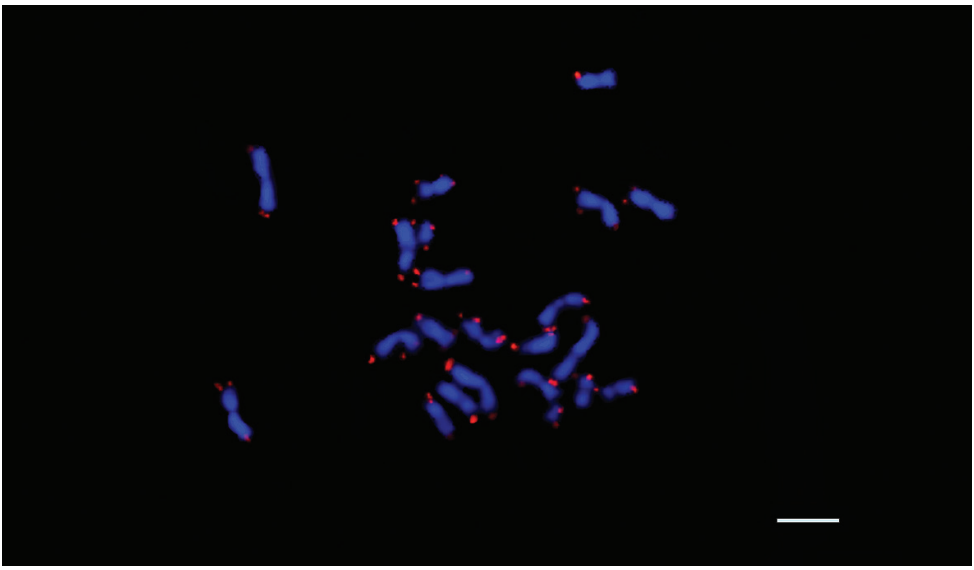


Figure 7. FISH mapping of *Atta sexdens* mitotic metaphase chromosomes using a $(TTAGG)_6$ telomeric probe; DAPI in blue and Cy3 in red. Scale bar: 5 μm.

some number as in previous data available from Uruguay (Goñi 1983), but distinct regarding the karyotype formula, overrepresented by subtelocentric and acrocentric chromosomes in the latter. These differences may be due the visual determination of chromosome morphology instead chromosome measurements applied here. The new chromosome counts reported in this study again corroborate the stable chromosomal number in *Acromyrmex* and the detailed karyomorphometry of the chromosomes suggests dynamism of chromosome morphology due to distinct karyotypic formulas.

Our FISH results add to the cytogenetic knowledge of new karyotypes and molecular cytogenetic analyses in leafcutter ants, and demonstrate that the pattern found in *Ac. striatus* seems to occur in *Atta* species and *Acromyrmex* species. Importantly, *Ac. striatus* is the sister clade of *Atta* and the remaining *Acromyrmex* species (Cristiano et al. 2013). The occurrence of telomeric regions marked positively by $(TTAGG)_n$ reinforces the premise that Formicidae presents high homology for the presence of the insect canonical sequence. This motif has been proposed to be a plesiomorphic chromosomal feature in Hymenoptera (Gokhman and Kuznetsova 2018). In fact, the canonical motif $(TTAGG)_n$ was observed in several branches of the clade of fungus-farming ants, from anciently diverged lineages such as *Mycetophylax* to recent lineages such as *Mycetomoellerius* (Micolino et al. 2019a, b, 2020). Besides, the alternative TCAGG motif present in insects seems to be restricted to some groups, but not to Formicidae (Kuznetsova et al. 2019), and we did not find any evidences for this in previously attempted experiments in our laboratory on the phylogenetic basis of fungus-farming ants (unpublished data).

Sahara et al. (1999) propose that $(TTAGG)_n$ is a sequence with high homology in Insecta because it is inherited from a common primitive ancestor of the class and the

fact that some families do not show the presence of canonical repetition is explained by the group evolutionary process, where $(TTAGG)_n$ has been lost and recovered several times. This theory is supported by Frydrychová et al. (2004) who studied 22 insect species from 20 different orders selected among the main phylogenetic group lineages and found that 15 species presented the $(TTAGG)_n$ on their telomeres, whereas only seven species did not have the sequence in their chromosomes. The authors compared their results with the available literature and concluded that 16 insect orders have the primitive telomeric region conserved and eight do not present it. In contrast, Menezes et al. (2017) evaluated the presence of the canonical repeats $(TTAGG)_n$ and $(TTAGGG)_n$ in 25 representative species of eight Hymenoptera families, and surprisingly none of them showed any signs of these repetitive sequences in their telomeres or in any chromosomal regions. Therefore, the hypothesis regarding multiple losses of the sequence inherited from a primitive ancestor appears unlikely to these authors, as the number of Insecta families without the $(TTAGG)_n$ sequence is higher than the number of those bearing it. Thus, the authors propose that the most probable evolutionary scenario is that the canonical repetition has been lost in the Apocrita ancestor or even in the Hymenoptera ancestor, whereas Apidae and Formicidae have recovered the region independently. On the contrary, the phylogenetic position and the presence of $(TTAGG)_n$ as the telomeric repeat in *Tenthredo omissa* (Förster, 1844) and *Taxonus agrorum* (Fallén, 1808) (Tenthredinidae: Symphyta) were suggested to be indicative of the ancestrality of this motif in Hymenoptera (Gokhman and Kuznetsova 2018).

Ants have high variability in their karyotypes; there are species with the haploid number of chromosomes n = 1 (Crosland and Crozier 1986; Taylor 1991) and species with n = 60 (Mariano et al. 2008). This variation exists with respect to the chromosome number as well as the morphology and classification. Robertsonian fissions result in two acrocentric chromosomes due to the breaking of a bi-armed chromosome, whereas Robertsonian fusions involve exactly the opposite process, where two acrocentric chromosomes unite to form a single bi-armed chromosome (Lorite and Palomeque 2010). These are possibly the two most important rearrangements for karyotype evolution in ants and support the minimum-interaction theory proposed by Imai et al. (1988, 1994, 2001). This theory defines that fission processes are more significant and common than fusion processes because higher chromosome numbers reduce the possibility of interaction between non-homologous chromosomes within the nucleus, minimizing the mutation rates. Thus, it is proposed that the chromosomal number of ant species usually tends to increase. In this sense, it is also proposed that the ancestral karyotype of ants would be composed of a small number of metacentric chromosomes whereas recently divergent lineages would have more chromosomes due to several chromosomal fission processes (Imai et al. 1977). Thus, it is plausible to state that in *Acromyrmex*, karyotypes with 38 chromosomes arose following several Robertsonian fissions, whereas the chromosome number of the iconic *Ac. striatus* is a plesiomorphic feature maintained in *Atta* spp. (Cristiano et al. 2013).

Establishment of the karyotype (the chromosome number and determination of their morphology) is very important for the knowledge of chromosomal variations and possible genetic barriers between phylogenetic groups (Cristiano et al. 2017; Cardoso

et al. 2018b). It is necessary to go further in describing the chromosome number and morphology, as more detailed karyomorphometric analyses may reveal additional and substantial variations not observed previously, mainly when accompanied with genome size estimates (Cardoso et al. 2018b). Tsutsui et al. (2008) state that closely related species, belonging to the same genus, may have very similar genome sizes, corroborating the pattern revealed by our karyomorphometric analyses in the *Acromyrmex* species studied here.

Acknowledgement

We are grateful to the many people that made this work possible. We thank all of our colleagues at the Lab and Research Group of Genetics and Evolution of Ants (GEF-UFOP) for their help with data or their assistance in the field of chromosome preparation. The authors thank Júlio Chaul and Rodrigo Feitosa of the Universidade Federal do Paraná that confirmed the identification of the ant species. We would like to thank Editage (www.editage.com) for English language editing. We also thank the editor and two reviewers for their helpful comments on the manuscript.

References

Barros LAC, de Aguiar H, Mariano CDF, Andrade-Souza V, Costa MA, Delabie JHC, Pompolo SD (2016) Cytogenetic data on six leafcutter ants of the genus *Acromyrmex* Mayr, 1865 (Hymenoptera, Formicidae, Myrmicinae): insights into chromosome evolution and taxonomic implications. Comparative Cytogenetics 10(2): 229–243. https://doi.org/10.3897/CompCytogen.v10i2.7612

Blackburn EH (1991) Structure and function of telomeres. Nature 350(6319): 569–573. https://doi.org/10.1038/350569a0

Bolton B (2020) An Online Catalog of the Ants of the World. http://antcat.org [accessed 23. March 2020]

Cardoso DC, Cristiano MP, Barros LA, Lopes DM, Pompolo SG (2012) First cytogenetic characterization of a species of the arboreal ant genus *Azteca* Forel, 1978 (Dolichoderinae, Formicidae). Comparative Cytogenetics 6(2): 107–114. https://doi.org/10.3897/compcytogen.v6i2.2397

Cardoso DC, Heinze J, Moura MN, Cristiano MP (2018b) Chromosomal variation among populations of a fungus-farming ant: implications for karyotype evolution and potential restriction to gene flow. BMC Evolutionary Biology 18(1): 1–146. https://doi.org/10.1186/s12862-018-1247-5

Cardoso DC, Santos HG, Cristiano MP (2018a) The Ant Chromosome database-(ACdb): an online resource for ant (Hymenoptera: Formicidae) chromosome researchers. Myrmecological News 27: 87–91. https://doi.org/10.25849/myrmecol.news_027:087

Cristiano MP, Cardoso DC, Fernandes-Salomão TM (2013) Cytogenetic and molecular analyses reveal a divergence between *Acromyrmex striatus* (Roger, 1863) and other congeneric species: taxonomic implications. PLoS ONE 8(3): e59784. https://doi.org/10.1371/journal.pone.0059784

Cristiano MP, Pereira TTP, Simões LP, Sandoval-Gómez VE, Cardoso DC (2017) Reassessing the Chromosome Number and Morphology of the Turtle Ant Cephalotes pusillus (Klug, 1824) Using Karyomorphometrical Analysis and Observations of New Nesting Behavior. Insects 8(4): 1–114. https://doi.org/10.3390/insects8040114

Crosland MWJ, Crozier RH (1986) Myrmecia pilosula, an ant with only one pair of chromosomes. Science 231(4743): 1278–1278. https://doi.org/10.1126/science.231.4743.1278

Crozier RH (1970) Karyotypes of twenty-one ant species (Hymenoptera: Formicidae), with reviews of the known ant karyotypes. Canadian Journal of Genetics and Cytology 12(1): 109–128. https://doi.org/10.1139/g70-018

Farji-Brener AG, Ghermandi L (2008) Leaf-cutting ant nests near roads increase fitness of exotic plant species in natural protected areas. Proceedings of the Royal Society of London B 275: 1431–1440. https://doi.org/10.1098/rspb.2008.0154

Frydrychová R, Grossmann P, Trubac P, Vítková M, Marec FE (2004) Phylogenetic distribution of TTAGG telomeric repeats in insects. Genome 47(1): 163–178. https://doi.org/10.1139/g03-100

Gokhman VE, Kuznetsova VG (2018) Presence of the canonical TTAGG insect telomeric repeat in the Tenthredinidae (Symphyta) suggests its ancestral nature in the order Hymenoptera. Genetica 146(3): 341–344. https://doi.org/10.1007/s10709-018-0019-x

Goñi B, De Zolessi LC, Imai HT (1983) Karyotypes of thirteen ant species from Uruguay (Hymenoptera, Formicidae). Caryologia 36(4): 363–371. https://doi.org/10.1080/00087114.1983.10797677

Imai HT, Crozier RH, Taylor RW (1977) Karyotype evolution in Australian ants. Chromosoma 59(4): 341–393. https://doi.org/10.1007/BF00327974

Imai HT, Satta Y, Takahata N (2001) Integrative study on chromosome evolution of mammals, ants and wasps based on the minimum interaction theory. Journal of Theoretical Biology 210(4): 475–497. https://doi.org/10.1006/jtbi.2001.2327

Imai HT, Taylor RW, Crozier RH (1988) Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. The Japanese Journal of Genetics 63(2): 159–185. https://doi.org/10.1266/jjg.63.159

Imai HT, Taylor RW, Crozier RH (1994) Experimental bases for the minimum interaction theory. I. Chromosome evolution in ants of the *Myrmecia pilosula* species complex (Hymenoptera: Formicidae: Myrmeciinae). The Japanese Journal of Genetics 69(2): 137–182. https://doi.org/10.1266/jjg.69.137

Kuznetsova V, Grozeva S, Gokhman V (2019) Telomere structure in insects: A review. Journal of Zoological Systematics of Evolutionary Research 58(1): 127–158. https://doi.org/10.1111/jzs.12332

Levan A, Fredga K, Sandberg A (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52(2): 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Loeck AE, Grutzmacher D, Coimbra S, Silvana M (2003) Occurrence of leaf-cutting ants of the genus *Acromyrmex* in the main agricultural regions of the Rio Grande do Sul state. Current Agricultural Science and Technology 9(2): 129–133.

Lorite P, Carrillo JA, Palomeque T (2002) Conservation of (TTAGG)n telomeric sequences among ants (Hymenoptera, Formicidae). Journal of Heredity 93(4): 282–285. https://doi.org/10.1093/jhered/93.4.282

Lorite P, Palomeque T (2010) Karyotype evolution in ants (Hymenoptera: Formicidae), with a review of the known ant chromosome numbers. Myrmecological News 13: 89–102.

Mariano CDSF, Pompolo SDG, Barros LAC, Mariano-Neto E, Campiolo S, Delabie JHCA (2008) A biogeographical study of the threatened ant *Dinoponera lucida* Emery (Hymenoptera: Formicidae: Ponerinae) using a cytogenetic approach. Insect Conservation and Diversity 1(3): 161–168. https://doi.org/10.1111/j.1752-4598.2008.00022.x

Menezes RST, Bardella VB, Cabral de Mello DC (2017) Are the TTAGG and TTAGGG telomeric repeats phylogenetically conserved in aculeate Hymenoptera? The Science of Nature 104(9–10): 1–85. https://doi.org/10.1007/s00114-017-1507-z

Meyne J, Hirai H, Imai HT (1995) FISH analysis of the telomere sequences of bulldog ants (Myrmecia: Formicidae). Chromosoma 104(1): 14–18. https://doi.org/10.1007/BF00352221

Micolino R, Cristiano MP, Cardoso DC (2019a) Population-Based Cytogenetic Banding Analysis and Phylogenetic Relationships of the Neotropical Fungus-Farming Ant *Trachymyrmex holmgreni* Wheeler, 1925. Cytogenetic and Genome Research 159(3): 151–161. https://doi.org/10.1159/000503913

Micolino R, Cristiano MP, Travenzoli NM, Lopes DM, Cardoso DC (2019b) Chromosomal dynamics in space and time: evolutionary history of *Mycetophylax* ants across past climatic changes in the Brazilian Atlantic coast. Scientific Reports 9(1): 1–13. https://doi.org/10.1038/s41598-019-55135-5

Micolino R, Cristiano MP, Cardoso DC (2020) Putative chromosomal inversion clue in the fungus-farming ant *Mycetomoellerius iheringi* Emery, 1888. Comparative Cytogenetics 14(2): 197–210. https://doi.org/10.3897/CompCytogen.v14i2.49846

Okazaki S, Tsuchida K, Maekawa H, Ishikawa H, Fujiwara H (1993) Identification of a pentanucleotide telomeric sequence, (TTAGG)n, in the silkworm *Bombyx mori* and in other insects. Molecular and Cellular Biology 13(3): 1424–1432. https://doi.org/10.1128/MCB.13.3.1424

Pereira TTP, dos Reis ACCC, Cardoso DC, Cristiano MP (2018) Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger, 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera). Comparative Cytogenetics 12(1): 13–26. https://doi.org/10.3897/CompCytogen.v12i1.21799

Sahara K, Marec F, Traut W (1999) TTAGG telomeric repeats in chromosomes of some insects and other arthropods. Chromosome Research 7(6): 449–460. https://doi.org/10.1023/A:1009297729547

Speicher MR, Carter NP (2005) The new cytogenetics: blurring the boundaries with molecular biology. Nature Reviews Genetics 6(10): 782–792. https://doi.org/10.1038/nrg1692

Taylor RW (1991) *Myrmecia croslandi* sp. n., a karyologically remarkable new Australian Jack-Jumper ant (Hymenoptera: Formicidae: Myrmeciinae). Australian Journal of Entomology 30(4): 288–288. https://doi.org/10.1111/j.1440-6055.1991.tb00438.x

Travenzoli NM, Lima BA, Cardoso DC, Dergam JA, Fernandes-Salomão TM, Lopes DM (2019) Cytogenetic analysis and chromosomal mapping of repetitive DNA in *Melipona* species (Hymenoptera, Meliponini). Cytogenetic and Genome Research 158(4): 213–224. https://doi.org/10.1159/000501754

Tsutsui ND, Suarez AV, Spagna JC, Johnston JS (2008) The evolution of genome size in ants. BMC Evolutionary Biology 8(1): 64–72. https://doi.org/10.1186/1471-2148-8-64

Vítková M, Král J, Traut W, Zrzavý J, Marec F (2005) The evolutionary origin of insect telomeric repeats (TTAGG)n. Chromosome Research 13(2): 145–156. https://doi.org/10.1007/s10577-007-1910-y

Wurm Y, Wang J, Riba-Grognuz O, Corona M, Nygaard S, Brendan G. Hunt, Ingram KK, Falquet L, Nipitwattanaphon M, Gotzek D, Dijkstra MB, Oettler J, Comtesse F, Shih C-J, Wu W-J, Yang C-C, Thomas J, Beaudoing E, Pradervand S, Flegel V, Cook ED, Fabbretti R, Stockinger H, Long L, Farmerie WG, Oakey J, Boomsma JJ, Pamilo P, Yi SV, Heinze J, Goodisman MAD, Farinelli L, Harshman K, Hulo N, Cerutti L, Xenarios I, Shoemaker DW, Keller L (2011) The genome of the fire ant *Solenopsis invicta*. Proceedings of the National Academy of Sciences USA 108(14): 5679–5684. https://doi.org/10.1073/pnas.1009690108

Zakian VA (1995) Telomeres: beginning to understand the end. Science 270(5242): 1601–1607. https://doi.org/10.1126/science.270.5242.1601

CompCytogen 14(3): 387–397 (2020)
doi: 10.3897/CompCytogen.v14i3.52727
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

First cytogenetic information for five Nilotic elephantfishes and a problem of ancestral karyotype of the family Mormyridae (Osteoglossiformes)

Sergey Simanovsky[1], Dmitry Medvedev[1], Fekadu Tefera[2], Alexander Golubtsov[1]

1 *Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, 33 Leninskij prosp., Moscow, 119071, Russia* **2** *National Fishery and Aquatic Life Research Center, Ethiopian Institute of Agricultural Research, Sebeta, P.O. Box 64, Ethiopia*

Corresponding author: Sergey Simanovsky (sergey.a.simanovsky@gmail.com)

Academic editor: Alicja Boroń | Received 7 April 2020 | Accepted 23 July 2020 | Published 19 August 2020

http://zoobank.org/56F3905D-E628-4D37-A60D-4D419EF0C37A

Citation: Simanovsky S, Medvedev D, Tefera F, Golubtsov A (2020) First cytogenetic information for five Nilotic elephantfishes and a problem of ancestral karyotype of the family Mormyridae (Osteoglossiformes). Comparative Cytogenetics 14(3): 387–397. https://doi.org/10.3897/CompCytogen.v14i3.52727

Abstract

The elephantfish family Mormyridae is the most diverse lineage of the primitive teleostean clade Osteoglossomorpha distributed in inland waters of all continents except Antarctica and Europe. The family Mormyridae is endemic to Africa and includes 22 genera and almost 230 species. The evolutionary radiation of mormyrids most probably should be attributed to their capability of both generating and receiving weak electric signals. Up-to-date cytogenetic studies have revealed substantial karyotype differentiation among the nine investigated elephantfish species and genera (a single species studied per each genus). In the present study, karyotypes of five species representing five mormyrid genera (four unexplored ones) collected from the White Nile system in southwestern Ethiopia are described for the first time. The results show substantial variety of the diploid chromosome and fundamental numbers: 2n = 48 and FN = 54 in *Brevimyrus niger* (Günther, 1866), 2n = 50 and FN = 72 in *Cyphomyrus petherici* (Boulenger, 1898), 2n = 50 and FN = 78 in *Hippopotamyrus pictus* (Marcusen, 1864), 2n = 50 and FN = 76 in *Marcusenius cyprinoides* (Linnaeus, 1758), 2n = 52 and FN = 52 in *Mormyrops anguilloides* (Linnaeus, 1758). Karyotype structure in the latter species seems to be close to the ancestral condition for the family. This hypothesis is discussed in the light of available data on karyotype diversity and phylogeny of mormyrids.

Keywords

Africa, chromosomes, karyotype evolution, *Brevimyrus*, *Cyphomyrus*, *Hippopotamyrus*, *Marcusenius*, *Mormyrops*

Introduction

The elephantfish family Mormyridae belongs to one of the most primitive groups of teleostean fishes, the cohort Osteoglossomorpha (Nelson et al. 2016). The family is endemic to the African continent and includes 22 genera and almost 230 species (Froese and Pauly 2019; Eschmeyer et al. 2020). In genus and species diversity it exceeds all other extant osteoglossomorph lineages. The evolutionary radiation of mormyrids most probably should be attributed to their ability of both generating and receiving weak electric signals that provides dual functions of 'electrolocation' and communication (Hopkins 2009, Carlson and Arnegard 2011).

First cytogenetic data on the osteoglossomorphs and particularly mormyrids were published by Hinegardner and Rosen (1972) and Uyeno (1973) almost half a century ago. Thereafter, the karyotype structure and cellular DNA content of osteoglossomorphs were progressively studied (reviewed by Arai 2011; Canitz et al. 2016; Barby et al. 2018; Cioffi et al. 2019). The recent works on mormyrids (Krysanov and Golubtsov 2014; Ozouf-Costaz et al. 2015; Canitz et al. 2016) raised to nine the number of mormyrid genera studied. The number of species studied is also nine because one species only has been karyotyped for all genera. The diploid chromosome numbers in most mormyrids are similar (2n = 48 or 50 excepting *Pollimyrus* Taverne, 1971 with 2n = 40). Nevertheless, the varying bi-armed chromosome numbers and 'amazing' diversity in NOR positions and C-banding patterns provide evidence for the substantial divergence in the karyotype structure with the dominating role of pericentric inversions (Ozouf-Costaz et al. 2015).

There is a coherent hypothesis about phylogenetic position of the family Mormyridae among other Osteoglossomorpha (Lavoué and Sullivan 2004; Inoue et al. 2009; Nelson et al. 2016). The phylogenetic structure of mormyrids themselves is not well-elaborated, but three basal groups in their radiation (the genera *Petrocephalus* Marcusen, 1854; *Myomyrus* Boulenger, 1898; *Mormyrops* Müller, 1843) are reliably defined (Alves-Gomes and Hopkins 1997; Sullivan et al. 2000; Lavoué et al. 2003). This makes it possible to hypothesize about the mormyrid karyotype evolution. Based on available data Canitz et al. (2016) suggested for Mormyridae the ancestral chromosome number 2n = 48–50, that is well-coordinated with the hypothetical ancestral karyotype for the teleostean fishes and early vertebrates in general (Ohno et al. 1969; Jaillon et al. 2004; Kohn et al. 2006; Nakatani et al. 2007).

Meanwhile, only a small fraction of the total mormyrid diversity (less than 5% of species) has been yet studied cytogenetically. New findings may correct the existing views on their karyotype evolution. In the present study, new data for five mormyrid species from northern East Africa are presented using cytogenetic analysis (chromosome number and morphology). Relevance of these data to undrstanding of karyotype evolution within the family Mormyridae is considered.

Material and methods

The fifteen individuals studied represent five species of different genera – *Brevimyrus niger* (Günther, 1866), *Cyphomyrus petherici* (Boulenger, 1898), *Hippopotamyrus pic-*

tus (Marcusen, 1864), *Marcusenius cyprinoides* (Linnaeus, 1758) and *Mormyrops anguilloides* (Linnaeus, 1758) – of the elephantfish family Mormyridae (Table 1). Fish were collected in southwestern Ethiopia under the umbrella of the Joint Ethiopian-Russian Biological Expedition (JERBE) at three sites in November of 2017: the Baro River downstream of the City of Itang (8°10'47"N, 34°15'2"E), the Tida River half way between the cities of Gambela and Itang (8°16'15"N, 34°25'52"E) and the Alvero River downstream of the Abobo Dam (7°52'23"N, 34°29'48"E). All three rivers belong to the Sobat River drainage discharging into the White Nile in South Sudan. Fish were caught with cast or gill nets, delivered in 80-l plastic containers into the field laboratory, where they were kept in permamently aerated water for several hours before treatment.

Before preparation fish were treated intraperitoneally with 0.1% colchicine for 3–4 hours. Then fish were euthanized with an overdose of tricaine methanesulfonate (MS-222), identified based on morphological key characters (Golubtsov et al. 1995, Levin and Golubtsov 2018), measured to an accuracy of 1 mm, dissected for gonad examination and tissue sampling, and preserved in 10% formaldehyde. Vouchers are deposited at the Institute of Ecology and Evolution (Moscow) under provisional labels of JERBE.

Chromosome preparations were obtained from anterior kidney according to Kligerman and Bloom (1977). Briefly, the anterior kidney tissue was incubated with 0.075M KCl hypotonic solution for 20–30 min at room temperature and fixed with 3:1 methanol : acetic acid. To prepare slides a fixed tissue was incubated with 50% glacial acetic acid, suspended, and dropped onto a hot slides. Air-dried chromosome spreads were stained conventionally with 4% Giemsa solution in phosphate buffer at pH 6.8 for 8 min.

Chromosome spreads were analysed under "Axioplan 2 Imaging" microscope (Carl Zeiss, Germany) equipped with "CV-M4+CL" camera (JAI, Japan) and "Ikaros" software (MetaSystems, Germany). Karyotypes were established according to the centromere position following the nomenclature of Levan et al. (1964). Chromosomes were classified as metacentric (a), submetacentric (sm) and acrocentric (a), including subtelocentric and telocentric chromosomes, and grouped according to their morphology in order of decreasing size. To determine the fundamental number (FN), metacentrics and submetacentrics were considered bi-armed and acrocentrics as uni-armed. The number of complete metaphase plates studied for each specimen is presented in Table 1.

Table 1. Species, fish standard length (SL), numbers of individuals (N) and metaphases (N_{mt}) studied, and collection site.

Species	SL, mm	N	N_{mt}	Collection site
Brevimyrus niger	81–87	3 (1♀, 2♂)	32	Tida River
Cyphomyrus petherici	69–153	5 (3♀, 2♂)	54	Alvero River
Hippopotamyrus pictus	197	1 (♂)	11	
Marcusenius cyprinoides	196–217	3 (2♀, 1♂)	30	
Mormyrops anguilloides	409–498	2 (1♀, 1♂)	21	
	413	1 (♀)	17	Baro River

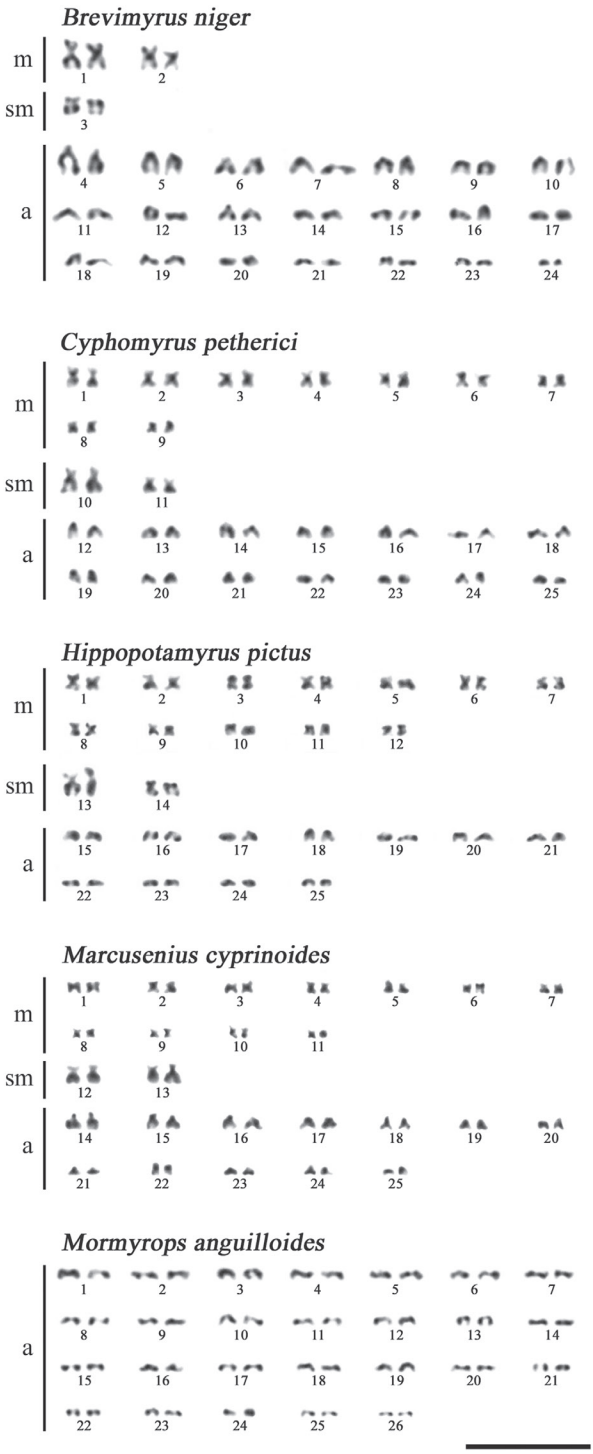


Figure 1. Karyotypes of five elephantfishes of the family Mormyridae. Scale bar: 10 μm.

Results and discussion

Brevimyrus niger has a karyotype with 2n = 48 (Fig. 1) consisting of 4 metacentrics (m), 2 submetacentrics (sm) and 42 acrocentrics (a). Three taxa share the same diploid numbers of chromosomes 2n = 50 but differ in karyotypic formula: *Cyphomyrus petherici* has 18m, 4sm and 28a, *Hippopotamyrus pictus* has 24m, 4sm and 22a, and *Marcusenius cyprinoides* has 22m, 4sm and 24a. Finally, *Mormyrops anguilloides* has karyotype with 2n = 52 consisting exclusively of acrocentrics gradually decreasing in size. In the other species studied by us one or two pairs of metacentrics or submetacentrics noticeably exceed in size most acrocentrics that admits an origin of the larger chromosomes via the centric fusions.

No distinguishable sex chromosomes were observed in complements of the four species in which individuals of both sexes were studied (*B. niger*, *C. petherici*, *M. cyprinoides*, and *M. anguilloides*), while the only male of *H. pictus* was karyotyped (Table 1). This is in agreement with the lack of reports on sex chromosomes in other mormyrids, but presence of heteromorphic sex chromosomes was supposed in the Asian arowana *Scleropages formosus* (Müller & Schlegel, 1840) from the family Osteoglossidae distantly related to Mormyridae (Bian et al. 2016; but see Cioffi et al. 2019).

Data for all mormyrid taxa studied cytogenetically in the present study and earlier are presented in Table 2. Taxa within the subfamily Mormyrinae are listed in alphabetical order. Recognition of the subfamily Petrocephalinae, as a sister group to all other mormyrids, is well-grounded by morphological (including structure of electrocytes) and molecular phylogenetic data (Taverne 1972; Alves-Gomes and Hopkins 1997; Sullivan et al. 2000; Lavoué et al. 2003). For the two earlier studied taxa names are changed in accordance with recent taxonomic arrangements (Eschmeyer et al. 2020): *Brienomyrus brachyistius* (Gill, 1862) was reported as "*Marcusenius brachistius* Gill" by Uyeno (1973) and *Campylomormyrus rhynchophorus* (Boulenger, 1898) as *C. compressirostris* (Pellegrin, 1924) by Canitz et al. (2016). *Brienomyrus* sp.7 of Ozouf-Costaz et al. (2015) is listed as *Paramormyrops* sp.7 following to Ráb et al. (2016).

Brevimyrus niger shares the karyotype with 2n = 48 with three other mormyrid taxa, but differs from two of them – *Campylomormyrus rhynchophorus* with FN = 78 and *Gnathonemus petersii* (Günther 1862) with FN = 64 or 68 – by a smaller number of biarmed elements (FN = 54). For third taxon, *Brienomyrus brachyistius*, the unbalanced karyotype with FN = 53 was described in a single specimen (Uyeno 1973). Apart from the unpaired metacentric chromosome of the unclear nature, its karyotype looks similar to that of *Brevimyrus niger*. Both species have two pairs of large biarmed chromosomes, while a pair of uni-armed chromosomes in *Brienomyrus brachyistius* might be substituted by a pair of submetacentrics in *Brevimyrus niger* lineage.

The karyotype with 2n = 50 was found to be dominating in both presently and previously studied mormyrids (three and five taxa, respectively). *Cyphomyrus petherici* (FN = 72), *Hippopotamyrus pictus* (FN = 78) and *Marcusenius cyprinoides* (FN = 76) have more biarmed elements in their compliment than any other mormyrid studied except *Campylomormyrus rhynchophorus* (FN = 78). Congeneric *Marcusenius cyprinoides* and *M. moorii* (Günther, 1867) sharing the same chromosome number differ substantially in

their karyotype structure. Up to recently *Cyphomyrus petherici* was considered as belonging to the genus *Pollimyrus* (Taverne 1971; Moritz et al. 2019). Substantial cytogenetic dissimilarity between the single studied species of the latter genus (2n = 40, FN = 42) and *C. petherici* corroborates the change of its generic position (Levin and Golubtsov 2018).

Mormyrops anguilloides has a karyotype unique for the mormyrids studied and composed of 52 uni-armed chromosomes. There are two mormyrids – *Petrocephalus microphthalmus* Pellegrin, 1909 and *Stomatorhinus walkeri* (Günther, 1867) – with 2n = 50 and FN = 52. Karyotypes of these three taxa dominated by the uni-armed elements seem to be close to each other and to a hypothetical ancestral karyotype of the family Mormyridae. Mutial trasnformation of these karyotypes could occur in a few evolutionary steps (Fig. 2). It is important that two of the three genera under consideration (*Petrocephalus* and *Mormyrops*) appear to be well-defined basal groups in the family phylogeny (Sullivan et al. 2000; Lavoué et al. 2003). Phylogenetic position of the third genera (*Stomatorhinus*) is unclear. Though it appears in the rather basal position (next to *Petrocephalus*) in the small cladogram by Ozouf-Costaz et al. (2015) based of the mitochondrial cytochrome *b* sequences, in the more extensive mormyrid phylogenies this genus is nested deeper in the phylogenetic trees but in varying and poorly surported positions (Lavoué et al. 2003; Sullivan et al. 2016; Levin and Golubtsov 2018). Unfortunatelly, cytogenetic data for one more genus with the well-defined basal position in the mormyrid phylogeny (*Myomyrus*, stemming out between *Petrocephalus* and *Mormyrops*) are absent.

Based on the simultaneous phylogenetic analysis of molecular data and chromosome number, Canitz et al. (2016) recognized karyotype with n = 24 as the most parsimonius ancestral state for the order Osteoglossiformes, while the haploid chromosome number of n = 24–25 was inferred for the most recent common ancestor of the family Mormyridae. Their analysis, however, did not include the most recent cytogenetic data for several osteoglossomorph clades (Ráb et al. 2016; Barby et al. 2018; Hatanaka et al. 2018; Jegede et al. 2018; Cioffi et al. 2019; de Oliveira et al. 2019). Moreover, the recent genomic data evidence for the ancestral Euteleostomi karyotype of 50 chromosomes with domination by acrocentric elements (Nakatani et al. 2007; Sacerdot et al. 2018; de Oliveira et al. 2019). If the ancestral karyotype of Mormyridae contained 50 uni-armed elements, three chromosomal rearrangements only might produce the observed karyotype structure in the three mormyrid genera (*Petrocephalus*, *Stomatorhinus* and *Mormyrops*) tentatively recognized by us as the least cytogenetically advanced (Fig. 2). The solitary submetacentic pairs in *Petrocephalus* and *Stomatorhinus* are suggested to be not syntenic because of some differences in chromosome morphology (Ozouf-Costaz et al. 2015). If the ancestral karyotype of Mormyridae contained 50 uni-armed elements, it is apparently not retained by any extant mormyrid or osteoglossomorph, in general. Although the karyotype with 2n = 50 is dominating among mormyrids, it contains from 1 to 14 pairs of bi-armed elements (Table 2).

Based on available data the most parsimonius scenarios of the early karyptype evolution in Mormyridae are presented in Figure 2. Three different ancestral karyotypes are considered: 2n = 50 and FN =50 (no bi-armed elements), 2n = 50 and FN = 52 (the only pair of bi-armed elements), 2n = 52 and FN = 52 (no bi-armed elements). The

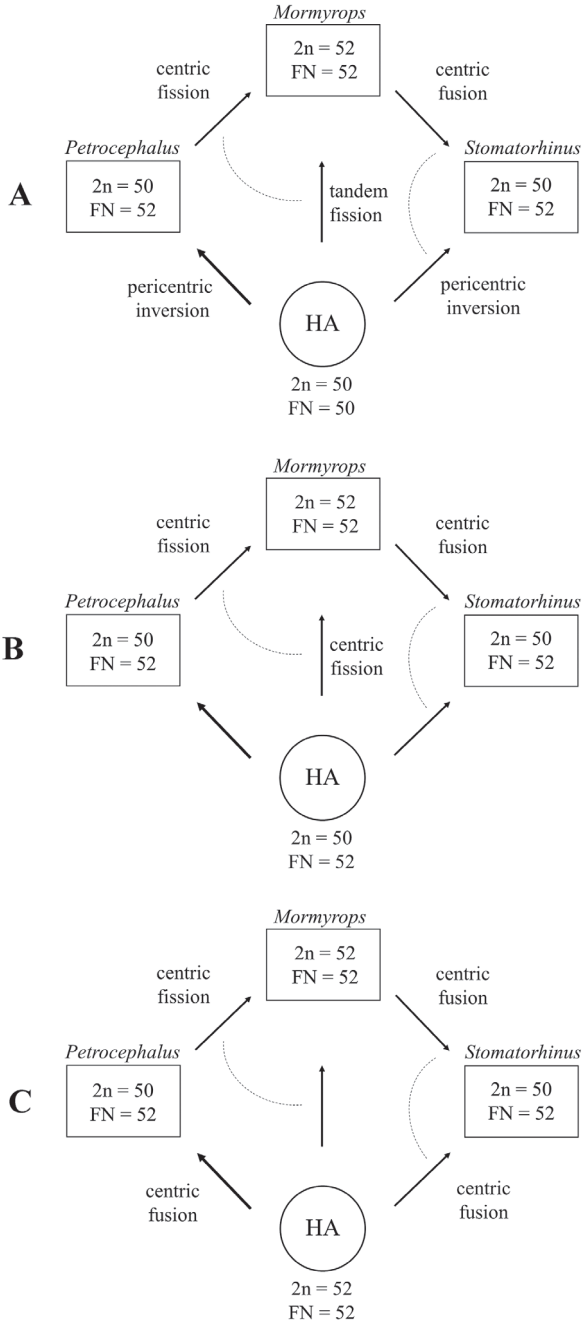


Figure 2. Most parsimonious scenarios of the early karyotype evolution within the family Mormyridae including three variants (**A–C**) of karyotype structure in a hypothetic ancestor (HA) and three studied lineages (the genera *Petrocephalus*, *Stomatorhinus* and *Mormyrops*) with least advanced karyotype structure within the family. The alternative transformations of karyotype structure are joint with a dashed line. The solitary submetacentric pairs in *Petrocephalus* and *Stomatorhinus* are suggested to be not syntenic.

Table 2. Cytogenetically studied elephantfishes of the family Mormyridae. Diploid chromosome number (2n), karyotypic formula, fundamental number (FN) and geographic origin.

Taxon	2n	Karyotypic formula	FN	Origin	References
Subfamily Petrocephalinae					
Petrocephalus microphthalmus Pellegrin, 1909	50	2sm + 48a	52	Ogooué Basin, Gabon	Ozouf-Costaz et al. 2015
Subfamily Mormyrinae					
Brevimyrus niger (Günther, 1866)	48	4m + 2sm + 42a	54	White Nile Basin, Ethiopia	This study
Brienomyrus brachyistius (Gill, 1862)	48	1m + 4sm + 2st + 41a	53	Unknown (fish store)	Uyeno 1973
Campylomormyrus rhynchophorus (Boulenger, 1898)	48	26m + 4sm + 18a	78	Unknown (laboratory stock)	Canitz et al. 2016
Cyphomyrus petherici (Boulenger, 1898)	50	18m + 4sm + 28a	72	White Nile Basin, Ethiopia	This study
Gnathonemus petersii (Günther, 1862)	48	10m + 6sm + 32a	64	Unknown (fish store)	Uyeno 1973
	48	18m + 2sm + 28a	68	Unknown (fish store)	Ozouf-Costaz et al. 2015
Hippopotamyrus pictus (Marcusen, 1864)	50	24m + 4sm + 22a	78	White Nile Basin, Ethiopia	This study
Ivindomyrus opdenboschi Taverne et Géry, 1975	50	10m + 2sm + 38a	62	Ntem River, Gabon	Ozouf-Costaz et al. 2015
Marcusenius cyprinoides (Linnaeus, 1758)	50	22m + 4sm + 24a	76	White Nile Basin, Ethiopia	This study
Marcusenius moorii (Günther, 1867)	50	4sm + 46a	54	Ntem River, Gabon	Ozouf-Costaz et al. 2015
Mormyrops anguilloides (Linnaeus, 1758)	52	52a	52	White Nile Basin, Ethiopia	This study
Paramormyrops sp.7	50	2m + 6sm + 42a	58	Ebeigne, Woleu River, Gabon	Ozouf-Costaz et al. 2015
Pollimyrus prope *nigricans* (Boulenger, 1906)	40	2m + 38a	42	White Nile and Omo-Turkana basins, Ethiopia	Krysanov and Golubtsov 2014
Stomatorhinus walkeri (Günther, 1867)	50	2sm + 48a	52	Ogooué Basin, Gabon	Ozouf-Costaz et al. 2015

karyotype structure suggested for a hypothetic ancestor could not be retained in any extant mormyrid lineage (Fig. 2A) or retained in *Petrocephalus* (Fig. 2B) or *Mormyrops* (Fig. 2C). It is impossible to judge which of the scenarios considered is more preferable. There are also plenty of less parsimonious scenarios that are not considered by us.

We believe that further cytogenetic studies of various mormyrid taxa may shape the existing views on the karyotype evolution within this diverse group of fish. Looking for the probable interspecific variation of the karyotype structure within the three phylogenetically basal groups (the genera *Petrocephalus*, *Myomyrus*, *Mormyrops*) is of special interest.

Acknowledgments

We gratefully acknowledge the JERBE coordinator Andrey A. Darkov (IEE RAS, Moscow) for logistic support, Sergey E. Cherenkov (IEE) for sharing field operations and assistance in collecting material, Eugeny Yu. Krysanov (IEE) for precious help at different stages of our work, Alicja Boroń (University of Warmia and Mazury in Olsztyn), Mikhail V. Mina (Koltsov Institute of Developmental Biology RAS, Moscow), Claudio Oliveira (São Paulo State University, São Paulo) for critical comments on the manu-

script. This work is financially supported by the Russian Foundation for Basic Research Project no. 18-34-00638 for SS and benefits also (at the stage of manuscript preparation) from the Russian Science Foundation Project no. 19-14-00218 for AG.

References

Alves-Gomes J, Hopkins CD (1997) Molecular insights into the phylogeny of mormyriform fishes and the evolution of their electric organs. Brain, Behavior and Evolution 49: 324–350. https://doi.org/10.1159/000113001

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

Barby FF, Ráb P, Lavoué S, Ezaz T, Bertollo LAC, Kilian A, Maruyama SR, de Oliveira EA, Artoni RF, Santos MH, Jegede OI, Hatanaka T, Tanomtong A, Liehr T, Cioffi MB (2018) From chromosomes to genome: insights into the evolutionary relationships and biogeography of Old World knifefishes (Notopteridae; Osteoglossiformes). Genes 96(6): 1–306. https://doi.org/10.3390/genes9060306

Bian C, Hu Y, Ravi V, Kuznetsova IS, Shen X, Mu X, Sun Y, You X, Li J, Li X, Qiu Y, Tay B-H, Thevasagayam NM, Komissarov AS, Trifonov V, Kabilov M, Tupikin A, Luo J, Liu Y, Song H, Liu C, Wang X, Gu D, Yang Y, Li W, Polgar G, Fan G, Zeng P, Zhang H, Xiong Z, Tang Z, Peng C, Ruan Z, Yu H, Chen J, Fan M, Huang Y, Wang M, Zhao X, Hu G, Yang H, Wang J, Wang J, Xu X, Song L, Xu G, Xu P, Xu J, O'Brien SJ, Orbán L, Venkatesh B, Shi Q (2016) The Asian arowana (*Scleropages formosus*) genome provides new insights into the evolution of an early lineage of teleosts. Scientific Reports 6: 1–17. https://doi.org/10.1038/srep24501

Canitz J, Kirschbaum F, Tiedemann R (2016) Karyotype description of the African weakly electric fish *Campylomormyrus compressirostris* in the context of chromosome evolution in Osteoglossiformes. Journal of Physiology-Paris 110: 273–280. https://doi.org/10.1016/j.jphysparis.2017.01.002

Carlson BA, Arnegard ME (2011) Neural innovations and the diversification of African weakly electric fishes. Communicative & Integrative Biology 4(6): 720–725. https://doi.org/10.4161/cib.17483

Cioffi MB, Ráb P, Ezaz T, Bertollo LAC, Lavoué S, de Oliveira EA, Sember A, Molina WF, Santos de Souza FH, Majtánová Z, Liehr T, Al-Rikabi ABH, Yano CF, Viana P, Feldberg E, Unmack P, Hatanaka T, Tanomtong A, Perez MF (2019) Deciphering the evolutionary history of arowana fishes (Teleostei, Osteoglossiformes, Osteoglossidae): insight from comparative cytogenomics. International Journal of Molecular Sciences 20 (17): 1–19. [4296]. https://doi.org/10.3390/ijms20174296

de Oliveira EA, Bertollo LAC, Rab P, Ezaz T, Yano CF, Hatanaka T, Jegede OI, Tanomtong A, Liehr T, Sember A, Maruyama SR, Feldberg E, Viana PF, Cioffi MB (2019) Cytogenetics, genomics and biodiversity of the South American and African Arapaimidae fish family (Teleostei, Osteoglossiformes). PLoS ONE 14(3): e0214225. https://doi.org/10.1371/journal.pone.0214225

Eschmeyer WN, Fricke R, van der Laan R [Eds] (2020) Catalog of Fishes: Genera, Species, References. http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp [Accesed 04.06.2020]

Froese R, Pauly D (2019) FishBase. http://www.fishbase.org [accessed 04.06.2020]

Golubtsov AS, Darkov AA, Dgebuadze YY, Mina MV (1995) An artificial key to fish species of the Gambela region (the White Nile basin in the limits of Ethiopia). Joint Ethio-Russian Biological Expedition, Addis Abeba, 84 pp.

Hatanaka T, de Oliveira EA, Ráb P, Yano CF, Bertollo LAC, Ezaz T, Jegede OOI, Liehr T, Olaleye VF, Cioffi MB (2018) First chromosomal analysis in *Gymnarchus niloticus* (Gymnarchidae: Osteoglossiformes): insights into the karyotype evolution of this ancient fish order. Biological Journal of the Linnean Society 125: 83–92. https://doi.org/10.1093/biolinnean/bly098

Hinegardner R, Rosen DE (1972) Cellular DNA content and the evolution of teleostean fishes. American Naturalist 106: 621–644. https://doi.org/10.1086/282801

Hopkins CD (2009) Electrical perception and communication. In: Squire LR (Ed.) Encyclopedia of neuroscience, volume 3, Academic Press, Oxford, 813–831. https://doi.org/10.1016/B978-008045046-9.01827-1

Inoue JG, Kumazawa Y, Miya M, Nishida M (2009) The historical biogeography of the freshwater knifefishes using mitogenomic approaches: a Mesozoic origin of the Asian notopterids (Actinopterygii: Osteoglossomorpha). Molecular Phylogenetics and Evolution 51: 486–499. https://doi.org/10.1016/j.ympev.2009.01.020

Jaillon O, Aury J-M, Brunet F, Petit J-L, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B, Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard V, Jubin C, Castelli V, Katinka M, Vacherie B, Biémont C, Skalli Z, Cattolico L, Poulain J, de Berardinis V, Cruaud C, Duprat S, Brottier P, Coutanceau J-P, Gouzy J, Parra G, Lardier G, Chapple C, McKernan KJ, McEwan P, Bosak S, Kellis M, Volff J-N, Guigó R, Zody MC, Mesirov J, Lindblad-Toh K, Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quétier F, Saurin W, Scarpelli C, Wincker P, Lander ES, Weissenbach JW, Crollius HR (2004) Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate protokaryotype. Nature 2004: 946–957. https://doi.org/10.1038/nature03025

Jegede O, Akintoye MA, Awopetu JI (2018) Karyotype of the African weakly electric fish, *Gymnarchus niloticus* (Osteoglossiformes: Gymnarchidae) from Oluwa River, Nigeria. Ife Journal of Science 20(3): 539–545. https://doi.org/10.4314/ijs.v20i3.8

Kohn M, Hogel J, Vogel W, Kehrer-Sawatzki H, Graves J, Hameister H (2006) Reconstruction of a 450-My-old ancestral vertebrate protokaryotype. Trends in Genetics 22: 203–210. https://doi.org/10.1016/j.tig.2006.02.008

Kligerman AD, Bloom SE (1977) Rapid chromosome preparations from solid tissues of fishes. Journal of the Fisheries Research Board of Canada 34(2): 266–269. https://doi.org/10.1139/f77-039

Krysanov EYu, Golubtsov AS (2014) Karyotypes of four fish species from the Nile and Omo-Turkana basins in Ethiopia. Journal of Ichthyology 54: 889–892. https://doi.org/10.1134/S0032945214100087

Lavoué S, Sullivan JP, Hopkins CD (2003) Phylogenetic utility of the first two introns of the S7 ribosomal protein gene in African electric fishes (Mormyroidea: Teleostei) and congruence with other molecular markers. Biological Journal of the Linnean Society 78: 273–292. https://doi.org/10.1046/j.1095-8312.2003.00170.x

Lavoué S, Sullivan JP (2004) Simultaneous analysis of five molecular markers provides a well-supported phylogenetic hypothesis for the living bony-tongue fishes (Osteoglossomorpha: Teleostei). Molecular Phylogenetics and Evolution 33: 171–185. https://doi.org/10.1016/j.ympev.2004.04.021

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

Levin BA, Golubtsov AS (2018) New insights into the molecular phylogeny and taxonomy of mormyrids (Osteoglossiformes, Actinopterygii) in northern East Africa. Journal of Zoological Systematics and Evolutionary Research 56(1): 61–76. https://doi.org/10.1111/jzs.12186

Moritz T, El Dayem ZM, Abdallah MA, Neumann D (2019) New and rare records of fishes from the White Nile in the Republic of the Sudan. Cybium 43(2): 137–151.

Nakatani Y, Takeda H, Kohara Y, Morishita S (2007) Reconstruction of the vertebrate ancestral genome reveals dynamic genome reorganization in early vertebrates. Genome Research 17: 1254–1265. https://doi.org/10.1101/gr.6316407

Nelson JS, Grande T, Wilson MVH (2016) Fishes of the World (5th Edn.,). John Wiley & Sons, Inc., Hoboken, New Jersey, 707 pp.

Ohno S, Muramoto J, Klein J, Atkin NB (1969) Diploid-tetraploid relationship in clupeoid and salmon fish. Chromozómes Today 2: 139–147.

Ozouf-Costaz C, Coutanceau J-P, Bonillo C, Belkadi L, Fermon Y, Agnèse J-F, Guidi-Rontani C, Paugy D (2015) First insights into karyotype evolution within the family Mormyridae. Cybium 39: 227–236.

Ráb P, Yano CF, Lavoué S, Jegede OI, Bertollo LAC, Ezaz T, Majtánová Z, de Oliveira EA, Marcelo B, Cioffi MB (2016) Karyotype and mapping of repetitive DNAs in the african butterfly fish *Pantodon buchholzi*, the sole species of the family Pantodontidae. Cytogenetic and Genome Research 149: 312–320. https://doi.org/10.1159/000450534

Sacerdot C, Louis A, Bon C, Berthelot C, Crollius HR (2018) Chromosome evolution at the origin of the ancestral vertebrate genome. Genome Biology 19: 1–166. https://doi.org/10.1186/s13059-018-1559-1

Sullivan JP, Lavoué S, Hopkins CD (2000) Molecular systematics of the African electric fishes (Mormyroidea: Teleostei) and a model for the evolution of their electric organs. Journal of Experimental Biology 203: 665–683.

Sullivan JP, Lavoué S, Hopkins CD (2016) *Cryptomyrus*: A new genus of Mormyridae (Teleostei, Osteoglossomorpha) with two new species from Gabon, West-Central Africa. ZooKeys 561: 117–150. https://doi.org/10.3897/zookeys.561.7137

Taverne L (1971) Note sur la systématique des poissons Mormyriformes. Le problème des genres *Gnathonemus*, *Marcusenius*, *Hippopotamyrus*, *Cyphomyrus* et les nouveaux genres *Pollimyrus* et *Brienomyrus*. Revue de Zoologie et de Botanique Africaines 84: 99–110.

Taverne L (1972) Ostéologie des genres *Mormyrus* Linné, *Mormyrops* Müller, *Hyperopisus* Gill, *Myomyrus* Boulenger, *Stomatorhinus* Boulenger et *Gymnarchus* Cuvier. Considérations générales sur la systématique des Poissons de l'ordre des Mormyriformes. Annales du Musée Royal de l'Afrique Centrale, Sciences Zoologiques 200: 1–194.

Uyeno T (1973) A comparative study of chromosomes in the teleostean fish order Osteoglossiformes. Japanese Journal of Ichthyology 20: 211–217.

CompCytogen 14(3): 399–416 (2020)
doi: 10.3897/CompCytogen.v14i3.56535
http://compcytogen.pensoft.net

REVIEW ARTICLE

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

Chromosomes of parasitic wasps of the superfamily Chalcidoidea (Hymenoptera): An overview

Vladimir E. Gokhman[1]

1 *Botanical Garden, Moscow State University, Moscow 119234, Russia*

Corresponding author: Vladimir E. Gokhman (vegokhman@hotmail.com)

Academic editor: D. C. Cardoso | Received 15 July 2020 | Accepted 15 August 2020 | Published 25 August 2020

http://zoobank.org/5BD67A46-B9EE-471A-AE9C-4DC8FD018845

Citation: Gokhman VE (2020) Chromosomes of parasitic wasps of the superfamily Chalcidoidea (Hymenoptera): An overview. Comparative Cytogenetics 14(3): 399–416. https://doi.org/10.3897/CompCytogen.v14i3.56535

Abstract

An overview of the current knowledge of chromosome sets of the parasitoid superfamily Chalcidoidea is given. Karyotypes of approximately 240 members of this group, i.e. just above one percent of described species, are studied up to now. Techniques for obtaining and analyzing preparations of chalcid chromosomes are outlined, including the so-called "traditional" and "modern" methods of differential staining as well as fluorescence in situ hybridization (FISH). Among the Chalcidoidea, the haploid chromosome number can vary from n = 3 to n = 11, with a clear mode at n = 6 and a second local maximum at n = 10. In this group, most chromosomes are either metacentric or submetacentric, but acrocentrics and/or subtelocentrics also can predominate, especially within karyotypes of certain Chalcidoidea with higher chromosome numbers. The following main types of chromosomal mutations are characteristic of chalcid karyotypes: inversions, fusions, translocations, polyploidy, aneuploidy and B chromosome variation. Although karyotype evolution of this superfamily was mainly studied using phylogenetic reconstructions based on morphological and/or molecular characters, chromosomal synapomorphies of certain groups were also revealed. Taxonomic implications of karyotypic features of the Chalcidoidea are apparently the most important at the species level, especially among cryptic taxa.

Keywords

base-specific fluorochromes, chalcid wasps, differential staining, FISH, karyotypes, phylogeny, taxonomy

Introduction

The superfamily Chalcidoidea is a very diverse, taxonomically complicated and economically important group of insects (Quicke 1997; Gokhman 2015b) that currently includes about 23 thousand described species (Huber 2017). Nevertheless, chromosomes of approximately 240 members of this group, i.e. just above one percent, are studied up to know (Gokhman 2009 onwards). The last detailed review of the chromosome study of Chalcidoidea was published more than a decade ago (Gokhman 2009, see also Gokhman and Gumovsky 2009), with only about 170 examined species. Consequently, important results of the karyotypic study of chalcids accumulated during this time, especially those obtained with the help of certain advanced techniques, substantially changed our views on the phylogenetic and taxonomic implications of chromosomal characters of this group (Gokhman 2013; Baur et al. 2014; König et al. 2019). An updated overview of the karyotypic study of the superfamily Chalcidoidea is therefore given below.

Techniques used for the chromosome study

Perhaps it is needless to mention that tissues with relatively large numbers of cell divisions should be examined to perform a successful chromosomal analysis of any given group. In the case of Hymenoptera, this for a long time meant studying immature stages (Crozier 1975; Imai et al. 1988; Gokhman 2009). Indeed, chromosome preparations made either from cerebral ganglia or from developing gonads of hymenopteran prepupae and early pupae apparently remain the best source of high-quality metaphase plates, which are the most suitable for morphometric analysis and application of advanced techniques of chromosome staining (Gokhman and Gumovsky 2009). However, obtaining that kind of preparation from many parasitic wasps, including chalcids, is impossible because the establishment of both host and parasitoid lab stocks is usually needed to get access to immature stages of parasitic wasps as well as to ensure reliable identification of this material based on a thorough morphological study of conspecific adults. Nevertheless, this limitation can be overcome in the case of gregarious species (Gokhman 2009). Ovaries of adult females of many parasitoid Hymenoptera can also provide certain numbers of mitotic divisions with discernible morphology of chromosomes, but this mainly applies to synovigenic species, in which oogonia generally continue to divide after eclosion of the female parasitoid from the host (Jervis et al. 2001). On the other hand, ovaries of chalcid wasps often contain meiotic divisions as well, although the number of these divisions is fairly low (Gokhman 2009). In addition, hymenopteran males, which are usually haploid, lack normal meiosis, including synapsis and the reductional division (Crozier 1975), and therefore many details of this process which are observed in diplo-diploid organisms, cannot be reported for parasitoid Hymenoptera. At present, examination of meiotic chromosomes is relatively scarce in Chalcidoidea (see e.g. Gokhman et al. 2014b), but, for example, it would be of considerable interest for studying hybrids between closely related forms with different karyotypes.

Nowadays, the technique developed by Imai et al. (1988) for obtaining air-drying chromosome preparations from prepupae and early pupae of ants, is generally used for karyotyping chalcids. However, stronger hypotonic treatment is usually needed to prevent overlapping of substantially longer chromosomes in the Chalcidoidea. In particular, I normally use 30 min incubation in the 0.5% sodium citrate solution before preparing cell suspension (e.g. Gokhman et al. 2017a), as opposed to 20 min treatment with the 1% solution recommended by Imai et al. (1988). The process also includes maceration of the tissue on the microscope slide in an aqueous solution containing both ethanol and acetic acid, and a subsequent treatment of the cells attached to the slide with an analogous although water-free fixative. However, the final step of chromosome preparation according to Imai et al. (1988), i.e. application of pure acetic acid as an additional fixative, is usually omitted in the case of Chalcidoidea and other parasitoids. I do not only consider this step redundant, but also suggest that the excessive amount of acids can hydrolyze DNA, which is crucial e.g. for performing fluorescence in situ hybridization (FISH). Nevertheless, to avoid washing the cells away from the slide during the subsequent treatment, post-fixation of the material, preferably by acid-free fixatives, is recommended (Gokhman et al. 2019a).

To visualize chromosomes of Chalcidoidea, modern optic microscopes are currently used. Additional epifluorescence modules are also needed to work with fluorochromes, including base-specific chromosome staining and FISH. Moreover, the resulting images must be captured by a modern digital camera, usually controlled through a computer. This camera should produce images with relatively high resolution (at least 300 dpi) and be sensitive enough to work with fluorescence. In turn, these images can be analyzed using specialized software, e.g. KaryoType (Altinordu et al. 2016), to determine absolute/relative lengths and centromere indices of particular chromosomes. As in all other Hymenoptera, chromosomes of chalcid wasps are monocentric, i.e. each of them carries a single centromere (Gokhman 2009). These chromosomes can be subdivided into four groups according to the centromere position, i.e., metacentrics (M), submetacentrics (SM), subtelocentrics (ST) and acrocentrics (A) generally following guidelines provided by Levan et al. (1964). In case of various types of differential staining, both localization and size of particular chromosomal segments have to be identified as well.

It is also noteworthy that precise species identifications are crucial for the karyotypic study of Chalcidoidea as well as of parasitoid Hymenoptera in general (Gokhman 2009). Bearing in mind an exceptional taxonomic complexity of this superfamily and the abundance of cryptic taxa (Gokhman 2018), expert identifications of the examined populations/strains and particular specimens should be obtained in every possible case.

Karyotypes of the overwhelming majority of chalcids were studied using only routine staining. Nowadays, chromosomes of Chalcidoidea are most often stained with Giemsa solution diluted in Sorensen's phosphate buffer (Gokhman 2009). Nevertheless, routinely stained karyotypes can be further studied using morphometric analysis which already proved its effectiveness for finding both similarities and differences between closely related forms of Chalcidoidea (Gokhman and Westendorff 2000; König et al. 2019). Use of this technique in chalcids is facilitated by the generally low chromosome numbers that are characteristic of most Chalcidoidea.

In addition, karyotypes of a few dozen members of the superfamily Chalcidoidea were examined using various methods of differential staining (Gokhman 2009). The latter techniques are often subdivided into the so-called "traditional" and "modern" ones (Gokhman 2015a). Among the former methods, various techniques of chromosome banding, i.e. C-, AgNOR- and sometimes also G-banding, are used. C- and AgNOR-banding respectively visualize constitutive heterochromatin and nucleolus organizing regions (NORs) (Sumner 1972; Howell and Black 1980). However, chromosomes of only few members of the superfamily Chalcidoidea were studied using either AgNOR- or C-banding. These species belong to the families Aphelinidae (Odierna et al. 1993; Baldanza et al. 1999; Baldanza and Giorgini 2001; Giorgini and Baldanza 2004), Eulophidae (Maffei et al. 2001; Gebiola et al. 2012), Pteromalidae (Reed 1993; Gokhman and Westendorff 2000) and Trichogrammatidae (Van Vugt et al. 2005). C-banding usually visualizes small to medium-sized pericentromeric and telomeric segments of the constitutive heterochromatin on chalcid chromosomes, but a few intercalary blocks were also revealed (Reed 1993; Baldanza et al. 1999; Gokhman and Westendorff 2000). As for AgNOR-banding, it most often detects a single NOR per haploid karyotype (Baldanza et al. 1999; Baldanza and Giorgini 2001 etc.), but two sites of this kind (and an additional NOR on a particular B chromosome) were visualized in the chromosome set of *Trichogramma kaykai* Pinto & Stouthamer, 1997 (Van Vugt et al. 2005). In the superfamily Chalcidoidea, subtelocentric/acrocentric chromosomes usually carry subterminal/terminal NORs, but these sites can be situated close to the centromeres of certain metacentrics (Baldanza et al. 1999; Giorgini and Baldanza 2004). The localization of NORs can vary among members of the same genus (Giorgini and Baldanza 2004), and this is further corroborated by FISH (see below).

G-banding is usually produced by treatment of chromosomes with certain proteolytic enzymes like trypsin (Chiarelli et al. 1972 onwards). Among chalcids, karyotypes of only three members of this group, i.e. *Encarsia berlesei* (Howard, 1906) and *E. inaron* (Walker, 1839) (Aphelinidae) as well as *Nasonia vitripennis* (Walker, 1836) (Pteromalidae) (Odierna et al. 1993; Baldanza et al. 1999; Rütten et al. 2004) were studied using G-banding. This technique identifies different chromosomes within karyotypes of the same species (Gadau et al. 2015), but apparently fails to highlight homologous elements among chromosome sets of closely related parasitoids (see e.g. Odierna et al. 1993; Baldanza et al. 1999), and therefore it cannot be used for a comparative cytogenetic study of parasitoid Hymenoptera.

The modern techniques of differential chromosome staining are mostly represented by using fluorochromes which specifically visualize AT- and GC-rich chromosome segments (Schweizer and Ambros 1994; Gokhman 2015a). Among the former dyes, 4', 6-diamidino-2-phenylindole (DAPI) is the most widely used. However, chromosomes of parasitoid Hymenoptera predominantly contain AT-rich DNA, and therefore staining chalcid karyotypes with DAPI and similar fluorochromes normally does not reveal any banding pattern (Odierna et al. 1993; Baldanza et al. 1999 etc.), sometimes except for a single negative band per haploid karyotype (Bolsheva et al. 2012). In turn, bands of this kind, which represent NORs, are usually GC-rich, and thus can be stained with chromomycin A_3 (CMA_3) or similar fluorochromes (Gokhman et

al. 2019b). Nevertheless, multiple CMA_3-positive and DAPI-negative terminal bands were recently discovered on every chromosome of a particular member of the family Eulophidae, *Trichospilus diatraeae* Cherian & Margabandhu, 1942, although it seems unlikely that they all represent NORs (Gokhman et al. 2017b). In addition, there are also several fluorochromes, like propidium iodide, which stain total DNA irrespective of its base composition (Bolsheva et al. 2012).

Nevertheless, FISH remains the most powerful tool for analyzing chromosomes of parasitoid Hymenoptera including chalcids (Gokhman 2015a). This technique seems to work particularly well with different DNA repeats (Van Vugt et al. 2005, 2009). Indeed, it is most frequently used, for example, to map clusters of ribosomal DNA (= NORs) in certain members of Chalcidoidea that belong to the families Eurytomidae, Torymidae, Eulophidae, Aphelinidae and Trichogrammatidae (Van Vugt et al. 2005, 2009; Bolsheva et al. 2012; Gokhman et al. 2014a, 2017a). Among other results, these data show that the number and localization of NORs vary within certain chalcid genera, e.g. *Eurytoma* Illiger, 1807 (Gokhman et al. 2014a; see above). Van Vugt et al. (2005, 2009) also mapped the whole fraction of repetitive DNA (C_0t-50) as well as the ITS2 and *Eco*RI repeats on chromosomes of *Trichogramma kaykai*. Analogously, Li et al. (2017) used the same approach to physically map a number of repeats on a particular B chromosome of *Nasonia vitripennis*. In addition, FISH revealed absence of the TTAGG telomeric repeat in all studied parasitoid Hymenoptera including chalcids (Gokhman et al. 2014a). Moreover, chromosome microdissection together with whole chromosome painting, a powerful technique for identifying particular chromosomes and their segments, was first applied to the karyotype of *N. vitripennis* more than 15 years ago (Rütten et al. 2004; Gadau et al. 2015). To prepare specific probes from each chromosome of this species which haploid karyotype contains five metacentrics of similar size, the chromosomes were first G-banded. Furthermore, Gokhman et al. (2019a) who applied the same technique to the chromosome sets of two cryptic species of the *Lariophagus distinguendus* (Förster, 1841) complex (Pteromalidae), were able to identify elements involved in a certain chromosomal fusion (see below).

Methods of immunocytochemistry also can be used for studying karyotypes of parasitoid Hymenoptera. Up to now, however, this technique was applied only to two closely related species, *Entedon cioni* Thomson, 1878 and *E. cionobius* Thomson, 1878 (Eulophidae) (Bolsheva et al. 2012). Specifically, chromosomes of these parasitoids were treated with antibodies against 5-methylcytosine, which visualized patterns of DNA methylation along different chromosomes.

Overview of known data

General notes

In the superfamily Chalcidoidea, haploid chromosome numbers (n) can vary from n = 3 to n = 11 (Table 1, Fig. 1). In fact, a few papers reporting n values outside of this range were also published during the previous century (Silvestri 1914;

Table 1. Chromosome numbers of different families of Chalcidoidea. Spalangiinae were earlier considered as a subfamily of Pteromalidae s.l., but they deserve the family rank (Heraty et al. 2013). Torymidae s.l. include Megastigmidae (Janšta et al. 2018), but they are treated here as a single taxon because relationships of the latter group with other chalcid families remain uncertain. Data from: Fusu 2008b, 2009, 2017; Gokhman 2009, 2010, 2015b; Gokhman and Gumovsky 2009, 2013; Bolsheva et al. 2012; Gebiola et al. 2012; Gokhman et al. 2014b, 2017a, 2019bc; Santos et al. 2015; Gokhman and Nishkomaeva 2018; Wu et al. 2019, the present paper and unpublished results of the author.

Family	No. species studied	Chromosome numbers (n)
Mymaridae	3	9, 11
Eulophidae	73	5, 6, 7, 8, 10
Trichogrammatidae	11	5
Aphelinidae	31	3, 4, 5, 6, 7, 8, 9, 10, 11
Agaonidae	8	5, 6
Encyrtidae	20	5, 8, 9, 10, 11
Eupelmidae	22	5, 6, 7, 8, 10
Eurytomidae	14	5, 6, 7, 8, 9, 10
Spalangiinae	2	4, 6
Leucospidae	1	6
Chalcididae	5	3, 5, 6
Ormyridae	2	5, 6
Torymidae s.l.	24	4, 5, 6, 10
Perilampidae	1	3
Eucharitidae	1	4
Pteromalidae	19	4, 5, 6, 7
Total	**237**	**3, 4, 5, 6, 7, 8, 9, 10, 11**

Muramoto 1993), but those results still need to be confirmed. Among chalcids, the distribution of chromosome numbers at the species level has a clear mode at n = 6, with a second local maximum at n = 10 (Fig. 1). Members of this superfamily with n = 5 are also very numerous, and the proportion of Chalcidoidea with other chromosome numbers is substantially smaller (Table 1, Fig. 1).

Just a decade ago (Gokhman 2009; Gokhman and Gumovsky 2009), chalcid families were generally subdivided into two groups according to their chromosome numbers, i.e. the so-called "low-numbered" and "high-numbered" families. Within these groups, n values ranged from 3 to 7 and 8 to 11 respectively, with just a few exceptions. Most families belonged to the first group (Fig. 2a–c), whereas higher chromosome numbers were characteristic of Mymaridae, Eurytomidae, and Encyrtidae (Table 1, Fig. 2d). In addition, Aphelinidae contained taxa with both lower and higher n values. Specifically, the subfamily Aphelininae harbored parasitoids with n = 4–5, whereas Coccophaginae often had n = 10–11 (Gokhman 2009). However, n = 3–10 was found in different species of the large genus *Encarsia* Förster, 1878 from the latter subfamily (Baldanza et al. 1999). Moreover, n = 10 was detected in *Podagrion pachymerum* (Walker, 1833) and *P. gibbum* Bernard, 1938 (Torymidae) (Fusu 2008a). Furthermore, the above-mentioned pattern also substantially changed during the last years. For example, parasitoids with lower chromosome numbers (n = 5 to 7) were found within both Encyrtidae and Eurytomidae (Gokhman and Mikhailenko 2008; Gokhman 2010). These

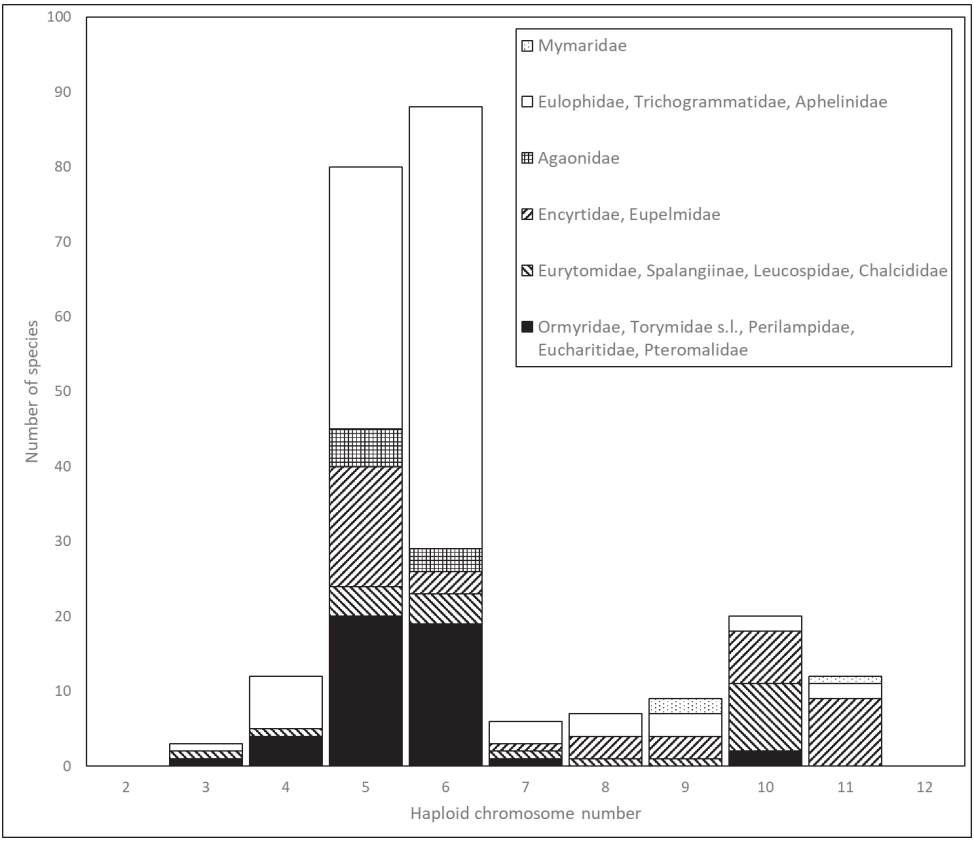


Figure 1. Distribution of main lineages of Chalcidoidea by the chromosome number at the species level (based on data from Table 1).

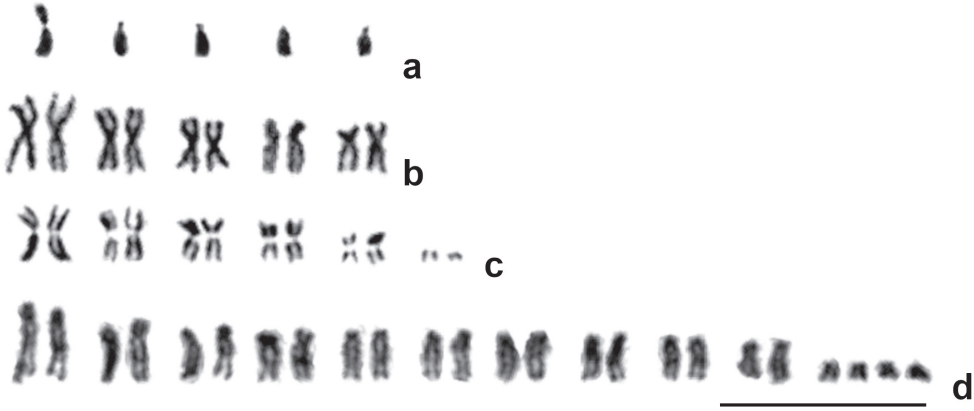


Figure 2. Representative karyotypes of Chalcidoidea **a** *Trichogramma principium* Sugonjaev & Sorokina, 1976 (Trichogrammatidae; n = 5) **b** *Mesopolobus mediterraneus* (Mayr, 1903) (Pteromalidae; 2n = 10) **c** *Oomyzus gallerucae* (Fonscolombe, 1832) (Eulophidae; 2n = 12) **d** *Eurytoma cynipsea* Boheman, 1836 (Eurytomidae; 2n = 20 + 4B). Scale bar: 10 μm.

lower n values could be attributed to independent chromosomal fusions which took place in these groups. Finally, n = 8 to 10 were also detected in certain Eupelmidae and Eulophidae (Fusu 2008b, 2017; Gokhman and Nishkomaeva 2018). As a result of these findings, most principal lineages of Chalcidoidea now include both "high-numbered" and "low-numbered" members (Table 1, Fig. 3).

Chromosomes of Chalcidoidea are generally longer than those found in many other parasitoid Hymenoptera, mainly due to lower chromosome numbers that are characteristic of most chalcids, with average chromosome lengths ranging from 5 to 7 μm (Gokhman 2009). In this group, chromosomes of the "low-numbered" taxa mostly have two distinct arms, i.e. they are either metacentric or submetacentric (Gokhman 2013; Fig. 2b, c). Nevertheless, acrocentric and/or subtelocentric chromosomes can predominate as well, often within karyotypes of certain "high-numbered" chalcids (Gokhman and Gumovsky 2009; Fig. 2d, but see also Fig. 2a). Transitions from the latter character state to metacentrics/submetacentrics usually accompany the process of consecutive chromosomal fusions (see e g. Gokhman and Mikhailenko 2008).

Among Chalcidoidea, meiotic chromosomes were examined in some detail in a few dozen members of the families Eulophidae, Aphelinidae, Encyrtidae, Eupelmidae, Eurytomidae, Torymidae s.l. (including Megastigmidae) and Pteromalidae (Fusu 2009, 2017; Gokhman 2009 and references therein, Gokhman and Gumovsky 2013; Gokhman et al. 2014b). Specifically, chalcid chromosomes can form rod-like, cross-like or ring-like bivalents in diplotene, as in other members of the order Hymenoptera. Each bivalent usually carries one or two terminal/subterminal chiasmata.

Chromosomal mutations

The following types of chromosomal mutations are characteristic of chalcid karyotypes: (Gokhman 2009): inversions, fusions (both central and tandem ones), translocations, polyploidy, aneuploidy and B chromosome variation. In addition, deletions/duplications probably also occur in this superfamily. Specifically, inversions were detected in certain members of the genus *Aphelinus* Dalman, 1820 (Aphelinidae) (Gokhman et al. 2017a). In this group, haploid karyotypes of most parasitoids that belong to the *varipes* species group with n = 4, contain two metacentric and two acrocentric chromosomes. However, *A. hordei* Kurdjumov, 1913 also has a similar karyotype structure, but the centromere of the second metacentric is significantly shifted towards the chromosome end, and in a certain sister species, *A. kurdjumovi* Mercet, 1930, this centromere becomes terminal, turning the particular chromosome into an acrocentric (Gokhman et al. 2017a). An inversion could also be involved in the process of karyotype transformation within the *Lariophagus distinguendus* species complex (König et al. 2019).

At present, direct evidence for translocations, which occur among Chalcidoidea, is generally scarce. For instance, reciprocal translocations are presumed in certain members of the family Eulophidae (Gokhman 2009). These rearrangements, together with deletions and duplications, are apparently responsible for the numerous size differences between chromosomes of related chalcid species with the same n values (Giorgini

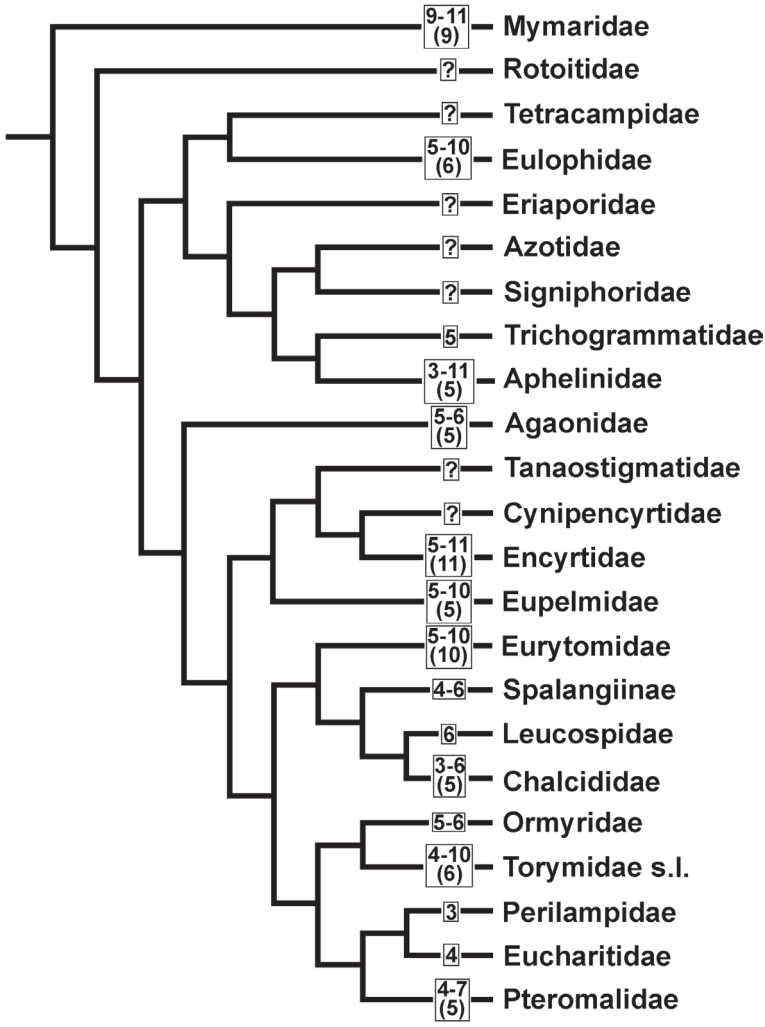


Figure 3. Variation ranges of chromosome numbers of Chalcidoidea mapped on the phylogenetic tree of chalcid families (simplified from Heraty et al. 2013). Most frequent chromosome numbers for certain taxa are given in brackets (redrawn from Gokhman 2013 and updated with data from Table 1).

and Baldanza 2004; Gebiola et al. 2012). Comparative studies of the genome size complemented with chromosome morphometrics can provide additional insights regarding possible deletions/duplications in closely related forms with similar karyotypes (Gokhman et al. 2017a). Nevertheless, detection of these mutations often requires sophisticated techniques of the chromosome study (see e.g. Gokhman et al. 2019a), and therefore more rearrangements of this kind are undoubtedly going to be discovered within chalcid karyotypes in the future.

Fortunately, other types of chromosomal mutations can be identified more easily among the Chalcidoidea, because these karyotypic changes usually affect the chromosome number of related forms. For example, this parameter decreases via chromosomal fusions,

and the products of these rearrangements can be instantly detected using e.g. chromosome morphometrics or whole chromosome painting (Gokhman et al. 2019a; König et al. 2019). Specifically, more or less well-documented consecutive chromosomal fusions were found in the Eurytomidae. Although parasitoids that belong to this group, and to the genus *Eurytoma* in particular, generally have n = 10 (Fig. 2d), but n = 5, 6 and 7 were found in *E. compressa* (Fabricius, 1794), *E. serratulae* (Fabricius, 1798) and *E. robusta* Mayr, 1878 respectively (Gokhman and Mikhailenko 2008). The number of larger metacentrics observed in these chalcids also corresponded with the above-mentioned scenario. Analogously, two studied members of the genus *Sycophila* Walker, 1871 from the same family, namely, *S. submutica* (Thomson, 1876) and *S. biguttata* (Swederus, 1795), have n = 8 and 9 respectively (Gokhman and Mikhailenko 2008; Gokhman and Gumovsky 2013). Furthermore, n = 10 is characteristic of both *Metaphycus flavus* (Howard, 1881) and *M. luteolus* (Timberlake, 1916) (Encyrtidae), but n = 9 and 5 were respectively found in *M. angustifrons* Compere, 1957 and *M. stanleyi* Compere, 1940 (Gokhman 2010). In addition, Gokhman et al. (2019a) who applied chromosome microdissection and whole chromosome painting to chromosome sets of two cryptic species of *Lariophagus distinguendus* complex with n = 5 and 6, were able to identify chromosomes involved in a particular fusion. During this process, the only acrocentric and a medium-sized metacentric in the chromosome set with n = 6 fused into the largest metacentric chromosome in the karyotype with n = 5. At present, however, it is difficult to distinguish between centric and tandem fusions in the superfamily Chalcidoidea. Nevertheless, since the haploid chromosome set containing eleven subtelocentrics or acrocentrics of similar size is considered ancestral for chalcids (Gokhman 2013), centric fusions could predominate in this group.

Polyploid individuals were found in a few groups of Chalcidoidea. For example, triploid females were found in *Nasonia vitripennis* and certain Aphelinidae (Gokhman 2009 and references therein). In the former species, diploid males and tetraploid females were also detected. However, various attempts to create a stable strain of *N. vitripennis* with tetraploid females and diploid males failed, probably due to the so-called preferential segregation of chromosomes (Crozier 1975). Nevertheless, a particular stock of *N. vitripennis* with triploid females/diploid males can be supported in the lab for many generations (Leung et al. 2019).

At present, the only reliable case of aneuploidy among chalcids is known in *Torymus bedeguaris* (Linnaeus, 1758) (Torymidae). In this species, which usually has 2n = 12, three copies of the smallest acrocentric chromosome carrying NORs were found in the only specimen with 2n = 13 (Gokhman et al. 2014a). In addition, Baldanza et al. (1999) reported n = 11 in a few male individuals of *Encarsia asterobemisiae* Viggiani & Mazzone, 1980 (Aphelinidae) normally having n = 10 and 2n = 20. However, this pattern was apparently caused by presence of a particular B chromosome (see below).

Up to now, B chromosomes were found in certain members of the superfamily Chalcidoidea. Specifically, the so-called PSR (paternal sex ratio) B chromosomes were detected in two distantly related chalcid species, i.e. *Nasonia vitripennis* and *Trichogramma kaykai* (Nur et al. 1988; Van Vugt et al. 2005). These paternally inherited chromosomes eliminate all other elements of the paternal genome from the diploid zygote, thus turning

it into the haploid one. In addition, B chromosomes which apparently do not carry sex-ratio distorting factors, were also found in a few members of the families Aphelinidae and Eulophidae (Baldanza et al. 1999; Gebiola et al. 2012; Gokhman et al. 2014b). For example, the highest number of B chromosomes among parasitoids was detected in *Pnigalio gyamiensis* Myartseva & Kurashev, 1990 (Eulophidae) with 2n = 12 + 0–6B (Gokhman et al. 2014b). Chromosomes of this kind have also been recently found in *Eurytoma cynipsea* Boheman, 1836 with 2n = 20 + 0–4B (Fig. 2d).

Phylogenetic implications of chromosomal characters

Chalcid karyotype evolution was previously studied using phylogenetic reconstructions that were based on morphological and/or molecular characters (Gokhman 2009, 2013, see also Gokhman and Gumovsky 2009). Together with other papers published during the last 10–15 years (Gokhman and Mikhailenko 2008; Gokhman 2010; Santos et al. 2015; Gokhman et al. 2017a), these studies revealed a number of synapomorphies of certain higher taxa (e.g. lower chromosome numbers shared by the Eucharitidae and Perilampidae, see Fig. 3) and related species. The best known synapomorphies of the latter kind are represented either by chromosomal fusions in the Eurytomidae and Encyrtidae or by inversions in the Aphelinidae (see above). However, understanding karyotype evolution of many supraspecific taxa of parasitic wasps is far from straightforward. For instance, a detailed molecular analysis suggests n = 6 as an ancestral chromosome number for the *Lariophagus distinguendus* complex (König et al. 2019), although n = 5 is currently considered as an ancestral value for the family Pteromalidae in general (Gokhman 2009).

The problem of phylogenetic reconstruction of karyotype evolution at the level of higher taxa can be illustrated by the example of the Eulophidae, apparently the best studied group of the superfamily Chalcidoidea (Table 1). Indeed, the haploid chromosome set containing five larger metacentrics and a smaller subtelocentric/acrocentric (n = 6) was long considered ancestral for the family, since it predominates in most previously examined lineages of Eulophidae (Gokhman 2009 and references therein). In that case, the karyotype of *Trichospilus diatraeae* which contains four longer metacentric and three shorter acrocentric chromosomes (n = 7), might originate from a centric fission from the apparently ancestral chromosome set (Gokhman et al. 2017b). However, a recent study of *Ophelimus maskelli* (Ashmead, 1900), the only member of the subfamily Opheliminae with the known karyotype, revealed n = 10 (Gokhman and Nishkomaeva 2018). Since this subfamily apparently represents a less derived group of Eulophidae (see e.g. Gumovsky 2008), n = 10 is likely to be considered ancestral for the family in general, with n = 7 and 6 arose from the preceding karyotype by consecutive chromosomal fusions (Gokhman and Nishkomaeva 2018).

In addition, numerous chromosomal fusions lead to independent origins of similar karyotypes within different lineages of Chalcidoidea (Gokhman 2013). Specifically, at least some chromosome sets with n = 10 originated from the apparently ancestral

karyotype containing eleven subtelocentrics/acrocentrics through pairwise fusions. Moreover, further consecutive rearrangements of this kind also led to the multiple origins of chalcid chromosome sets with n = 6 (five larger metacentrics/submetacentrics and a smaller subtelocentric/acrocentric; Fig. 2c). In turn, numerous karyotypes with five metacentric chromosomes (n = 5; Fig. 2b) also can originate through independent fusions of the above-mentioned subtelocentrics/acrocentrics to certain metacentric chromosomes (Gokhman 2013). These parallel transitions apparently occurred in a few distantly related chalcid families, including Eulophidae, Agaonidae, and Torymidae s.l. plus Ormyridae (Fig. 3).

Taxonomic implications of chromosomal characters

In the superfamily Chalcidoidea, karyotypic features can have substantial taxonomic implications, and these implications are the most important at the species level (Gokhman 2015b). Specifically, in a few cases different karyotypes were reported for the same parasitoids. Although some of those reports apparently resulted from misidentifications of well-defined different species (see Gokhman 2009 and references therein), cryptic taxa were also involved in certain cases. For example, a chromosome study of the supposedly well-known synanthropic parasitoid of many stored-product pests, *Anisopteromalus calandrae* (Howard, 1881) (Pteromalidae), eventually resulted in the detection and description of a new cosmopolitan species, *A. quinarius* Gokhman & Baur, 2014, with these species respectively having n = 7 and 5 (Baur et al. 2014). Analogously, two morphologically indistinguishable cryptic species with n = 5 and 6 were found in the *Lariophagus distinguendus* complex from the same family (König et al. 2019). In addition, two newly described members of the genus *Eupelmus* Dalman, 1820 (Eupelmidae), *E. barai* Fusu, 2017 and *E. vladimiri* Fusu, 2017, were earlier misidentified as *E. vesicularis* (Retzius, 1783) and *E. impennis* Nikol'skaya, 1952, although the first, the last, and the two remaining species have n = 6, 9, and 5 respectively (Fusu 2017). Similar cases are summarized and discussed in the recent review on integrative taxonomy of parasitoid Hymenoptera (Gokhman 2018).

Variation of chromosome morphology between routinely stained karyotypes of related species with the same n values was also revealed. For instance, two reproductively isolated populations of *Encarsia sophia* (Girault & Dodd, 1915) (Aphelinidae) from Spain and Pakistan have structurally different karyotypes with n = 5 (Giorgini and Baldanza 2004). We also found that chromosome sets of two members of the genus *Trichogramma* Westwood, 1833 with n = 5, i.e. *T. pretiosum* Riley, 1879 and *T. principium* Sugonjaev & Sorokina, 1976, substantially differ in their morphometric parameters (Gokhman et al. 2017b and the present paper; Fig. 2a), contrary to some previous reports for this genus (Hung 1982). Up to now, various techniques of differential staining did not reveal karyotypic differences between closely related species with the same morphology of chromosomes, but this seems possible, given the fact that members of the same genus, for instance, can differ in the number and localization of NORs (Baldanza and Giorgini 2001; Giorgini and Baldanza 2004; Gokhman et al. 2014a).

Future directions

In the coming decades, karyotypic study is undoubtedly going to become an important tool of taxonomic and cytogenetic research on many groups of parasitic wasps, including chalcids. However, this investigation can be effective only if complemented by other modern approaches and techniques. For example, it should be used in combination with a thorough morphological analysis for detecting and identifying cryptic species of parasitoids (Gokhman 2018). This is especially true for the families with a relatively high variation in chromosomal characters, e.g. Encyrtidae, Aphelinidae, Eurytomidae, Pteromalidae etc. (Gokhman 2015a). Since the genome size is generally correlated with the total length of chromosomes, but not necessarily with the overall karyotype structure (Gokhman et al. 2017a), a combined study can highlight hidden chromosomal rearrangements among closely related forms (see e.g. Moura et al. 2020). On the other hand, cytogenetic research of the superfamily Chalcidoidea per se will also benefit from using molecular and similar approaches, which include microdissection and chromosome painting (Gokhman et al. 2019a), immunochemical techniques (Bolsheva et al. 2012) and other applications. In turn, some of these techniques could be used to investigate fine structure of meiotic chromosomes of hybrids between closely related chalcid species (see e.g. König et al. 2019). Finally, modern efforts for genome sequencing can also be supported by cytogenetic studies of the Chalcidoidea in a number of ways – from providing direct estimates of the number of linkage groups (which equals to the n value) to the physical mapping of various DNA sequences, especially repetitive ones, using FISH (Gokhman 2009; Gokhman et al. 2017a).

Conclusion

Although a considerable amount of new data of the karyotypic study of the superfamily Chalcidoidea were collected and summarized during the last decade (see e.g. Gokhman 2015a), chromosomes of many chalcid taxa remain totally unknown. Nevertheless, conclusions based on the accumulated data already have important implications for genetics, taxonomy and phylogeny of this enormous group, as well as for its use in biological pest control (Gokhman 2015b, 2018). In turn, phylogenetic and taxonomic research provides essential information which enables better understanding of various cytogenetic phenomena occurring in the Chalcidoidea (Baur et al. 2014; Fusu 2017; Gokhman et al. 2017a; König et al. 2019), and I am sure both these trends are certainly going to continue in the observable future.

Acknowledgements

The author is grateful to many Russian and foreign colleagues for providing living material for the karyotypic study and for identifying specimens of Chalcidoidea as well as for the useful discussion. The present work was partly supported by a research grant no. 18-04-00611 from the Russian Foundation for Basic Research.

References

Altinordu F, Peruzzi L, Yu Y, He X (2016) A tool for the analysis of chromosomes: KaryoType. Taxon 65(3): 586–592. https://doi.org/10.12705/653.9

Baldanza F, Gaudio L, Viggiani G (1999) Cytotaxonomic studies of *Encarsia* Förster (Hymenoptera: Aphelinidae). Bulletin of Entomological Research 89: 209–215. https://doi.org/10.1017/S0007485399000322

Baldanza F, Giorgini M (2001) Karyotype and NOR localization differences between *Encarsia formosa* Gahan and *Encarsia luteola* Howard (Hymenoptera: Aphelinidae). Bollettino del Laboratorio di Entomologia agraria 'Filippo Silvestri' 56: 33–41.

Baur H, Kranz-Baltensperger Y, Cruaud A, Rasplus J-Y, Timokhov AV, Gokhman VE (2014) Morphometric analysis and taxonomic revision of *Anisopteromalus* Ruschka (Hymenoptera: Chalcidoidea: Pteromalidae) – an integrative approach. Systematic Entomology 39: 691–709. https://doi.org/10.1111/syen.12081

Bolsheva NL, Gokhman VE, Muravenko OV, Gumovsky AV, Zelenin AV (2012) Comparative cytogenetic study on two species of the genus *Entedon* Dalman, 1820 (Hymenoptera: Eulophidae) using DNA-binding fluorochromes and molecular and immunofluorescent markers. Comparative Cytogenetics 6(1): 79–92. https://doi.org/10.3897/compcytogen.v6i1.2349

Chiarelli B, Sarti Chiarelli M, Shafer DA (1972) Chromosome banding with trypsin. Genetica 43(2): 190–194. https://doi.org/10.1007/BF00123624

Crozier RH (1975) Animal Cytogenetics 3(7). Gebrüder Borntraeger, Berlin-Stuttgart, 95 pp.

Fusu L (2008a) Chromosomes of two *Podagrion* species (Hymenoptera, Chalcidoidea, Torymidae) and the evolution of high chromosome numbers in Chalcidoidea. Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza". Secţiunea Genetică şi Biologie Moleculară 9: 61–64.

Fusu L (2008b) The usefulness of chromosomes of parasitic wasps of the subfamily Eupelminae (Hymenoptera: Chalcidoidea: Eupelmidae) for subfamily systematics. European Journal of Entomology 105: 823–828. https://doi.org/10.14411/eje.2008.109

Fusu L (2009) Romanian Eupelmidae (Hymenoptera, Chalcidoidea): new cytogenetic, faunistic and host records. North-Western Journal of Zoology 5(2): 307–320.

Fusu L (2017) An integrative taxonomic study of European *Eupelmus* (*Macroneura*) (Hymenoptera: Chalcidoidea: Eupelmidae), with a molecular and cytogenetic analysis of *Eupelmus* (*Macroneura*) *vesicularis*: several species hiding under one name for 240 years. Zoological Journal of the Linnean Society 181: 519–603. https://doi.org/10.1093/zoolinnean/zlw021

Gadau J, Rütten K, Neusser M (2015) Parasitoid wasps (Hymenoptera). In: Sharakhov IV (Ed.) Protocols for Cytogenetic Mapping of Arthropod Genomes. CRC Press, Boca Raton, 257–284. https://doi.org/10.1201/b17450-8

Gebiola M, Giorgini M, Navone P, Bernardo U (2012) A karyological study of the genus *Pnigalio* Schrank (Hymenoptera: Eulophidae): Assessing the taxonomic utility of chromosomes at the species level. Bulletin of Entomological Research 102: 43–50. https://doi.org/10.1017/S0007485311000356

Giorgini M, Baldanza F (2004) Species status of two populations of *Encarsia sophia* (Girault & Dodd) (Hymenoptera: Aphelinidae) native to different geographic areas. Biological Control 30: 25–35. https://doi.org/10.1016/j.biocontrol.2003.09.013

Gokhman VE (2009) Karyotypes of Parasitic Hymenoptera. Springer, 183 pp. https://doi.org/10.1007/978-1-4020-9807-9

Gokhman VE (2010) Chromosomes of parasitic wasps of the genus *Metaphycus* (Hymenoptera: Chalcidoidea: Encyrtidae). Comparative Cytogenetics 4: 21–25. https://doi.org/10.3897/compcytogen.v4i1.29

Gokhman VE (2013) Parallel pathways of karyotype evolution in the superfamily Chalcidoidea (Hymenoptera). Russian Entomological Journal 22(3): 177–179.

Gokhman VE (2015a) Comparative karyology of parasitic Hymenoptera: between the past and the future. Proceedings of the Russian Entomological Society 86(2): 31–40. [in Russian]

Gokhman VE (2015b) Results and prospects of the chromosomal study of the main groups of economically important Chalcidoidea (Hymenoptera). Entomological Review 95(4): 450–455. https://doi.org/10.1134/S0013873815040077

Gokhman VE (2018) Integrative taxonomy and its implications for species-level systematics of parasitoid Hymenoptera. Entomological Review 98(7): 834–864. https://doi.org/10.1134/S0013873818070059

Gokhman VE, Anokhin BA, Kuznetsova VG (2014a) Distribution of 18S rDNA sites and absence of the canonical TTAGG insect telomeric repeat in parasitoid Hymenoptera. Genetica 142(4): 317–322. https://doi.org/10.1007/s10709-014-9776-3

Gokhman VE, Cioffi MdB, König C, Pollmann M, Gantert C, Krogmann L, Steidle JLM, Kosyakova N, Liehr T, Al-Rikabi A (2019a) Microdissection and whole chromosome painting confirm karyotype transformation in cryptic species of the *Lariophagus distinguendus* (Förster, 1841) complex (Hymenoptera: Pteromalidae). PLoS ONE 14(11): e0225257. https://doi.org/10.1371/journal.pone.0225257

Gokhman VE, Gumovsky AV (2009) Main trends of karyotype evolution in the superfamily Chalcidoidea (Hymenoptera). Comparative Cytogenetics 3(1): 63–69. https://doi.org/10.3897/compcytogen.v3i1.1

Gokhman VE, Gumovsky AV (2013) New data on chromosomes of Chalcidoidea (Hymenoptera). Entomological Review 93(1): 30–34. https://doi.org/10.1134/S0013873813010053

Gokhman VE, Kuhn KL, Woolley JB, Hopper KR (2017a) Variation in genome size and karyotype among closely related aphid parasitoids (Hymenoptera, Aphelinidae). Comparative Cytogenetics 11(1): 97–117. https://doi.org/10.3897/CompCytogen.v11i1.10872

Gokhman VE, Mikhailenko AP (2008) Karyotypic diversity in the subfamily Eurytominae (Hymenoptera: Eurytomidae). Folia biologica (Kraków) 56(3–4): 209–212. https://doi.org/10.3409/fb.56_3-4.209-212

Gokhman VE, Nishkomaeva EM (2018) Chromosome sets of two chalcid wasps (Hymenoptera, Eulophidae), invasive pests of eucalypts (*Eucalyptus* spp.) on the Black Sea coast of Krasnodar Territory. Entomological Review 98(6): 674–677. https://doi.org/10.1134/S0013873818060039

Gokhman VE, Nugnes F, Bernardo U (2019b) A cytogenetic study of *Baryscapus silvestrii* Viggiani & Bernardo, 2007 (Hymenoptera: Eulophidae) using base-specific fluorochrome staining. Russian Entomological Journal 28(2): 180–182. https://doi.org/10.15298/rusentj.28.2.10

Gokhman VE, Nugnes F, Bernardo U (2019c) Chromosomes of *Eupristina verticillata* Waterston, 1921 and an overview of known karyotypes of chalcid wasps of the family Agaonidae (Hymenoptera). Journal of Hymenoptera Research 71: 157–161. https://doi.org/10.3897/jhr.71.35951

Gokhman VE, Pereira FF, Costa MA (2017b) A cytogenetic study of three parasitic wasp species (Hymenoptera, Chalcidoidea, Eulophidae, Trichogrammatidae) from Brazil using chromosome morphometrics and base-specific fluorochrome staining. Comparative Cytogenetics 11(1): 179–188. https://doi.org/10.3897/CompCytogen.v11i1.11706

Gokhman VE, Westendorff M (2000) The chromosomes of three species of the *Nasonia* complex (Hymenoptera, Pteromalidae). Beiträge zur Entomologie 50: 193–198.

Gokhman VE, Yefremova ZA, Yegorenkova EN (2014b) Karyotypes of parasitic wasps of the family Eulophidae (Hymenoptera) attacking leaf-mining Lepidoptera (Gracillariidae, Gelechiidae). Comparative Cytogenetics 8(1): 31–41. https://doi.org/10.3897/compcytogen.v8i1.6537

Gumovsky AV (2008) Chalcidoid wasps of the families Eulophidae and Tetracampidae (Hymenoptera: Chalcidoidea): morpho-biological features and phylogeny. D.Sc. Thesis, Kyiv, Ukraine: Zoological Institute, National Academy of Sciences of Ukraine, 235 pp. [In Ukrainian]

Heraty JM, Burks RA, Cruaud A, Gibson GAP, Liljeblad J, Munro J, Rasplus JY, Delvare G, Jansta P, Gumovsky A, Huber J, Woolley JB, Krogmann L, Heydon S, Polaszek A, Schmidt S, Darling DC, Gates MW, Mottern J, Murray E, Dal Molin A, Triapitsyn S, Baur H, Pinto JD, van Noort S, George J, Yoder M (2013) A phylogenetic analysis of the megadiverse Chalcidoidea (Hymenoptera). Cladistics 29: 466–542. https://doi.org/10.1111/cla.12006

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

Huber JT (2017) Biodiversity of Hymenoptera. In: Foottit RG, Adler PH (Eds) Insect Biodiversity: Science and Society (2nd edn.). Wiley Blackwell, Oxford, 419–461. https://doi.org/10.1002/9781118945568.ch12

Hung ACF (1982) Chromosome and isozyme studies in *Trichogramma* (Hymenoptera: Trichogrammatidae). Proceedings of the Entomological Society of Washington 84: 791–796.

Imai HT, Taylor RW, Crosland MWJ, Crozier RH (1988) Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. Japanese Journal of Genetics 63: 159–185. https://doi.org/10.1266/jjg.63.159

Janšta P, Cruaud A, Delvare G, Genson G, Heraty J, Křížková B, Rasplus J-Y (2018) Torymidae (Hymenoptera, Chalcidoidea) revised: molecular phylogeny, circumscription and reclassification of the family with discussion of its biogeography and evolution of life-history traits. Cladistics 34(6): 627–651. https://doi.org/10.1111/cla.12228

Jervis MA, Heimpel GE, Ferns PN, Harvey JA, Kidd NAC (2001) Life-history strategies in parasitoid wasps: a comparative analysis of 'ovigeny'. Journal of Animal Ecology 70(3): 442–458. https://doi.org/10.1046/j.1365-2656.2001.00507.x

König C, Paschke S, Pollmann M, Reinisch R, Gantert C, Weber J, Krogmann L, Steidle JLM, Gokhman VE (2019) Molecular and cytogenetic differentiation within the *Lariophagus distinguendus*

(Förster, 1841) species complex (Hymenoptera, Pteromalidae). Comparative Cytogenetics 13(2): 133–145. https://doi.org/10.3897/CompCytogen.v13i2.34492

Leung K, van de Zande L, Beukeboom LW (2019) Life-history traits of the Whiting polyploid line of the parasitoid *Nasonia vitripennis*. Entomologia Experimentalis et Applicata 167: 655–669. https://doi.org/10.1111/eea.12808

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

Li Y, Jing XA, Aldrich JC, Clifford C, Chen J, Akbari OA, Ferree PM (2017) Unique sequence organization and small RNA expression of a "selfish" B chromosome. Chromosoma 126: 753–768. https://doi.org/10.1007/s00412-017-0641-x

Maffei EMD, Pompolo SG, Silva-Junior JC, Caixeiro APA, Rocha MP, Dergam JA (2001) Silver staining of nucleolar organizer regions (NOR) in some species of Hymenoptera (bees and parasitic wasp) and Coleoptera (lady-beetle). Cytobios 104: 119–125.

Moura MN, Cardoso DC, Lima Baldez BC, Cristiano MP (2020) Intraspecific variation in the karyotype length and genome size of fungus-farming ants (genus *Mycetophylax*), with remarks on procedures for the estimation of genome size in the Formicidae by flow cytometry. PLoS ONE 15(8): e0237157. https://doi.org/10.1371/journal.pone.0237157

Muramoto N (1993) A chromosome study of five hemipterans (Hemiptera) and two hymenopterans (Hymenoptera). La Kromosomo 69: 2336–2341.

Nur U, Werren JH, Eickbush DG, Burke WD, Eickbush TH (1988) A "selfish" B chromosome that enhances its transmission by eliminating the paternal genome. Science 240: 512–514. https://doi.org/10.1126/science.3358129

Odierna G, Baldanza F, Aprea G, Olmo E (1993) Occurrence of G-banding in metaphase chromosomes of *Encarsia berlesei* (Hymenoptera: Aphelinidae). Genome 36: 662–667. https://doi.org/10.1139/g93-088

Quicke DLJ (1997) Parasitic Wasps. Chapman & Hall, London, 480 pp.

Reed KM (1993) Cytogenetic analysis of the paternal sex ratio chromosome of *Nasonia vitripennis*. Genome 36: 157–161. https://doi.org/10.1139/g93-020

Rütten KB, Pietsch C, Olek K, Neusser M, Beukeboom LW, Gadau J (2004) Chromosomal anchoring of linkage groups and identification of wing size QTL using markers and FISH probes derived from microdissected chromosomes in *Nasonia* (Pteromalidae: Hymenoptera). Cytogenetic and Genome Research 105: 126–133. https://doi.org/10.1159/000078019

Santos IS, Delabie JHC, Costa MA, Mariano CSF, Silva JG (2015) First description of the karyotype of a eucharitid wasp (Hymenoptera, Chalcidoidea, Eucharitidae). Comparative Cytogenetics 9(4): 607–612. https://doi.org/10.3897/CompCytogen.v9i4.5201

Schweizer D, Ambros PF (1994) Chromosome banding. Stain combinations for specific regions. Methods in Molecular Biology 29: 97–112. https://doi.org/10.1385/0-89603-289-2:97

Silvestri F (1914) Prime fasi sviluppo del *Copidosoma Buyssoni* (Mayr), imenottere calcidide. Anatomischer Anzeiger 47: 45–56.

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

Van Vugt JJFA, de Jong H, Stouthamer R (2009) The origin of a selfish B chromosome triggering paternal sex ratio in the parasitoid wasp *Trichogramma kaykai*. Proceedings of the Royal Society B 276: 4149–4154. https://doi.org/10.1098/rspb.2009.1238

Van Vugt JJFA, de Nooijer S, Stouthamer R, de Jong H (2005) NOR activity and repeat sequences of the paternal sex ratio chromosome of the parasitoid wasp *Trichogramma kaykai*. Chromosoma 114: 410–419. https://doi.org/10.1007/s00412-005-0026-4

Wu S-B, Liu Q, Yang P, Yang D-R, Peng Y-Q, Song J (2019) Karyotype analysis of a pollinator, and a non-pollinator, of *Ficus auriculata* Loureiro. Chinese Journal of Applied Entomology 56(4): 819–825. [in Chinese]

CompCytogen 14(3): 417–435 (2020)
doi: 10.3897/CompCytogen.v14i3.54422
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Rapid chromosomal evolution in the bush-cricket *Gonatoxia helleri* Hemp, 2016 (Orthoptera, Phaneropterinae)

Elżbieta Warchałowska-Śliwa[1], Beata Grzywacz[1], Anna Maryańska-Nadachowska[1], Klaus-Gerhard Heller[2], Claudia Hemp[3]

1 *Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland* **2** *Magdeburg, Germany* **3** *University of Bayreuth, Dept. Plant Systematics, Bayreuth, Germany*

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

Academic editor: Alexander G. Bugrov | Received 18 May 2020 | Accepted 27 July 2020 | Published 28 August 2020

http://zoobank.org/FE323E4D-F956-459E-9B53-2DEDF78182D9

Citation: Warchałowska-Śliwa E, Grzywacz B, Maryańska-Nadachowska A, Heller K-G, Hemp C (2020) Rapid chromosomal evolution in the bush-cricket *Gonatoxia helleri* Hemp, 2016 (Orthoptera, Phaneropterinae). Comparative Cytogenetics 14(3): 417–435. https://doi.org/10.3897/CompCytogen.v14i3.54422

Abstract

Gonatoxia helleri Hemp, 2016 is one of the most widespread bush-crickets of the genus *Gonatoxia* Karsch, 1889 in East Africa. This species with seven large chromosomes ($2n♂ = 7$) differs from other representatives of the genus *Gonatoxia* drastically by its reduced chromosome number, the asymmetrical karyotype including karyomorphs rarely found in tettigoniids, as well as in irregularities in the course of meiosis. To better understand the origin of such an exceptional karyotype, chromosomes of 29 specimens from four populations/localities were studied using classical techniques, such as C-banding, silver impregnation, fluorochrome double staining and fluorescence *in situ* hybridization (FISH) technique with 18S rDNA and $(TTAGG)_n$ telomeric probes. FISH showed many 18S rDNA loci as well as interstitial telomeric sequences, where chromosome morphology varied in these components in terms of quantity and distribution. The 18S rDNA loci coincided with active NORs and C-banding patterns. We suggest that a combination of Robertsonian rearrangements and/or multiple common tandem fusions involving the same chromosomes contributed to the formation of this karyotype/karyomorphs. The results are the first step towards a better understanding of chromosomal reorganization and evolution within the genus *Gonatoxia*. Low chromosome number, together with the incidence of chromosomal polymorphism that is higher in *G. helleri* than previously reported in bush-crickets, implies that this species can be a valuable new model for cytogenetic and speciation studies. Our findings suggest that chromosomal translocations lead to diversification and speciation in this species and could be the driving force of adaptive radiation.

Keywords
18S rDNA, adaptive radiation, C-banding, chromosome rearrangements, FISH, fluorochrome staining, NOR, Phaneropterinae, Tanzania, telomeric DNA

Introduction

Chromosome number and structure, including their size and morphology, are important aspects of genome organization, because chromosomal variation may lead to species divergence. The analysis of the karyotype is also a useful feature in the systematic and evolutionary analysis because closely related species tend to have more similar karyotypes than more distinctly related ones (Sumner 2003). Changes in chromosome numbers (karyotype variability) or chromosome polymorphism within species as observed in many plant and animal groups may be involved in adaptation (e.g. Potter et al. 2017). The role of chromosomal rearrangements (translocations, inversions, changes in chromosome number) in the formation of reproductive barriers has been investigated and found to play a causal role in the isolation of species or populations in some genera of Hemiptera (Mills and Cook 2014, Chirino et al. 2017), Diptera (Coluzzi et al. 2002), Coleoptera (Kobayashi et al. 2000, Xavier et al. 2018), Lepidoptera (Vershinina and Lukhtanov 2017, Lucek 2018), and Orthoptera (e.g. Kawakami et al. 2011, Taffarel et al. 2015, Buleu et al. 2019, Silva et al. 2019).

Comparative cytogenetics, as a powerful tool to study karyotype variation, is based on accurate chromosome identification. Physical mapping involves fluorescence *in situ* hybridization (FISH) of specific segments of genomic DNA to their physical location on chromosomes, and it is useful in terms of gaining an insight into structural arrangements within the genome. The presence of repetitive DNA clusters in some genomic regions may represent fragile breakage sites that are associated with rearrangements during chromosome evolution (e.g. Schneider et al. 2013). Recently, series of works with FISH and conventional chromosome banding showed that the number and location of rDNA and heterochromatin sites can be useful markers for the study of tettigoniid karyotype evolution, and for the identification of genus/species-specific patterns (e.g. Grzywacz et al. 2011; Warchałowska-Śliwa et al. 2011, 2013a; Grzywacz et al. 2014a, b).

East Africa is a region of exceptional diversity of Orthoptera including Tettigoniidae bush-crickets (e.g. Hemp et al. 2013a, b, 2017). In the last few years, numerous papers have been published about East African Phaneropterinae taxa including descriptions of new genera and species combined with genetic studies, mainly on chromosome level (e.g. Hemp et al. 2010a, b, 2014, 2015a, b, c; Warchałowska-Śliwa et al. 2015; Hemp et al. 2016a, b, 2018). *Gonatoxia* Karsch, 1889 is a poorly known genus occurring in East Africa from which to date four species have been described. From *G. maculata* Karsch, 1889, little is yet known, although it is probably widely distributed throughout Tanzania, Kenya and Somalia, inhabiting deciduous dry forests and savanna woodlands in northern Tanzania; also little is known for *G. immaculata* Karsch, 1889, a species

adapted to wet lowland forest in the East Usambara Mountains and along the Tanzanian coast; *G. furcata* Hemp, 2016 is probably endemic to the Udzungwa Mountains; *G. helleri* Hemp, 2016 was found syntopically at some localities with *G. immaculata* and *G. furcata*. The ecological niche of *G. helleri* seems to be broader than in the other species of the genus, as it occurs from coastal and lowland wet forests (e.g. East Usambara Mountains) up to montane elevations (e.g. Uluguru Mountains).

Gonatoxia is a very unusual genus within the subfamily Phaneropterinae, characterized by rarely observed high variability of chromosomes (both chromosome number and structure, 2n♂ = 7, 27 or 29) in bush-crickets (Hemp et al. 2016a). Our preliminary analysis of *Gonatoxia* showed that compared to other investigated East African Phaneropterinae, *Gonatoxia helleri* had the lowest number of chromosomes (2n♂ = 7). Our cytogenetic studies indicated that such dramatic chromosomal rearrangements probably took place during a relatively early stage of speciation in *G. helleri* which is one of the most wide-spread and intriguing species of the genus so far (Hemp et al. 2016a). In the present study, a detailed cytogenetic characterization of *G. helleri* was performed using different techniques including mapping of repetitive DNA sequences characterizing chromosomal diversity. Based on the markers obtained we try to clarify the rearrangements responsible for intra- and inter-specimen chromosomal variability. It is important to investigate the potential role of repetitive DNA in the chromosomal evolution of this species.

Material and methods

Cytogenetic analysis was conducted on 19 males and 10 females of *G. helleri* collected from four populations/localities in northern Tanzania: Morogoro District, Udzungwa Mountains [Ud], National Park Headquarters, Mangula Gate, lowland wet forest, 300 m (males: CH7949, CH8048, CH8087, CH8088, CH8089, CH8144, CH8145 CH8247; females: CH8072, CH8073, CH8138, CH8139, CH8146, CH8147), and Uluguru Mountains [Ul], forest above Morningside, 1800–2100 m (males: HE89, HE96, HE105, CH8246, CH8251, CH8252, CH8253; females: CH8250, CH8289) as well as East Usambara, Nilo [Ni] forest reserve, lowland wet to a submontane forest, 450–1150 m; (male CH8134, HE97, HE104; female CH8135) and Sigi Trail [Si], 450 m, East Usambara Mountains (male CH862; female CH8136)

Testes, ovaries, and somatic hepatic caeca were dissected, incubated in hypotonic solution (0.9% sodium citrate) and fixed in Carnoy's solution [ethanol – acetic acid (3:1, *v/v*)], squashed in 45% acetic acid, followed by removal of coverslips using the dry ice technique and air-drying. For karyotyping and the identification of chromosome rearrangements, the preparations from all specimens were used for C-banding according to Sumner (1972). Additionally, some slides were analysed qualitatively by CMA_3 (chromomycin A_3) and DAPI (4,6-diamidino-2-phenylindole) staining (Schweizer 1976) as well as by $AgNO_3$ (silver nitrate) staining to visualize active nucleolus organizer regions (NORs) (Warchałowska-Śliwa and Maryańska-Nadachowska 1992).

The best preparations (for individuals Ud: CH7949, CH8048, CH8088; Ul: HE89, HE96, CH8252; Si: CH621; Ni: HE97) were used for fluorescence *in situ* hybridization (FISH). All FISH experiments with 18S rDNA and telomeric probes were carried out according to the protocol described in Grzywacz et al. (2018). Unlabelled 18S rDNA probe was generated by PCR, using the genomic DNA of bush-crickets as templates. The probe was labelled with biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany). The telomeric probe was generated by non-template PCR. The unlabelled telomeric probe was labelled with digoxigenin-11-dUTP (Roche Diagnostics GmbH). The detection of biotin-16-dUTP and digoxigenin-11-dUTP was carried out by avidin-FITC (Invitrogen, USA) and anti-digoxigenin rhodamine (Roche Diagnostics GmbH), respectively. Finally, the slides were counterstained with DAPI and mounted in the DABCO-based antifade solution. Preparations from FISH experiments were observed under a fluorescence microscope. Color images were recorded with a CCD DS-U1 camera using the NIS-Elements BR 3.0 software package. For each individual, at least 10 mitotic metaphase (oogonial/spermatogonial) and/or 20 meiotic divisions were analyzed using different markers.

Results

The study of mitotic metaphase spermatogonial, oogonial, and somatic gastric caeca cells showed 2n = 7 (6+X), FN = 10–13 in most cells of the male and 2n = 8 (6+XX), FN = 11–14 in the female. In the karyotype, the first long pair of autosomes was metacentric, whereas the second long (three main karyomorphs) and small third pairs (four main karyomorphs) were polymorphic with respect to the morphology of homologous chromosomes in specimens of the analyzed localities. The 2nd chromosome pair showed three main karyomorphs: homozygous metacentric (2A) [18 specimens: Udzungwa (Ud) 9, Uluguru (Ul) 3, Nilo (Ni) 4, Sigi (Si) 2], heterozygous – subacro/ acro (2B) [9 specimens: Ud 5, Ul 4] and homozygous acrocentric (2C) [2 individuals from Ul]. The 3rd chromosome pair was greatly polymorphic and was observed in both Ud and Ul populations. It should be noted that in individuals from Si and Ni populations (few individuals analyzed), the 1st and 2nd chromosome pairs were homozygous (both bi-armed) in terms of chromosome morphology. The acrocentric sex chromosome (X) was the largest element of the set (Figs 1–3).

Constitutive heterochromatin blocks with pericentromeric thick C-bands were found in all chromosomes. Additionally, the bi-armed first pair possessed thin telomeric and two interstitial (near the centromeric region in one arm and thin near the end in the second arm) C-bands, which are a feature in distinguishing this pair from the 2nd pair, more or less similar in size. The heterochromatin in the 1st pair revealed a discrete size polymorphism in the C-patterns. Also, the 2nd (karyomorphs 2A, 2B, 2C) and 3rd chromosome pairs showed heteromorphism in terms of the size/locality of bands on respective homologous chromosomes. Pericentromeric, interstitial and terminal C-bands with differences in size were observed on the acrocentric X chromosome (Figs 1a, a', 2a, e, 4). The secondary constriction (not always seen) of the 2nd chromosome and the

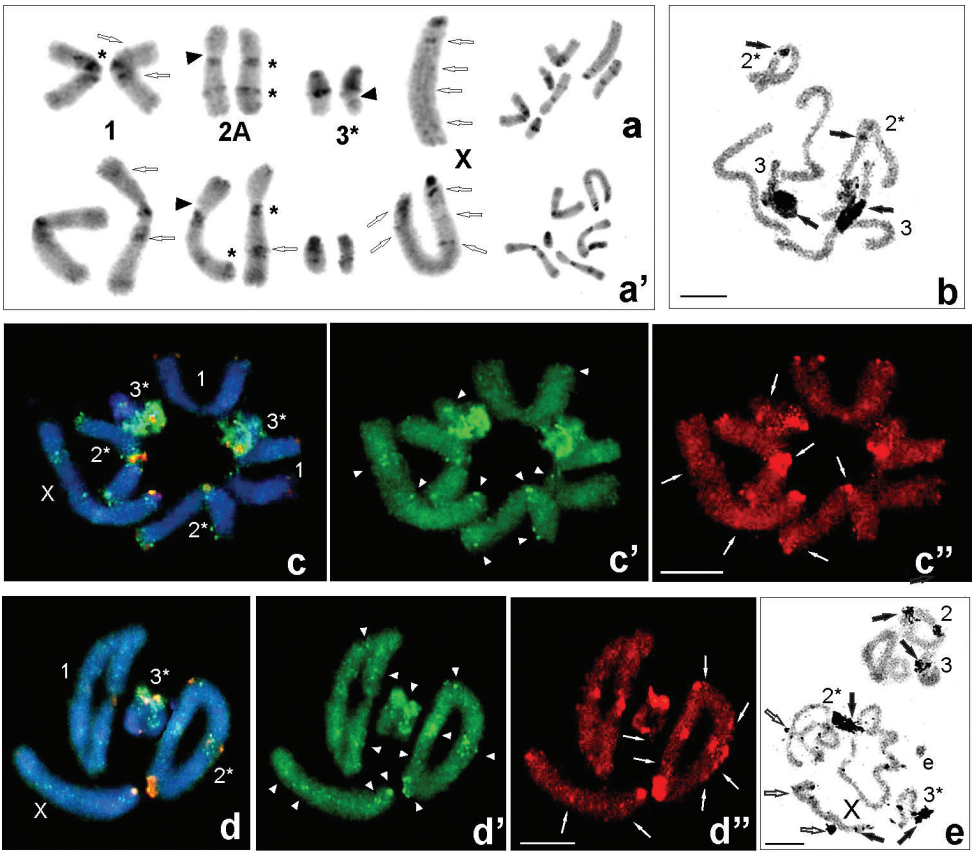


Figure 1. Examples of C-banding (**a, a'**), silver nitrate staining (**b, e**) and fluorescence *in situ* hybridization (**c–c''**, **d–d''**) in individuals with karyomorph 2A from populations: Udzungwa Mts (Ud CH8089) (**a**), Sigi (Si CH8621) (**a'**), Nilo (Ni HE97) (**b, c–c'', d–d'', e**). C-banding karyotypes of males chromosome complement (arranged from mitotic metaphase – right side); open arrows point to interstitial C-bands in the X chromosome and chromosome pairs 1st and 2nd; black arrowheads indicate secondary constriction (**a, a'**). $AgNO_3$ staining in male spermatogonial metaphases (**a**) and metaphase I/ diplotene (**e**) revealed medium sized and large active NORs of the bivalents 2nd and 3rd (black arrows) and very small NORs seen in the X (open arrows). FISH using 18S rDNA (green – **c, c', d, d'**) and telomeric DNA (red – **c, c'', d, d''**) probes in mitotic metaphase (**c**) and metaphase I (**f**); white arrowheads point to rDNA clusters near centromeric, interstitial and telomeric regions of the chromosomes (**c', d'**) and white arrows ITS signals (**c'', d''**). Heterochromatin (**a, a', b, e**) and hybridization areas (**c, d**) vary in size between homologous chromosomes, which are marked with asterisks (*). Elements (e) arisen from rearrangements were found (**e**). The X chromosome is indicated. Scale bars: 10 μm.

X chromosome were located near the C-band (Figs 1a, a', 2a, e). Besides a large active NOR in 3rd chromosomes/bivalent and a smaller in the 2nd, being coincident with secondary constrictions, a site with faint silver nitrate staining was observed, indicating the occurrence of small NORs, probably "secondary NORs", in bivalents and the X chromosome (Figs 1b, e, 2b). Positive C-blocks in the 1st chromosome pair were neutral for G+C (DAPI+) and A+T (CMA_3+) base pairs, whereas the 2nd and 3rd pair

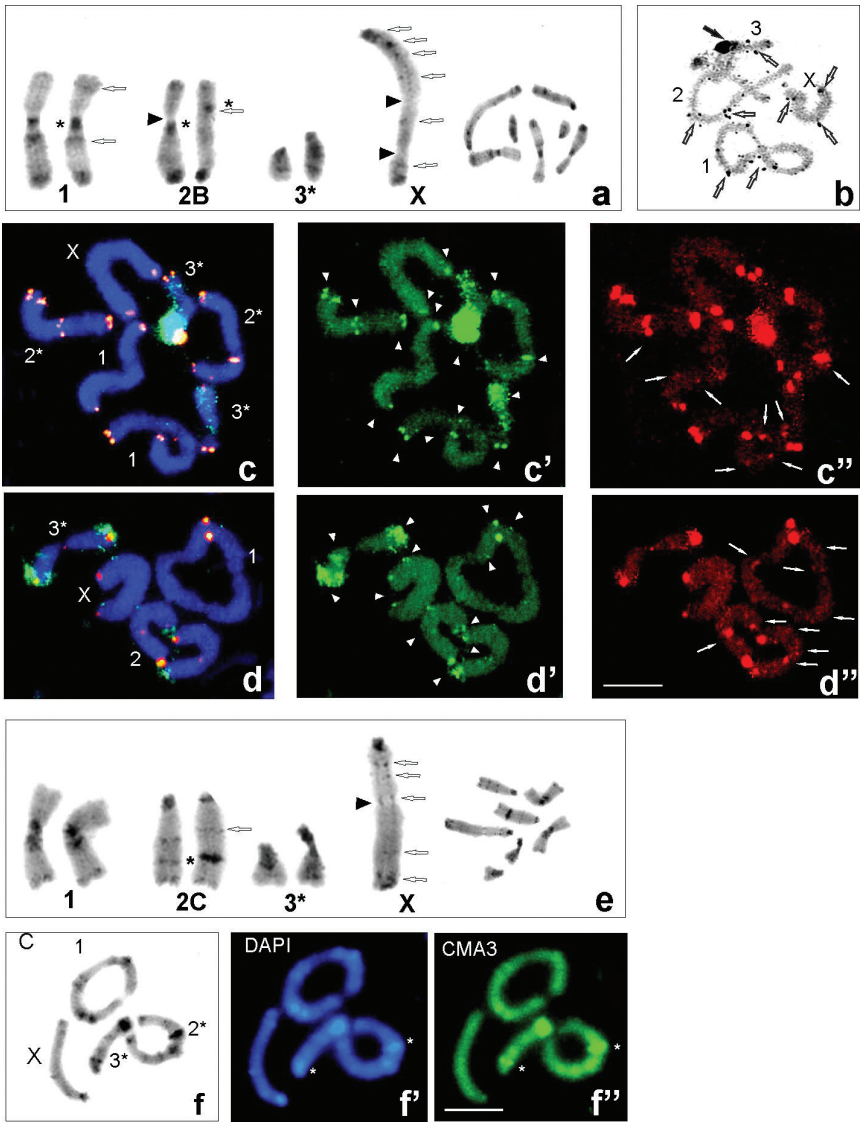


Figure 2. Examples of C-banding (**a, e**), silver nitrate staining (**b**), C-, DAPI and CMA_3 stained heterochromatin (**f–f''**) and FISH (**c–c'', d–d''**) in individuals with karyomorph 2B (**a–d''**) and 2C (**e, f''**) from populations: Udzungwa Mts (Ud CH7949 and CH8088) (**a, b),** Ud CH8088 **(c–c'')**, Uluguru Mts (Ul HE89) (**d–d'')**. C-banding karyotypes of males chromosome complement (arranged from mitotic metaphase – right side); open arrows point to interstitial C-bands in the X chromosome and chromosome pairs 1st and 2nd; black arrowheads indicate secondary constriction (**a, e**). $AgNO_3$ staining (**b**) at diplotene revealed large active NOR of the 3rd bivalent (black arrows) and very small NORs seen in the X and bivalents (open arrows). FISH using 18S rDNA (green – **c, c', d, d'**) and telomeric DNA (red – **c, c'', d, d''**) probes in mitotic metaphase (**c**) and diakinesis (**d**); white arrowheads point to rDNA clusters near centromeric, interstitial and telomeric regions of the chromosomes (**c', d'**) and white arrows ITS signals (**c'', d''**). C/DAPI/CMA_3 blocks were located very close to each other, but bright CMA_3 signals coincided with active NORs. Heterochromatin (**a, e, f**) and hybridization areas (**c, d**) vary in size between homologous chromosomes, which are marked with asterisks (*). The X chromosome is indicated. Scale bars: 10 μm.

of chromosomes with interstitial C-bands revealed clearly seen CMA_3+ block (for example Fig. 2f, f', f") which coincided with active NORs.

Physical mapping by FISH with the 18S rDNA and telomeric probes was performed in eight individuals from four analyzed populations (Ud: CH7949, CH8048, CH8088; Ul: HE89, HE96, CH8252; Si: CH621; Ni: HE97). Generally, all examined specimens demonstrated similar rDNA signals located in the centromeric, interstitial and telomeric regions and usually were connected with C-positive regions. The acrocentric/ subacrocentric 3rd chromosome pair carried major rDNA located near the centromeric region and interstitial minor 18S rDNA clusters. Additionally, low-intensity/small clusters on the 2nd chromosome pair and the X chromosome, both near the centromeric regions were observed (Figs 2c, c', d, d', 3c, c', d, d', 4). Heteromorphism of rDNA-signals (marked with an asterisk), similar to C-heterochromatin bands (Figs 1a, a', 2a), was observed in terms of the size/ strength or presence/ absence on the homologous chromosome depending on the karyomorphs in the 1st, 2nd and 3rd chromosome pairs. Some interstitial C-positive regions in the sex chromosome contained rDNA (Figs 1c', d', 2c',d', 4).

FISH analyses with the $(TTAGG)_n$ probe generated signals in telomeres of all chromosomes but the size of the clusters on different arms of some chromosomes and between individuals, with different karyomorphs varied as well (Figs 1c", d", 2c", d"). In addition to the typical telomeric, of the so-called interstitial telomeric sequences (ITSs) in the inner parts of all chromosomes were observed at both mitotic metaphase (Figs 1c", 2c") and diakinesis/ metaphase I (Figs 1d", 2d"). The signals within secondary constrictions were much larger than those at the chromosome ends. The 2nd chromosome pair in the karyomorph A exhibited heteromorphic signals of ITS in both the bi-armed chromosomes in the centromeric region (Fig. 1c", d"). Whereas bright hybridization signals were detected in the centromeric region in the bi-armed homologue and interstitially located in the acrocentric homologue of the 2nd pair in karyomorph B (Fig. 2c", d"). Besides that, additional week ITS signals, present in a low copy number, in subterminal/ medial position in autosomes and sex chromosome were observed (Figs 1c", d", 4).

In some individuals with a chromosome number close to the diploid count, intra-individual variability cells with 14 (male) and 16 (female) chromosomes were observed, probably corresponding to tetraploid levels. Thus, based on the analysis of 50 metaphase cells per individual, about 11% oogonial (Ud: CH8073, CH8138, CH8147, Ni: CH8135, Ul: CH8250) and about 5% spermatogonial cells (Ud: CH7949, CH8048, CH8088, CH8145, Ul: HE89, HE96, HE105, CH8251, CH8251, Ni: CH8134) had tetraploid chromosome numbers (Fig. 3a, c, d). However, in somatic gastric caeca mitotic metaphase in both male (Ud: CH7949, CH8088, Ul: CH8252, Si: CH8621) and female (Ud: CH8146, CH8147, Ul: CH8289, Ni: CH8135) cells with intra-individual variability of chromosome numbers were not observed.

Cytogenetic preparations of the females did not show cells in meiotic division. Male meiosis was classified as synaptic and chiasmate because the chromosomes were generally paired during early pachytene stage and bivalents exhibited a chromosomal configuration indicating crossing over (Fig. 3f upper right corner). In anaphase I, the

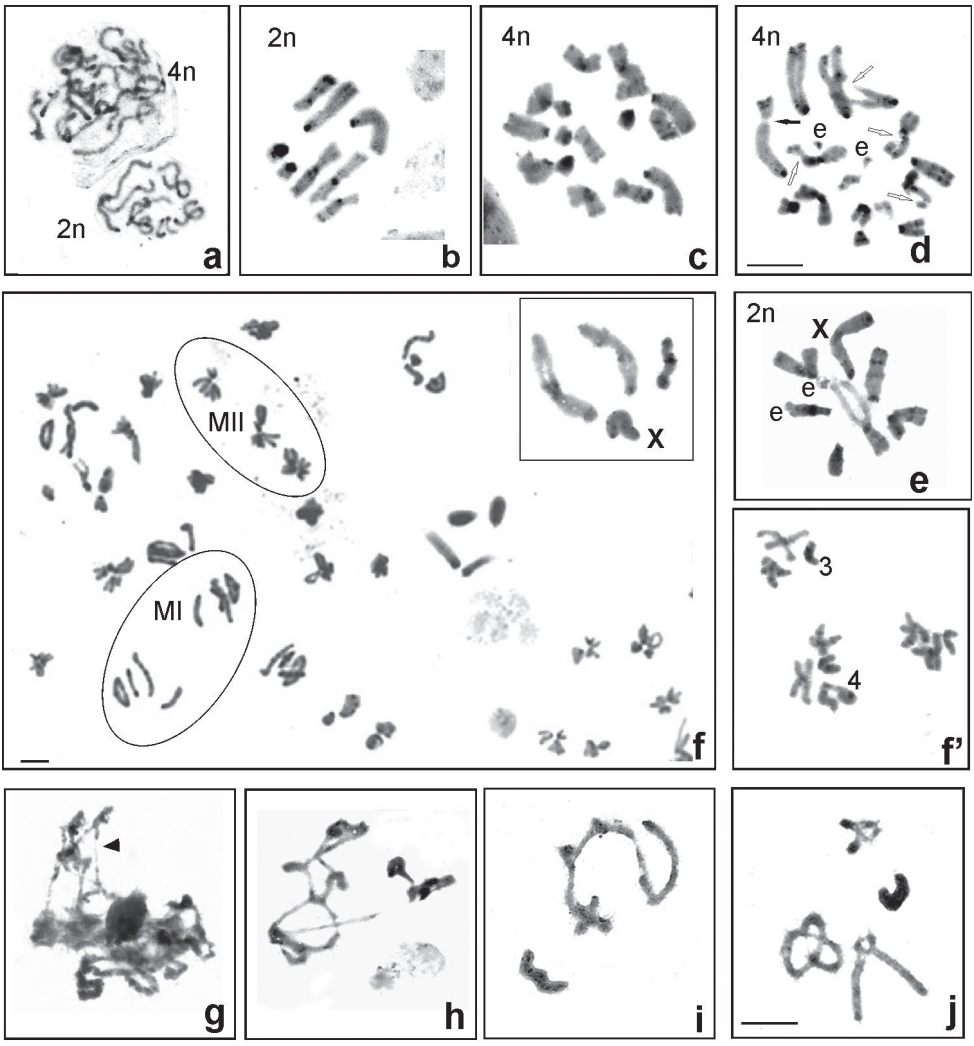


Figure 3. C-banded mitosis and meiosis (**a–j**). Spermatogonial early metaphase (Uluguru Mts [Ul] HE96) (**a**), oogonial metaphase (Udzungwa Mts [Ud] CH8138) (**b, c, d**) and spermatogonial metaphase (Ud CH8088) (**e**) with diploid and tetraploid cells. Black arrow indicates secondary constriction and open arrows point of deletions in one of homologous chromosomes (**d**). Elements (e) resulting from rearrangements were found in both female and male cells (**d, e**). During meiosis, bivalents show crossing over in metaphase I (**f** – in the right corner) and normal metaphase II complements with 3 (3+0) or 4 (3+X) chromosomes (**f, f'**). Arrowhead indicates asynapsis in early prophase I (**g**). In diplotene/diakinesis, a multivalent-like chain (**h**) or end-to-end association comprising three autosomal elements (**i**) as well as asynapsis in individuals Ul CH8246 (**g**) and Ud CH8088 (**h–j**) were observed. The X chromosome is indicated. Scale bars: 10 μm.

X chromosome segregated to one pole and in metaphase II, there were 3 (3+0) or 4 (3+X) chromosomes, confirming the regular segregation of all chromosomes during the first meiotic division. (Fig. 3f, f'). However, in some specimens from different populations: Ud (CH8088, CH8089, CH8144, CH8138, CH8147, CH8247),

Ni (HE97) and Ul (HE96, CH8246) sporadically in pachytene spermatocytes, the chromosomal elements showed asynapsed interstitial and/ or terminal chromosomal segments (Fig. 3g, j) and in the prophase I a multivalent-like chain or end-to-end association comprising three autosomal elements (Fig. 3h, i). Furthermore, lightly stained, less condensed chromatin in some regions on the bivalents (chromosome pair 3rd) or one of homologous chromosomes in mitotic metaphase, deletions, or fragments of chromosomes/ elements were observed in both male (Fig. 3d, j) and female (Fig. 3e) cells. Additionally, in these populations, very rarely mitotic spermatogonial cells showed intra-individual variability since one autosome was lacking.

Discussion

The result obtained here is in accordance with the previous study about diploid chromosome numbers in *Gonatoxia helleri* (Hemp et al. 2016a). It is probably a young species with specific karyotypic macrostructure, a very interesting case with the lowest number of chromosomes (2n♂ = 7, X0 type of sex determination) compared to other species of the genus *Gonatoxia* (2n♂ = 29, 27, X0) and Phaneropterinae as a whole. Such a low number of chromosomes, in addition to morphological differences, allowed describing the analyzed individuals as a new species in this genus (Hemp et al. 2016a). To date, a similar karyotype has been described only in the Australian *Yutjuwalia sallyae* Rentz, 2001 (Listroscelidinae), with 7 metacentric chromosomes in the male, but without additional information (Ueshima 2001). Most East African bush-crickets of Phaneropterinae, e.g. *Parapyrrhicia* Brunner von Wattenwyl, 1891, *Eurycorypha* Stål, 1873, and *Plangia* Stål, 1873 have uniform karyotypes (Hemp et al. 2013a, 2015a, 2017) with a diploid chromosome number (2n♂) 31 or 29 and the X0 type of sex determination.

The main characteristic of the karyotype of *G. helleri* is the presence of very large autosomes compared to the other species of this genus, based on the analysis of the main relative lengths of the autosomes (Warchałowska-Śliwa et al. in preparation). This reflects the derivation from multiple rearrangements. Even under the assumption that fusions or inversions are frequent in orthopteran chromosomal evolutionary history (e.g. White 1973, Hewitt 1979, Warchałowska-Śliwa 1998) leading to a reduced diploid number, the karyotype of *G. helleri* is an extreme example compared to other phaneropterines and *Gonatoxia* species (Warchałowska-Śliwa et al. in preparation). In specimens analyzed in this paper, probably Robertsonian translocations (centric fusion) and tandem fusions and/or inversion were the reason of the observed morphological arrangements in the 1st chromosome pair in all investigated populations of *G. helleri*. However, in the 2nd chromosome pair, such types of chromosomal rearrangements occurred only in the Sigi (Si) and Nilo (Ni) populations of the species. In some individuals, the 2nd chromosome pair [Udzungwa Mts (Ud) and Uluguru Mts (Ul)] and the 3rd chromosome pair (Ud, Ul, and Ni) were polymorphic with respect to the morphology (bi-armed or acrocentric) of homologous chromosomes. Unfortunately, not much can be said about the variability of the karyotype in individuals from the Sigi population due to the insufficient number of individuals analyzed. Robertsonian trans-

locations are the basic mechanism of rearrangements of chromosomes in the evolution of the orthopteran karyotype, especially in Acrididae grasshoppers (e.g. Taffarel et al. 2015) and some katydids Tettigoniinae, Saginae and Bradyporinae (Warchałowska-Śliwa et al. 2009, 2013a; Grzywacz et al. 2017). These rearrangements are not usually found in Phaneropterinae, as here tandem fusions dominate in karyotype evolution (e.g. Warchałowska-Śliwa 1998). Due to such extensive reorganization, it is very difficult to determine the order and evolutionary causes of these specific rearrangements that led to the extreme karyotype evolution of *G. helleri*, compared to the probable ancestral number of chromosomes (2n♂ = 29) in the genus *Gonatoxia*.

Physical mapping involving fluorescence *in situ* hybridization of specific segments of genomic DNA is extremely useful in terms of making an insight into structural rearrangements within the genome. Repetitive sequences change rapidly during evolution, providing excellent markers for the identification of chromosomes, chromosome segments and the resulting evolutionary chromosome rearrangements (e.g. in crickets: Palacios-Gimenez et al. 2015). Generally, Phaneropterinae usually carry one rDNA/ NOR (per haploid genome), found in the pericentromeric region or rarely located interstitially (e.g. Grzywacz et al. 2011; Warchałowska-Śliwa et al. 2011, 2013b; Grzywacz et. al. 2014a, b). In other orthopterans, a high number and variation of rDNA was observed mainly in the grasshopper taxa *Podisma sapporensis* Shiraki, 1910 and *P. pedestris* (Linnaeus, 1758) (Veltsos et al. 2009, Grzywacz et al. 2019), *Eyprepocnemis plorans* (Charpentier, 1825) (Cabrero et al. 2003), or *Abracris flavolineata* (De Geer, 1773) (Ferretti et al. 2019). Compared to phaneropterines and other tettigoniids, the chromosomal distribution of major 18S rDNA in *G. helleri* described in this paper is unique because: (i) it occurs in a high number of sites with different intensity of hybridization signals, (ii) it is located pericentromerically, interstitially and near the telomeric regions, (iii) clusters of various size or heteromorphic structures occur in the same chromosome pair. The 3rd chromosome pair (most variable in the amount of heterochromatin) carried large/ high intensity clusters near the pericentromeric region, whereas in the 2nd chromosome pair and the X chromosome this cluster was very small. Additionally, interstitial regions with a very low intensity of hybridization signals of 18S rDNA were seen located in different chromosomes (Fig. 4). Generally, our results demonstrate a coincidence between the location of rDNA loci, C-positive (Fig. 4) and GC-rich heterochromatin regions as well as active NORs (occurring in secondary constrictions and small ones in bivalents and the X chromosome). It should also be noted that heterochromatin amplification or loss could be responsible for the variation in the morphology among karyomorphs in all three pairs of chromosomes. In some grasshoppers a large variety of localization of 18 rDNA and tDNA clusters is not associated with structural rearrangements of chromosomes in the karyotype but to the evolution/massive amplification of repetitive DNA (Bugrov et al. 2016, Buleu et al. 2019).

Both conventional heterochromatin staining and rDNA-FISH revealed size heteromorphism/polymorphism between homologous chromosomes, indicating either recent or rapid evolution in this species. The presence of individuals with heteromorphic pairs may be a result by unequal meiotic cross-over, tandem duplication of ribosomal

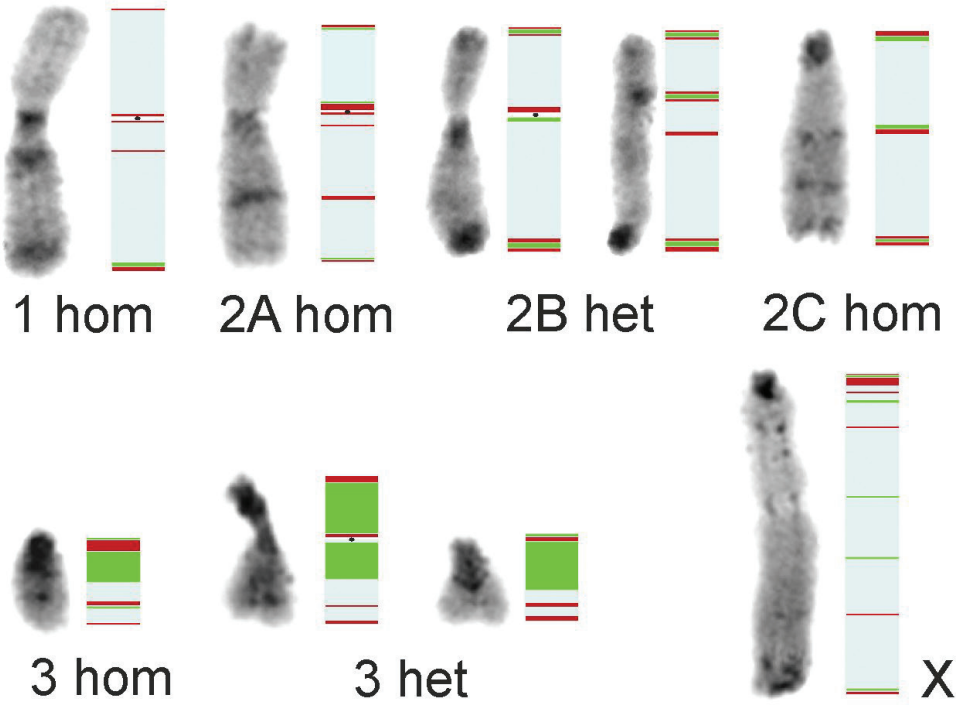


Figure 4. Scheme summary the distribution of C-banding pattern (represented in left side), 18S rDNA (green) and as well as true telomeres (at the ends) and interstitial telomeric (ITS – in the inner parts) repeats (red) of *Gonatoxia helleri*. Three chromosome pairs (1st and polymorphic 2nd and 3rd) in main karyomorphs [homozygous metacentric (1A hom, 2A hom, 3 hom), heterozygous – submeta/ acrocentric (2B het, 3 het), homozygous acrocentric (2C hom)] and X chromosome showed differences in the size and position of rDNA and tDNA signals detected by FISH and generally demonstrate a coincidence between the location of rDNA loci and C-positive heterochromatin regions. The presence of ITSs near the pericentromeric and interstitial and/ or near telomeric region suggest that karyomorphs could be the result of different chromosomal rearrangements.

sequences and related to sister chromatid exchange or translocation rearrangements or homologous recombination (e.g. Cabral-de-Mello et al. 2011; Warchałowska-Śliwa et al. 2013a,b; Grzywacz et al. 2014a, b).

Another universal probe is the telomeric sequence [tDNA, $(TTAGG)_n$]) that itself is an ideal marker for the identification of chromosome ends (Kuznetsova et al. 2020) and a marker for chromosome rearrangements, being conserved in many groups of insects (Vítková et al. 2005). Interstitial telomeric sequences (ITSs) reflect remnants of multiple chromosome fusions of ancestral chromosomes e.g. in moths (Lepidoptera, Noctuidae: Rego and Marec 2003), giant water bugs (Hemiptera: Chirino et al. 2017) or grasshoppers (Jetybayev et al. 2012, Grzywacz et al. 2019). The individuals examined in this study showed differences in the intensity and position of the hybridization signals of the $(TTAGG)_n$ probes in both autosomes and sex chromosomes (Fig. 4). Variation in the intensity of signals in chromosomes, including sex chromosomes, could

result from differences in the length of target TTAGG sequences. Sometimes the lack of hybridization signals in some ends of chromosomes suggests a low number of copies of telomeric repeats (López-Fernández et al. 2004).

The karyotype described here for *G. helleri* is different from that described for other species of this genus since we found a reduction in the number of acrocentric pairs (Hemp et al. 2016a), and probably an inter-population variation (more individuals should be analyzed). In this species several sites with ITS were identified in addition to the terminal/true telomeric sequences. The presence of ITSs near the pericentromeric and interstitial and/ or near telomeric region (Fig. 4) suggest that this karyotype /these karyomorphs could be the result of telomere-telomere fusions of the chromosomes, inversions (intra-chromosomal rearrangements), unequal crossing over, or the insertion of telomeric DNA into unstable sites during the repair of double-strand breaks (e.g. Bolzán and Bianchi 2006). ITS repeats in the pericentric C-positive block of the biarmed chromosome pairs in the 2nd and the smaller 1st chromosome pairs of *G. helleri* created through centric fusion has never been recorded in any phaneropterine so far. Thus, it cannot be excluded that these centric fusions are not the result of Robertsonian rearrangements (i.e. fusion-fission cycle) but are true fusions that left remnants of telomeric DNA in the arms of the acrocentric chromosomes involved in the fusion. A similar observation was described in the grasshopper species *Chorthippus jacobsoni* (Jetybayev et al. 2012). In this case, the chromosome break points are localized near the centromere, which are less frequently involved in the formation of dicentric chromosomes. Additionally, it is also worth noting that the acrocentric X chromosome with some interstitial ITSs thin C-bands (Figs 1c', d', 4) and sometimes secondary NORs (Figs 1a, a', e, 2a, b, e) might have undergone sequential inversions and/or end-to-end fusions during the chromosome evolution of *G. helleri*.

Some individuals of *G. helleri* exhibited multivalent chromosome associations during meiosis I, asynapsed and/or heterosynapsed chromosome segments and bivalents with distinctly associated regions (gaps and less condensed chromatin) in postpachytene nuclei. These findings indicate that certain chromosome regions were non-homologous and carried heterozygous chromosomal rearrangements. Various degrees of heterosynapsis/asynapsis have also been described in other organisms, which were heterozygous for paracentric or pericentric inversions in grasshoppers or scorpions (e.g. Díez and Santos 1993, Mattos et al. 2013). Variation as the chromosome polymorphism observed in *G. helleri* indicates that the meiotic segregation of these chromosomes has not led to the production of gametes with unbalanced chromosomes and consequent fertility loss. It suggests that some of the chromosome mutations had no negative impact on the carriers and were neutral or may have even increased fitness (Menezes et al. 2013). In *G. helleri*, based on the orientation of the chromosomes in metaphase I and the haploid complement observed in metaphase II, the chromosomes analyzed in the present work probably underwent balanced segregation. A fascinating intra-individual variability of chromosome numbers in some mitotic spermatocytes/oogonia and in meiotic cells was observed as tetraploid cells. This observation could indicate the occurrence of endopolyploidy. The absence of polyploid cells in metaphase II nuclei can be assumed

that the "aberrant cells" degenerated during the cellular cycle as errors in chromosome segregation (Mattos et al. 2013). The formation of polyploid mitotic cells can be probably associated with problems in cytokinesis that disrupt chromosome segregation.

Based on the results presented in this paper, we suggest that the change in chromosome numbers associated with multiple chromosomal rearrangements and observed heterozygous chromosomes may have presented a precondition to colonize new habitats and might be a case of adaptive radiation in *G. helleri*. Generally, the occurrence of chromosomal changes may be the result of ancestral allopatry, sympatry, and/or hybridization (meiotic and mitotic instability), demographic processes associated with colonization (founder effect), environmental fragmentation or a combination of these factors, and it may also point to recent speciation processes and hybridization (e.g. Dion-Côté et al. 2017; Gould et al. 2017). An excellent example for the role of chromosomal rearrangement in speciation in orthopteran insects is the Australian genus of morabinae grasshoppers *Vandiemenella* Key, 1976, specifically the *viatica*-species group. All taxa of this genus show extensive chromosomal variation, parapatric distribution patterns, and narrow zones at their boundaries. A number of population genetic and phylogenetic studies showed "extensive non-monophyly of chromosome races and suggest that geographical isolation leading to the fixation of chromosomal variants in different geographic regions, followed by secondary contact, resulted in the present day parapatric distribution of chromosomal races" (review Kawakami et al. 2011).

Gonatoxia helleri is the only *Gonatoxia* species able to inhabit almost the complete offer of ecological niche forests in eastern Africa, while most other species of this genus are restricted to certain forested types. Many or maybe even most bush-cricket taxa probably were first forest dwellers and later adapted to open land habitats in Africa (Voje et al. 2009) due to the beginning aridification of Africa about 8 Mya (deMenocal 2004; Cohen et al. 2007). It cannot be excluded that inter-population differences in the genome may be a sign that species in high elevations of the Uluguru Mts and Udzungwa Mts have a different karyomorph compared to lowland populations of *G. helleri* along the coast, East and West Usambara (Nilo and Sigi populations). On the other hand, there may already have been a selection of different populations because they are now isolated from each other. Our results suggest that forest dwelling *Gonatoxia* species with a restricted area of occurrence, such as *G. furcata* and *G. immaculata*, are more basal taxa, and species which are wide-spread and inhabiting a broad ecological niche, such as *G. maculata* and *G. helleri*, might be evolutionary more recent species. *Gonatoxia helleri* is probably the most plastic and adaptive species as well as has the most dramatically rearranged genome and inter-specific differences even within one population and might be the youngest species of the genus.

In conclusion, the cytogenetic analysis of *G. helleri* provides a new example of chromosomal evolution by multiple rearrangements. Several rearrangements, probably including primary (insertion, deletion or duplication, peri- or paracentric inversion, and intra- or interchromosomal reciprocal translocation) or secondary translocations were responsible for the formation of the karyotype and karyomorphs in *G. helleri*. The bi-armed chromosomes of the 1st pair occurring in individuals from all populations

probably originated by Robertsonian fusion, whereas the other two pairs in the set are still subject to continuous rearrangements. At this moment, the insufficient number of individuals analyzed from the Nilo Forest Reserve and the Sigi Trail populations in the East Usambaras does not allow to determine possible differences of individuals within and between the populations. Nevertheless, we suggest that these chromosome mutations had no negative impact on the fitness of carriers.

The present study demonstrates that molecular cytogenetic techniques as useful tools for understanding chromosomal organization and evolutionary history in the genus *Gonatoxia*. The chromosome number is lower and the degree of chromosomal polymorphism is greater in *G. helleri* than previously reported in bush-crickets. Our results suggest that this species may be a valuable new model system for further studying the potential role of morphological rearrangements of chromosomes in speciation. We determine the possibility that chromosomal rearrangements might be a driver of adaptive radiation enabling a species to broaden its ecological niche and thus higher adaptability to changing climatic conditions. The adaptive significance of chromosomal rearrangements for *G. helleri* and the origin of such low diploid chromosome numbers require additional genetic analyses, especially the development of multiple cytogenetic markers and molecular studies.

References

Bolzán AD, Bianchi MS (2006) Telomeres, interstitial telomeric repeat sequences, and chromosomal aberrations. Mutation Research 612: 189–214. https://doi.org/10.1016/j.mrrev.2005.12.003

Bugrov AG, Jetybayev IE, Karagyan GH, Rubtsov NB (2016) Sex chromosome diversity in Armenian toad grasshoppers (Orthoptera, Acridoidea, Pamphagidae). Comparative Cytogenetics 10(1): 45–59. https://doi.org/10.3897/CompCytogen.v10i1.6407

Buleu OG, Jetybayev IY, Chobanov DP, Bugrov AG (2019) Comparative analysis of C-heterochromatin, ribosomal and telomeric DNA markers in chromosomes of Pamphagidae grasshoppers from Morocco. Comparative Cytogenetics 13(1): 61–74. https://doi.org/10.3897/CompCytogen.v13i1.32039

Cabral-de-Mello DC, Martins C, Souza MJ, Moura RC (2011) 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

Cabrero J, Perfectti F, Gómez R, Camacho JPM, Lopéz-Leon MD (2003) Population variation in the A chromosome distribution of satellite DNA and ribosomal DNA in the grasshopper *Eyprepocnemis plorans*. Chromosome Research 11(4): 375–381. https://doi.org/10.1023/A:1024127525756

Chirino MG, Dalíková M, Marec F, Bressa MJ (2017) Chromosomal distribution of interstitial telomeric sequences as signs of evolution through chromosome fusion in six species of the giant water bugs (Hemiptera, Belostoma). Ecology and Evolution 7(14): 5227–5235. https://doi.org/10.1002/ece3.3098

Cohen AS, Stone JR, Beuning KRM, Park LE, Reinthal PN, Dettman D, Scholz CA, Johnson TC, King JW, Talbot MR, Brown ET, Ivory SJ (2007) Ecological consequences of early Late Pleistocene megadroughts in tropical Africa. Proceedings of the National Academy of Sciences of the United States of America 104(42): 16422–16427. https://doi.org/10.1073/pnas.0703873104

Coluzzi M, Sabatini A, Torre A, di Deco MA, Vinzenzo P (2002) A polytene chromosome analysis of the *Anopheles gambiae* species complex. Science 298: 1415–1418. https://doi.org/10.1126/science.1077769

Díez M, Santos JL (1993) Synapsis in a paracentric inversion heterozygote of *Chrothippus jacobsi* (grasshopper). Heredity 70: 231–236. https://doi.org/10.1038/hdy.1993.34

Dion-Côté A-M, Symonová R, Lamaze FC, Pelikánová S, Ráb P, Bernatchez LB (2017) Standing chromosomal variation in Lake Whitefish species pairs: the role of historical contingency and relevance for speciation. Molecular Ecology 26: 178–192. https://doi.org/10.1111/mec.13816

Ferretti ABSM, Ruiz-Ruano FJ, Milani D, Loreto V, Marti DA, Ramos E, Martins C, Cabral-de-Mello DC (2019) How dynamic could be the 45S rDNA cistron? An intriguing variability in a grasshopper species revealed by integration of chromosomal and genomic data. Chromosoma 128: 165–175. https://doi.org/10.1007/s00412-019-00706-8

Gould BA, Chen Y, Lowry DB (2017) Pooled ecotype sequencing reveals candidate genetic mechanisms for adaptive differentiation and reproductive isolation. Molecular Ecology 26: 163–177. https://doi.org/10.1111/mec.13881

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. https://doi.org/10.1186/1471-2148-14-48

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

Grzywacz B, Tatsuta H, Bugrov AG, Warchałowska-Śliwa E (2019) Cytogenetic markers reveal a reinforcement of variation in the tension zone between chromosome races in the brachypterous grasshopper *Podisma sapporensis* Shir. on Hokkaido Island. Scientific Reports 9: 16860. https://doi.org/10.1038/s41598-019-53416-7

Grzywacz B, Tatsuta H, Shikata K, Warchałowska-Śliwa E (2018) A comparative chromosome mapping study in Japanese Podismini Grasshoppers (Orthoptera: Acrididae: Melanoplinae). Cytogenetic and Genome Research 154: 37–44. https://doi.org/10.1159/000487063

Hemp C, Grzywacz B, Warchałowska-Śliwa E, Hemp A (2016b) Topography and climatic fluctuations boosting speciation: biogeography and a molecular phylogeny of the East African genera *Afroanthracites* Hemp & Ingrisch and *Afroagraecia* Ingrisch & Hemp (Orthoptera, Tettigoniidae, Conocephalinae, Agraeciini). Organisms Diversity and Evolution 16(1): 211–223. https://doi.org/10.1007/s13127-015-0244-4

Hemp C, Heller K-G, Warchałowska-Śliwa E, Grzywacz B, Hemp A (2013a) Biogeography, ecology, acoustics and chromosomes of East African *Eurycorypha* Stål species (Orthoptera, Phaneropterinae) with the description of new species. Organisms Diversity and Evolution 13: 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, Grzywacz B, Hemp A (2015b) Ecology, acoustics and chromosomes of the East African genus *Afroanthracites* Hemp & Ingrisch (Orthoptera, Tettigoniidae, Conocephalinae, Agraeciini) with the description of new species. Organisms Diversity and Evolution 15(2): 351–368. https://doi.org/10.1007/s13127-014-0194-2

Hemp C, Heller K-G, Warchałowska-Śliwa E, Grzywacz B, Hemp A (2017) Review of the African species of the phaneropterine genus *Parapyrrhicia* Brunner von Wattenwyl, 1891 (Insecta: Orthoptera); secret communication of a forest-bound taxon. Organisms Diversity and Evolution 17: 231–250. https://doi.org/10.1007/s13127-016-0303-5

Hemp C, Heller K-G, Warchałowska-Śliwa E, Grzywacz B, Hemp A (2018) New genera and new species of Acrometopini (Orthoptera: Tettigonioidea Phaneropterinae) from East Africa and a review of all known stridulatory organs, songs and karyotypes of the tribe. Insect Systematics and Evolution 49: 241–298. https://doi.org/10.1163/1876312X-00002170

Hemp C, Heller K-G, Warchałowska-Śliwa E, Hemp A (2010a) 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 (2013b) The genus *Aerotegmina* (Orthoptera, Tettigoniidae, Hexacentrinae): chromosomes, morphological relations, phylogeographical patterns and description of a new species. Organisms, Diversity and Evolution 13: 521–530. https://doi.org/10.1007/s13127-013-0133-7

Hemp C, Heller K-G, Warchałowska-Śliwa E, Hemp A (2014) Description of the female and notes on distribution, habitat, nymphal development, song and chromosomes of *Tripidonotacris grandis* Ragge (Orthoptera: Phaneropterinae). Zootaxa 3893(4): 569–578. https://doi.org/10.11646/zootaxa.3893.4.6

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

Hemp C, Heller K-G, Warchałowska-Śliwa E, Hemp A (2016a) Spotted males, uniform females and the lowest chromosome number in Tettigoniidae recorded: review of the genus *Gonatoxia* Karsch (Orthoptera, Phaneropterinae). Deutsche Entomologische Zeitschrift 63(2): 271–286.

Hemp C, Voje KL, Heller K-G, Warchałowska-Śliwa E, Hemp A (2010b) 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 (2012) Chromosomal localization of ribosomal and telomeric DNA provides new insights on the evolution of gomphocerinae grasshoppers. Cytogenetic and Genome Research 138: 36–45. https://doi.org/10.1159/000341571

Kawakami T, Butlin RK, Cooper JB (2011) Chromosomal speciation revisited: modes of diversification in Australian morabine grasshoppers (*Vandiemenella*, *viatica* species group). Insecta 2: 49–61. https://doi.org/10.3390/insects2010049

Kobayashi N, Shirai Y, Tsurusaki N, Tamura K, Aotsuka T, Katakura H (2000) Two cryptic species of the phytophagous ladybird beetle *Epilachna vigintioctopunctata* (Coleoptera: Coccinellidae) detected by analyses of mitochondrial DNA and karyotypes, and crossing experiments. Zoological Science 17: 1159–1166. https://doi.org/10.2108/zsj.17.1159

Kuznetsova V, Grozeva S, Gokhman V (2020) Telomere structure in insects: A review. Journal of Zoological Systematics and Evolution 58: 127–158. https://doi.org/10.1111/jzs.12332

López-Fernández C, Pradillo E, Zabal-Aguirre M, Fernández JL, Garcia de la Vega C, Gisálvez J (2004) Telomeric and interstitial telomeric-like DNA sequence in Orthoptera genomes. Genome 47: 757–763. https://doi.org/10.1139/g03-143

Lucek K (2018) Evolutionary mechanisms of varying chromosome numbers in the radiation of *Erebia* butterflies. Genes 9: 166. https://doi.org/10.3390/genes9030166

Mattos VF, Cella DM, Carvalho DMC, Schneider MC (2013) High chromosome variability and the presence of multivalent associations in buthid scorpions. Chromosome Research 21: 121–136. https://doi.org/10.1007/s10577-013-9342-3

Menezes RST, Silva TM, Carvalho AF, Andrade-Souza V, Silva JG, Costa MA (2013) Numerical and structural chromosome variation in the swarm-founding wasp *Metapolybia decorata* Gribodo 1896 (Hymenoptera, Vespidae). Genetica 141: 273–280. https://doi.org/10.1007/s10709-013-9726-5

deMenocal PB (2004) African climate change and faunal evolution during the Pliocene Pleistocene. Earth and Planetary Science Letters 220: 3–24. https://doi.org/10.1016/S0012-821X(04)00003-2

Mills PJ, Cook LG (2014) Rapid chromosomal evolution in a morphologically cryptic radiation. Molecular Phylogenetics and Evolution 77: 126–135. https://doi.org/10.1016/j.ympev.2014.03.015

Palacios-Gimenez OM, Carvalho CR, Soares FAF, Cabral-de-Mello DC (2015) Contrasting organization of repetitive DNAs in two Gryllidae crickets with highly divergent karyotypes. PloS ONE: 10(12) e0143540. https://doi.org/10.1371/journal.pone.0143540

Potter S, Bragg JG, Blom MPK, Deakin JE, Kirkpatric M, Eldridge MDB, Moritz JE (2017) Chromosomal speciation in the genomic era: disentangling phylogenetic evolution of rock-wallabies. Frontiers in Genetics 8: 10. https://doi.org/10.3389/fgene.2017.00010

Rego A, Marec F (2003) Telomeric and interstitial telomeric sequences in holokinetic chromosomes of Lepidoptera: Telomeric DNA mediates association between postpachytene

bivalents in achiasmatic meiosis of females. Chromosome Research 11: 681–694. https://doi.org/10.1023/A:1025937808382

Schneider CH, Gross MC, Terencio ML, Artoni RA, Vicari MR, Feldberg E (2013) Chromosomal evolution of neotropical cichlids: the role of repetitive DNA sequences in the organization and structure of karyotype. Reviews in Fish Biology and Fisheries 23: 201–214. https://doi.org/10.1007/s11160-012-9285-3

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58(4): 307–324. https://doi.org/10.1007/BF00292840

Silva BC, Souza LHB, Chamorro-Rengifo J, Araujo D (2019) Karyotypes of three species of *Hyperophora* Brunner von Wattenwyl, 1878 (Tettigoniidae, Phaneropterinae) enable morphologically similar species to be distinguished. Comparative Cytogenetics 13(1): 87–93. https://doi.org/10.3897/CompCytogen.v13i1.31803

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

Sumner AT (2003) Chromosome organization and function. Blackwell, North Berwick.

Taffarel A, Bidau CJ, Marti DA (2015) Chromosome fusion polymorphisms in the grasshopper, *Dichroplus fuscus* (Orthoptera: Acrididae: Melanoplinae): Insights on meiotic effects. European Journal of Entomology 112(1): 11–19. https://doi.org/10.14411/eje.2015.010

Ueshima N (2001) Karyotypes and meiosis in the subfamilies Listroscelidinae, Meconematinae and Microtettigoniinae. In: Rentz DCF(Ed.) Tettigoniidae of Australia Vol.3. CSIRO Publishing, Melbourne, 482–511.

Veltsos P, Keller I, Nichols RA (2009) Geographically localized bursts of ribosomal DNA mobility in the grasshopper *Podisma pedestris*. Heredity 103: 54–61. https://doi.org/10.1038/hdy.2009.32

Vershinina AO, Lukhtanov VA (2017) Evolutionary mechanisms of runaway chromosome number change in *Agrodiaetus* butterflies. Scientific Reports 7: 8199. https://doi.org/10.1038/s41598-017-08525-6

Vítková M, Král J, Traut W, Zrzavý J, Marec F (2005) The evolutionary origin of insect telomeric repeats, $(TTAGG)_n$. Chromosome Research 13: 145–156. https://doi.org/10.1007/s10577-005-7721-0

Voje K, Hemp C, Flagstad Ø, Sætre GP, Stenseth, NC (2009) Climatic change as an engine for speciation in flightless Orthoptera species inhabiting African mountains. Molecular Ecology 18: 93–108. https://doi.org/10.1111/j.1365-294X.2008.04002.x

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, 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, 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. https://doi.org/10.1139/gen-2013-0119

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: 1–9. https://doi.org/10.14411/eje.2009.001

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. https://doi.org/10.1080/00087114.1992.10797213

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

White MJD (1973) Mode of Speciation. (ed. W.H. Freeman). San Francisco, CA, USA.

Xavier C, Soares RVSS, Amorim IC, Cabral-de-Mello DC, de Cassia de Moura R (2018) Insights into the evolution and speciation of the beetle *Euchroma gigantea* (Coleoptera: Buprestidae). Chromosome Research 26: 163–178. https://doi.org/10.1007/s10577-018-9576-1

CompCytogen 14(3): 437–451 (2020)
doi: 10.3897/CompCytogen.v14i3.55279
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 cytogenetic of six species of Amazonian Peacock bass (*Cichla*, Cichlinae): intrachromosomal variations and genetic introgression among sympatric species

Janice Quadros[1], Alex M. V. Ferreira[1], Patrik F. Viana[1], Leandro Marajó[1], Ezequiel Oliveira[3], Efrem Ferreira[2], Eliana Feldberg[1]

1 *Laboratório de Genética Animal, Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia, Av. André Araújo 2936, Petrópolis, 69067-375, Manaus, AM, Brazil* **2** *Laboratório de Ecologia de peixes, Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia, Av. André Araújo 2936, Petrópolis, 69067-375, Manaus, AM, Brazil* **3** *Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, São Paulo 13565-905, Brazil*

Corresponding author: Eliana Feldberg (feldberg@inpa.gov.br)

Academic editor: R. Noleto | Received 9 June 2020 | Accepted 2 September 2020 | Published 17 September 2020

http://zoobank.org/8EEA88F2-8218-48C8-9F6C-F3A4CADB42CD

Citation: Quadros J, Ferreira AMV, Viana PF, Marajó L, Oliveira E, Ferreira E, Feldberg E (2020) Comparative cytogenetic of six species of Amazonian Peacock bass (*Cichla*, Cichlinae): intrachromosomal variations and genetic introgression among sympatric species. Comparative Cytogenetics 14(3): 437–451. https://doi.org/10.3897/CompCytogen.v14i3.55279

Abstract

Cytogenetic data for the genus *Cichla* Bloch et Schneider, 1801 are still very limited, with only four karyotype descriptions to date. The sum of the available cytogenetic information for *Cichla* species, points to a maintenance of the diploid number of 48 acrocentric chromosomes, considered a typical ancestral feature in cichlids. In the current study, we performed molecular and classical cytogenetic analyses of the karyotype organization of six species of *Cichla*, the earliest-diverging genus of Neotropical cichlids. We cytogenetically analysed *Cichla kelberi* Kullander et Ferreira, 2006, *Cichla monoculus* Agassiz, 1831, *Cichla piquiti* Kullander et Ferreira, 2006, *Cichla temensis* Humboldt, 1821, *Cichla vazzoleri* Kullander et Ferreira, 2006 and *Cichla pinima* Kullander et Ferreira, 2006, including three individuals that showed mixed morphological characteristics, likely from different species, suggesting they were hybrid individuals. All individuals analysed showed 2n = 48 acrocentric chromosomes, with centromeric heterochromatic blocks on all chromosomes and a terminal heterochromatic region on the *q* arm of the 2nd pair. Mapping 18S rDNA gave hybridization signals, correlated with the nucleolus organizer regions, on the 2nd pair for all analyzed individuals. However, we found distinct patterns for 5S rDNA: interstitially at the proximal position on

6th pair of four species (*C. kelberi*, *C. pinima*, *C. piquiti* and *C. vazzoleri*), and on the distal of the 4th pair in two (*C. monoculus* and *C. temensis*). Accordingly, we present here new data for the genus and discuss the evolutionary trends in the karyotype of this group of fish. In addition, we provide data that supports the occurrence of hybrid individuals in the Uatumã River region, mainly based on 5S rDNA mapping.

Keywords
5S rDNA, FISH, Heterochromatin, Hybridization, karyotype

Introduction

The genus *Cichla* Bloch et Schneider, 1801 belongs to the subfamily Cichlinae that, jointly with *Retroculus* Eigenmann et Bray, 1894, makes up the tribe Cichlini, and is the earliest-diverging lineage of Neotropical cichlids (Leo Smith et al. 2008). This taxon is widely distributed within the Amazon, Tocantins, and Orinoco River basins, and in the smaller rivers draining the Guianas to the Atlantic Ocean. Most *Cichla* species follow an allopatric distribution pattern, although some species are sympatric or even syntopic (Kullander and Ferreira 2006). However, some species, such as *C. monoculus* Agassiz, 1831, *C. kelberi* Kullander et Ferreira, 2006 and *C. piquiti* Kullander et Ferreira, 2006, have been introduced into other areas, where they are well established, due to their generalist habit. *Cichla* are very emblematic fish in South America, with high economic and ecological importance, especially since they are predators in Amazonian rivers and used widely for sport fishing (Nascimento et al. 2001; dos Santos et al. 2016; Diamante et al. 2017).

Representatives of the genus *Cichla* are easily distinguished from all other Neotropical cichlids by the shape of the dorsal fin, and the presence of 1 to 4 dark vertical bars along the body. However, the species are very similar, and while their color patterns still provide the best species diagnostic characters, in some cases these may complicate accurate identification, since key characters may show ontogenetic changes (Kullander and Ferreira 2006).

According to Kullander and Ferreira (2006), the genus comprises 15 morphologically distinct species, and recently another species has been described (*Cichla cataractae* Sabaj et al. 2020) from the Essequibo River basin, where it is endemic). However, Willis et al. (2012), based on multilocus data, recognized only eight species. The species often have restricted natural distributions, but to variable extents. For example, while *C. monoculus* is found all over the Amazon River and low tributary course, *C. temensis* Humboldt, 1821 is found only in black water rivers, whereas *C. piquiti* and *C. kelberi* are restricted to the Tocantins River.

Cytogenetic data concerning the family Cichlidae points to a remarkable trend in the maintenance of the diploid number 2n = 48, mostly in the acrocentric form (Thompson 1979). However, as more species were karyotyped, a huge chromosomal diversity was observed in the derived clades (ranging from 32 to 60 chromosomes), but with predominance of 2n = 48 in most lineages, which is considered an ancestral trait for this group (Feldberg et al. 2003; Gross et al. 2009; Poletto et al. 2010; da Costa et al. 2019). For the genus *Cichla*, only *C. monoculus*, *C. temensis*, *C. kelberi* and *C. piquiti* have had their karyotypes described, all exhibiting a diploid number com-

posed of 48 acrocentric chromosomes, as the species from earliest-diverging Cichlinae tribes (Retroculini, Astronotini and Chaetrobranchini) (Feldberg et al. 2003; Alves-Brinn et al. 2004; Poletto et al. 2010; Mourão et al. 2017).

Interestingly, Alves-Brinn et al. (2004), based on cytogenetic data, reported the occurrence of hybridization between *C. monoculus* and *C. temensis* in the Uatumã River (Balbina Hydroelectric Dam). In addition, interspecific hybridization and introgression between species has been much discussed in relation to the adaptive advantages and increase of genetic variability (Willis et al. 2012). For some authors, hybridization may be related to diversification and speciation, or the extinction of populations or species (Mourão et al. 2017). Under either species concept, the phylogenetic breadth of introgression in this group is clear, with both sister species and species from different mtDNA clades exhibiting genetic introgression (Willis et al. 2012).

In the current study, we used different classical and molecular cytogenetic markers to characterize *Cichla* species, from different river drainages within the Amazon basin and investigate the likely existence of hybrid individuals, where more than one species occurs, such as at Uatumã River (Balbina Hydroelectric Dam).

Material and methods

In the current study, we sampled 50 individuals of the genus *Cichla* from five locations in the Brazilian Amazon basin (Table 1, Figs 1, 2) under ICMBIO (Instituto Chico Mendes de Conservação da Biodiversidade) permit number: 28095-1. Voucher specimens were deposited in the Fish Collection of the National Institute of Amazonian Research (Instituto Nacional de Pesquisas da Amazônia – INPA) (Table 1). Dr. Efrem Ferreira and Dr. Jansen Zuanon, following description of Kullander and Ferreira (2006), identified the *Cichla* species included in the current study. However, three individuals had mixed characteristics of more than one species, and were thus considered possible hybrids by specialists.

Chromosomal preparations were obtained from the kidney, following the protocol of Gold et al. (1990). The active nucleolus-organizing region (NOR) was detected with

Table 1. The *Cichla* species included in the current study, collecting localities, the number of individuals analyzed, and Voucher number. ♂ = male, ♀ = female. AM = Amazonas State, PA = Pará State, MT = Mato Grosso State.

Species	Number of individuals	Collecting localities	Coordinates	Voucher
C. kelberi	4♂ 3♀	Araguaia River – São Félix, MT	11°39'03.9"S, 50°52'59.4"W	MZUSP125273
C. monoculus	5♀	Anavilhanas (Negro River), AM (Black water)	2°33'28.4"S, 60°46'29.7"W	INPA-ICT059045
C. monoculus	3♀	Uatumã River (Balbina Hydroelectric Dam) AM, Black water)	1°55'02.2"S, 59°28'23.7"W	INPA-ICT059046
C. monoculus	1♀	Tapajós River – Santarém, PA (Clear water)	2°24'53.0"S, 54°46'48.3"W	INPA-ICT059047
C. monoculus	4♂ 1♀	Catalão Lake, AM (Mix of white and black water)	3°10'30.8"S, 59°56'30.3"W	INPA-ICT059044
C. pinima	7♂ 6♀	Tapajós River (Mix of white and clear water)	24°21'16.4"S, 54°70'23.16"W	INPA-ICT059045
C. piquiti	2♂ 2♀	Araguaia River – São Félix, MT	11°38'01.7"S, 50°40'11.3"W	MZUSP125272
C. temensis	2♂ 2♀	Uatumã River (Balbina Hydroelectric Dam). AM, Black water)	1°55'02.2"S, 59°28'23.7"W	INPA-ICT059043
C. vazzoleri	2♂ 3♀	Uatumã River (Balbina Hydroelectric Dam, AM, Black water)	1°55'02.2"S, 59°28'23.7"W	INPA-ICT059048
Hybrids	3♂	Uatumã River (Balbina Hydroelectric Dam, AM, Black water)	1°55'02.2"S, 59°28'23.7"W	INPA-CT059047

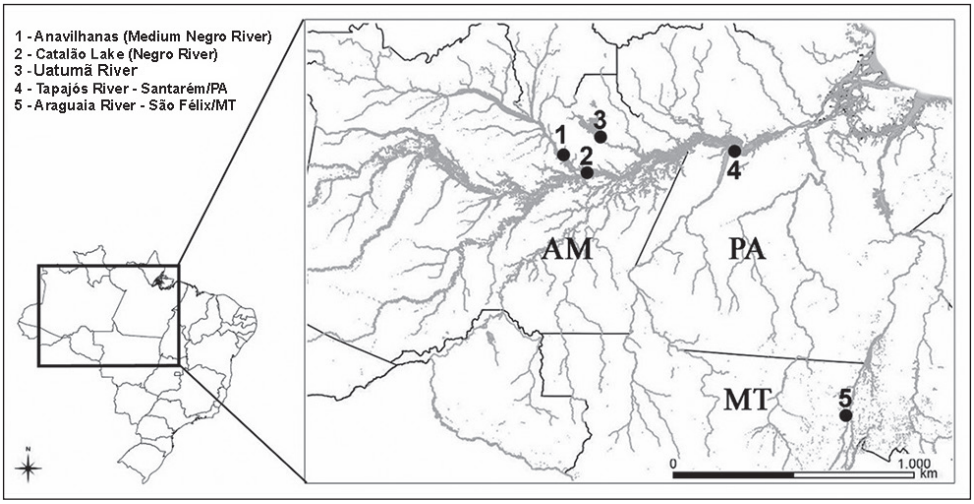


Figure 1. Map showing the collection points of *Cichla* species analyzed in current study.

silver nitrate impregnation (Ag-NOR), following Howell and Black (1980), while constitutive heterochromatin was detected following Sumner (1972). DNA was extracted using the Wizard Extraction Kit (Promega), following manufacturer's recommendations, and quantified using a NanoVue Plus spectrophotometer (GE Healthcare).

Amplification of 18S and 5S rDNA used the Polymerase Chain Reaction (PCR) with primers 18S F(5' -CCG CTT TGG TGA CTC TTG AT-3') and R(5' -CCG AGG ACC TCA CTA AAC CA-3') (Gross et al. 2010), and the primers 5S F(5' -TAC GCC CGA TCT CGT CCG ATC-3') and R(5' -CAG GCT GGT ATG GCC GTA AGC-3') (Martins and Galetti 1999).

All PCRs were performed with a final volume of 25 µL, containing genomic DNA of each species (200 ng), 10× buffer with 1.5 mM $MgCl_2$, DNA polymerase (5 U/µL), dNTPs (1 mM), primers (5 mM) and Milli-Q. The reaction profile for 18S rDNA was 1 min. at 95 °C, 35 cycles of 1 min. at 94 °C, 1 min. at 56 °C and 1 min. and 30 s at 72 °C, followed by 5 min. at 72 °C. The reaction profile for 5S rDNA amplification was 1 min. at 95 °C, followed by 30 cycles of 1 min. at 94 °C, 1 min. at 59 °C and 1 min. and 30 s at 72 °C. The final extension was 5 min. at 72 °C. PCR products were checked on 1% agarose gel, quantified on a NanoVue Plus spectrophotometer (GE Healthcare). PCR products were labeled with digoxigenin (Dig-Nick Translation mix; Roche) and biotin (Bio-Nick Translation mix; Roche), and used as probes for the fluorescent *in situ* hybridization technique (FISH).

Hybridizations were performed according to the protocol described by Pinkel et al. (1986), with a stringency of 77% (2.5 ng/µL) for 18S rDNA, 5S rDNAr, 50% formamide, 10% dextran sulfate and 2xSSC at 37 °C for 18 h), post-hibridization washes were made with formamide 15% and 2xSSC Tween 0.5%. Chromosomes were counterstained with DAPI (2 mg/mL) using the Vectashield (Vetor) mounting medium. Telomeric segments were generated using non-templated PCR with *primers* (TTAGGG)5 and (CCCTAA)5 (Ijdo et al. 1991).

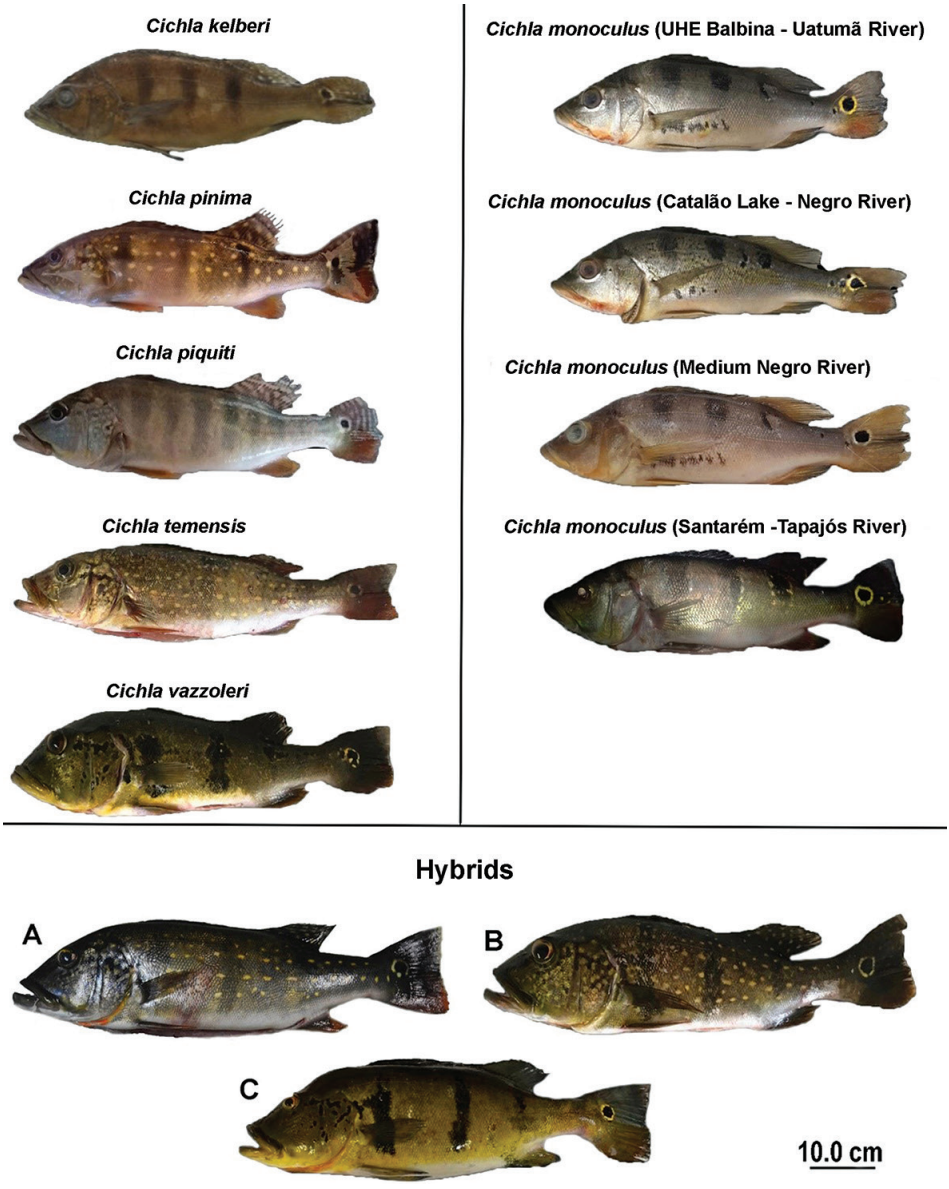


Figure 2. *Cichla* species and individuals considered morphologically hybrid (**A–C**). *C. kelberi* SL = 170.0 mm; *C. pinima* SL = 190.5 mm; *C. piquiti* SL = 200.0 mm; *C. temensis* SL = 210.0 mm; *C. vazzoleri* SL = 250.0 mm; *C. monoculus* (Uatumã River) SL = 160.0 mm; *C. monoculus* (Catalão Lake, Negro River) SL = 150.0 mm; *C. monoculus* (Anavilhanas, Medium Negro River) SL = 180.0 mm; *C. monoculus* (Santarém, Tapajós River) SL = 180.0 mm; Hybrid **A** SL = 280.0 mm; Hybrid **B** SL = 230.0 mm; Hybrid **C** SL = 320.0 mm.

We analyzed at least 30 metaphase per individual to confirm the diploid number and karyotype structure. Images were captured using an Olympus BX51 epifluorescence microscope, and processed using Image-PRO MC 6.0 softwares. Chromosomes

were measured using the Image J program, arranged in descending order of chromosome size, and classified according to Levan et al. (1964).

All methodological procedures in the current study were performed in accordance with the guidelines of the Ethics Committee of the National Institute of Amazonian Research (Instituto Nacional de Pesquisas da Amazônia – INPA), protocol: CEUA No. 009/2018.

Results

The six species analyzed (*C. kelberi*, *C. monoculus*, *C. pinima*, *C. piquiti*, *C. temensis* and *C. vazzoleri*) all had a diploid number equal to 48 acrocentric chromosomes, and a fundamental number (FN) equal to 48. The NORs (Ag-NORs and 18S rDNA) were located in a distal position on the *q* arms of pair n° 2 in all species (Figs 3, 4). *Cichla monoculus* was the sole species sampled in more than one location, and it showed no difference when compared to data in Alves-Brinn et al. (2004) and Schneider et al. (2013) (data not shown). The 5S rDNA site was located interstitially at the proximal position of pair n° 6 in four species (*C. kelberi*, *C. pinima*, *C. piquiti* and *C. vazzoleri*), and on distal portion of pair n° 4 in two (*C. monoculus* and *C. temensis*) (Fig. 4).

The six species had centromeric heterochromatic blocks on all chromosomes and a terminal heterochromatic region on pair 2, which corresponds to the same position as the NORs. However, some blocks were species-specific: terminal blocks were observed on the *q* arm of *C. kelberi* pairs 1, 3 and 4; pair 3 of *C. pinima* and *C. temensis*; pairs 1 and 3 of *C. piquiti*; and in *C. vazzoleri* pairs 3 and 6 (Fig. 3). *C. monoculus*, which was sampled in four different locations, showed variable constitutive heterochromatin patterning, where individuals from the Uatumã River (Balbina Hydroelectric Dam) also had terminal blocks on the *q* arms of the chromosomal pairs 1, 6, 9, 12, 15, 19. Catalão Lake individuals appeared to have terminal pale blocks on all pairs, with conspicuous ones on 1, 5, 6, 8, 10 chromosomal pairs. This also occurred for individuals from Anavilhanas, but in these, the blocks were more conspicuous in practically all chromosomes, and still had interstitial markings on pairs 1, 3 and 6. Individuals from the Tapajós River had terminal blocks on pairs 1, 3, 5, 11, 14, 15, and interstitials on pairs 14 and 15 (Fig. 5).

The three individuals morphologically considered hybrids also had 2n = 48 acrocentric chromosomes and FN = 48, Ag-NOR and 18S rDNA on the second pair at terminal position on the *q* arm, collocated with a conspicuous heterochromatic portion (Figs 6, 7). Constitutive heterochromatin was present in the centromeric region of all chromosomes in the three individuals and the first pair had an interstitial block. Additionally, individual A (Fig. 6b) had terminal blocks on pairs 1 and 5; individual B (Fig. 6e) had terminal blocks on most chromosomes and interstitials on pairs 3 and 6; individual C (Fig. 6h) had terminal pale blocks on pairs 3 and 10.

5S rDNA was detected interstitially on one pair 4 homolog, and on one pair 6 homolog in two hybrid individuals (A and C). Individual B showed 5S rDNA sites on both pair 4 chromosomes (Fig. 7).

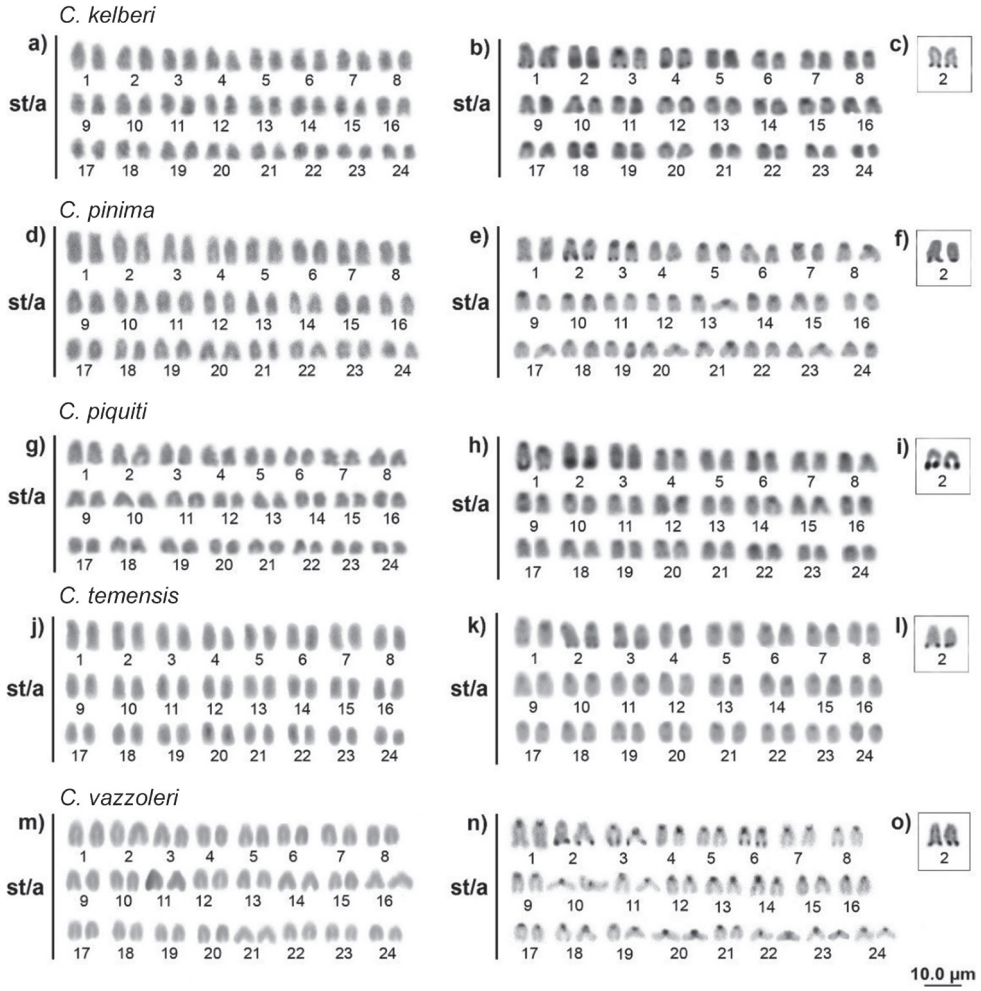


Figure 3. Karyotypes analyzed by conventional Giemsa staining, C banding and Ag-NOR: *Cichla kelberi* (**a, b, c**) *C. pinima* (**d, e, f**) *C. piquiti* (**g, h, i**) *C. temensis* (**j, k, l**) *C. vazzoleri* (**m, n, o**).

For all analysed species, hybridization with telomeric probes showed, as expected, only markings on the terminal portions of both arms (data not shown).

Discussion

For Cichlidae species, a diploid number equal to 48 acrocentric-like chromosomes is considered an ancestral feature (Thompson 1979), and chromosomal evolution in this family was thought to be conserved from the karyotype macrostructure point of view (Feldberg et al. 2003). However, as more Cichlidae species were cytogenetically studied and more accurate techniques were applied (e.g. mapping of different molecular

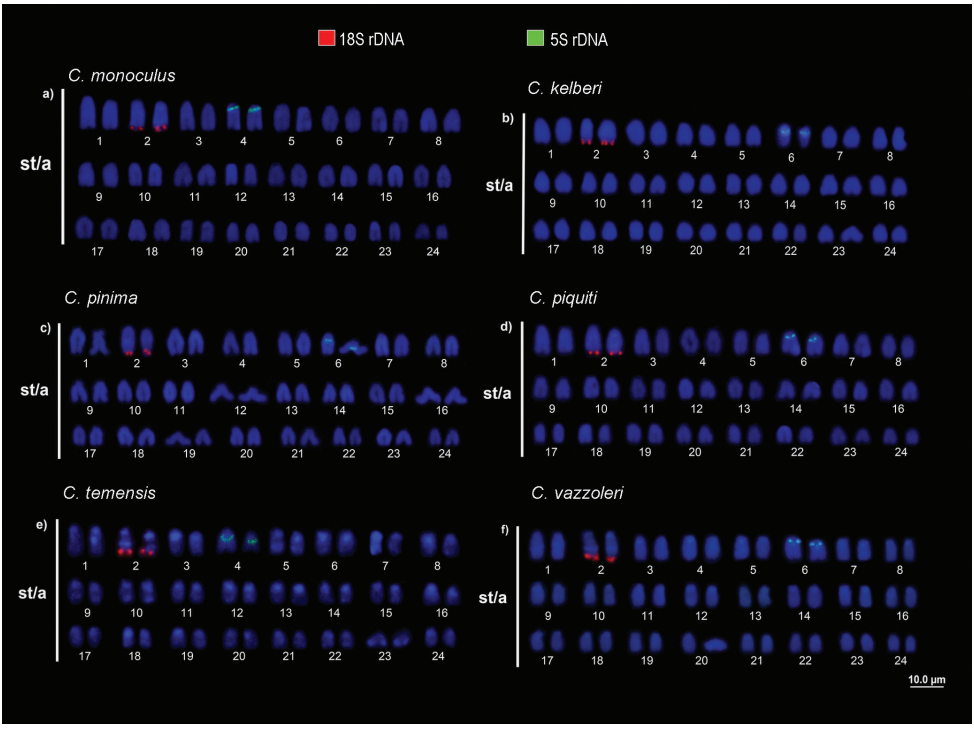


Figure 4. Karyotypes analyzed with molecular chromosome markers. Double FISH with 18S (red) and 5S (green) rDNA probes. *Cichla monoculus* (**a**) *C. kelberi* (**b**) *C. pinima* (**c**) *C. piquiti* (**d**) *C. temensis* (**e**) *C. vazzoleri* (**f**).

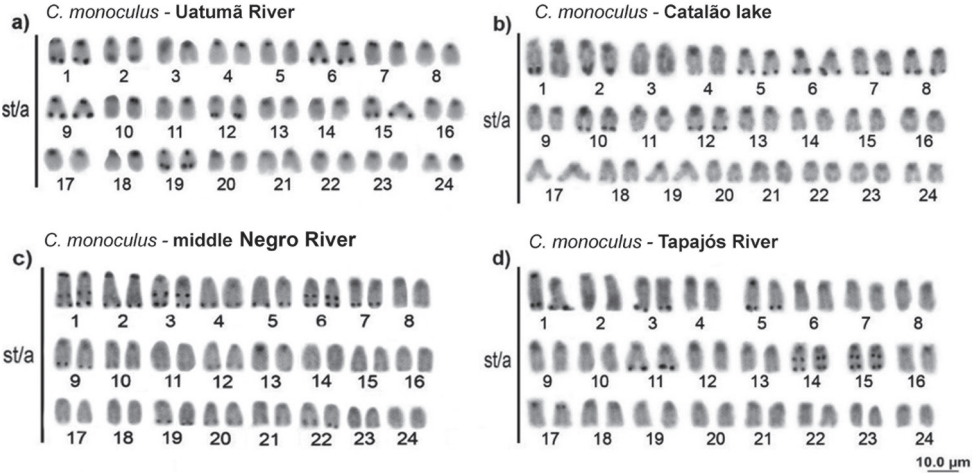


Figure 5. *Cichla monoculus* karyotype from different locations with conventional Giemsa staining, C. banding: **a** Uatumã River **b** Catalão Lake (Negro River) **c** Anavilhanas (middle Negro River) **d** Tapajós River.

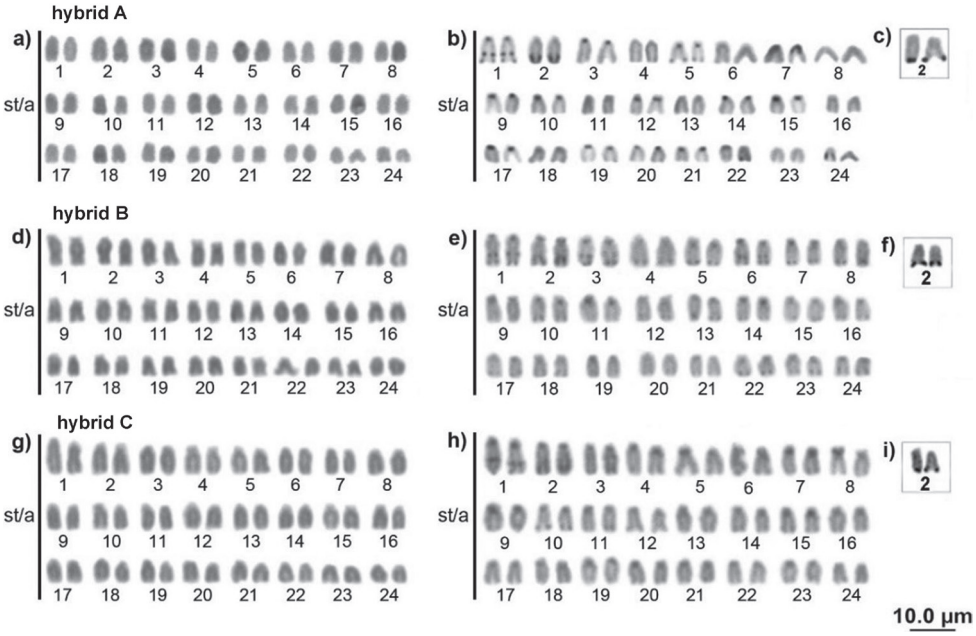


Figure 6. Karyotype of hybrid individuals A, B and C (as in Fig. 2) respectively, with conventional Giemsa staining (**a, d, g**), C-banding (**b, e, h**) and Ag-NOR (**c, f, i**).

chromosomal markers), several karyotypic formulas and configurations have been found (Gross et al. 2009; Poletto et al. 2010; Schneider et al. 2013), suggesting that this fish group experienced multiple non-robertsonian chromosomal rearrangements during its evolution, since the 2n = 48 is retained in most Cichlinae lineages.

In the current study, analyzes focused on the genus *Cichla*, which represents one of the most basal lineages of Neotropical cichlids (Leo Smith et al. 2008). To date, all species karyotyped possess a complement of 48 acrocentric-like chromosomes, with very similar karyotypes between species, including the NOR pattern, which is usually found on the 2nd pair (Alves Brinn et al. 2004; Schneider et al. 2013; Mourão et al. 2017; current study). In addition, some studies examining morphological-mitochondrial divergences (Andrade et al. 2001; Willis et al. 2010), as well as chromosome features (Alves Brinn et al. 2004; Oliveira et al. 2006), and electrophoretic esterase comparisons (Teixeira and Oliveira 2005) have inferred hybridization in natural and in artificial or disturbed environments/populations.

For constitutive heterochromatin, the distribution pattern can often be used as a species-specific or population marker (Feldberg et al. 2003; Vicari et al. 2006; Benzaquem et al. 2008; Perazzo et al. 2011). In our analyses, for instance, we found four different heterochromatin patterns for *C. monoculus* (Fig. 5). This is one of the most widely-distributed species in the Amazon River basins (Kullander and Ferreira 2006), and the

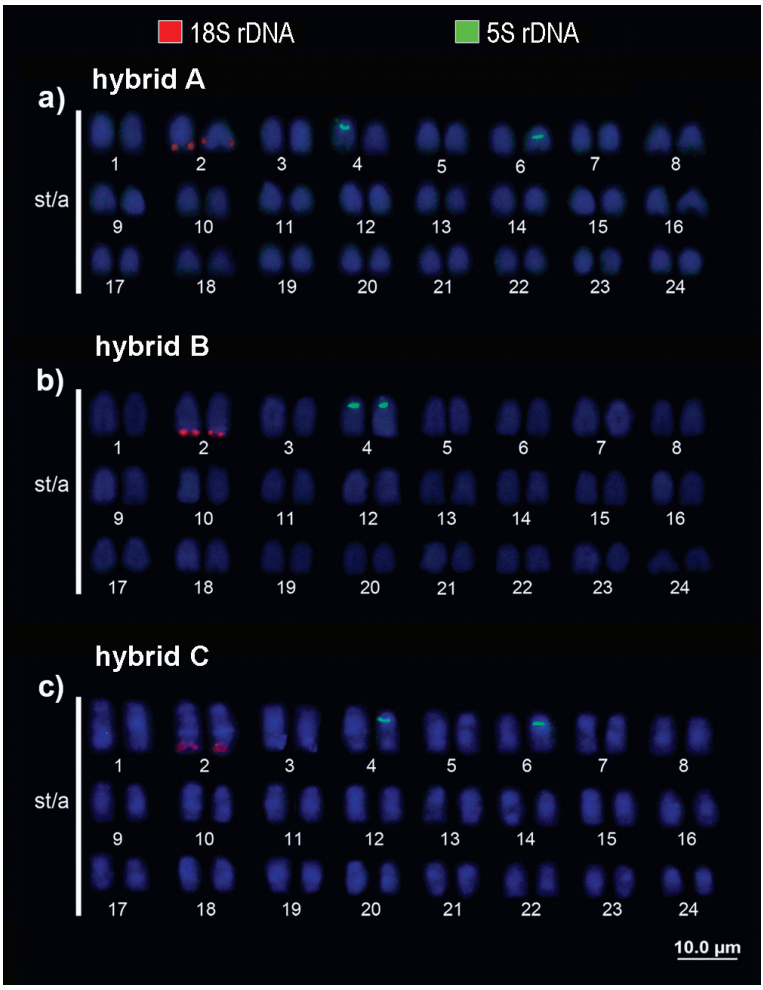


Figure 7. Karyotypes of hybrid individuals **A**, **B** and **C** (as shown in Fig. 2) respectively with molecular chromosomal markers. Double FISH with 18S (red) and 5S (green) rDNA probes.

commonly introduced into dam resevoirs throughout Brazil (dos Santos et al. 2016; Diamante et al. 2017). Heterochromatin is known to play important roles in the chromosomal architecture and karyotype organization, such as assisting in chromosomal segregation, nuclear organization and expression of gene regulation, associated with responses to environmental changes (Grewal and Jia 2007; Varriale et al. 2008; Bühler 2009; Ribeiro et al. 2017; Viana Ferreira et al. 2019). This seems to be the case for the different *C. monoculus* populations analyzed in our study, where individuals from black and acid waters (Negro and Uatumã rivers), white and black mixed waters (confluence of Negro and Solimões rivers), and in the confluence of white and clear waters (confluence of Amazonas and Tapajós rivers), showed intraspecific variability in their heterochromatic patterns, possibly reflecting chromatin adaptation and/or epigenomic responses to changes in the specific environment inhabited by these different populations.

Interestingly, the heterochromatic patterns of the three probable hybrids was very similar and much closer to the pattern described for the Negro River (*C. monoculus* from Anavilhanas) with some interstitial blocks. Could it be heterochromatinization? Such heterochromatin variability can also be explained by stressors, such as environmental changes, or even hybridization processes (Richards et al. 2010; Ribeiro et al. 2017), which would explain the heterochromatin distribution differences found in *C. monoculus* and in the probable hybrids.

Besides the conservation of the karyotype macrostructure, Schneider et al. (2013) reported that 12 out of 13 Cichlinae species analyzed in their study had only one chromosome pair harboring 5S rDNA sites, but at different karyotypic positions, indicating that 5S rDNA sites are a robust molecular chromosomal marker in cichlid species. 5S rDNA is an important cytotaxonomic and evolutionary marker, since it helps provide a better understanding of fish chromosomal diversity (Bellafronte et al. 2005; Teixeira et al. 2009; Vicari et al. 2010). For instance, a study by Ferreira et al. (2016), mapping of 5S rDNA sequences in *Bunocephalus coracoideus* Cope, 1874, revealed an association between this rDNA site and a multiple sex chromosome system previously unknown in Siluriformes ($X_1X_1X_2X_2/X_1Y_1X_2Y_2$). Repetitive 5S and 18S rDNA sequences are the most well-studied in fish, and have been gaining prominence mainly in studies of between-species evolutionary relationships, population characterization and genome structure (Martins et al. 2004; Terencio et al. 2012; Schneider et al. 2013).

In the current study, individuals of all six species, including the hybrids, had 18S rDNA on terminal position of the *q* arm of the 2nd chromosomal pair (same position as NORs). Meanwhile, 5S rDNA mapping in *Cichla* species showed two patterns: on the 4th pair (*C. monoculus* and *C. temensis*), and on the 6th pair (*C. kelberi*, *C. pinima*, *C. piquiti* and *C. vazzoleri*). However, in the individuals morphologically considered hybrids, we found two distinct patterns: two of them (hybrids A and C) having 5S rDNA in one homologue of the 4th pair and one homologue of the 6th pair, while the other hybrid (individual B) had 5S rDNA on both homologues of the 4th pair. Since these probable hybrids were captured in the Uatumã River (Balbina Hydroelectric Dam), where *C. monoculus*, *C. temensis* and *C. vazzoleri* all occur (Kullander and Ferreira 2006), we believe that these species might be hybridizing.

Interestingly, the karyotypes of *C. pinima* and *C. vazzoleri* (current study) are very similar, except for a heterochromatic terminal block on the *q* arms of the 6th pair in *C. vazzoleri*. It is notable that *C. pinima* was sampled in the Tapajós River and *C. vazzoleri* in the Uatumã River, very distant locations with no history of sympatry or migration (Ferreira, personal communication). However, according to Willis (2017), *C. pinima sensu lato* includes *C. pinima*, *C. vazzoleri*, *C. jariina*, Kullander et Ferreira, 2006 and *C. thyrorus* Kullander et Ferreira, 2006 (*sensu* Kullander and Ferreira 2006), and reports that the evolutionary relationships in this group are more complex than previously thought. Willis (2017) suggest that this separation into four species does not correspond to its evolutionary history and contemporary dynamics of the genus.

In addition, Willis et al. (2012) reported that genetic introgression is a common phenomenon in *Cichla* species. Introgression can be defined as the movement of DNA from the genetic pool of one species into that of another species by repeated backcross-

ing of hybrid individuals with one or both parent species. Such hybridization events are expected to occur most commonly in modified habitats, but interestingly, most of the hybridization cases known for *Cichla* species, were found in undisturbed natural environments (Willis et al. 2012), suggesting that introgression forms a natural part of the evolution of many tropical species, so increasing genetic diversity. In this sense, we cannot rule out hybridization and genetic introgression among the likely parental species, especially taking in account that all three probable hybrid individuals used here had male gonads.

Conclusions

Our data supports the tendency in the maintenance of the 2n = 48 chromosomes for *Cichla* species, as well as the conservation of the karyotypic formula and simple NOR, but reveals 5S rDNA to be an important cytogenetic marker for this group. In additon, here we provide, for the first time, the karyotype for *C. pinima* and *C. vazzoleri*. Furthermore, our data shows that the heterochromatin pattern may differentiate populations of *C. monoculus*, suggesting that this variation might be the result of epigenetic events triggered by different water types.

Acknowledgements

The authors are grateful to Jansen Zuanon for identifying the individual fish. Brazilian institutions: INPA/BADPI the post-graduate program in Freshwater Biology and Continental Fisheries, the Laboratory of Animal Genetics (LGA/INPA). This study was financed by the Centro de Estudos de Adaptação às Mudanças Ambientais da Amazônia (INCT ADAPTA II, FAPEAM/CNPq 573976/2008-2), Conselho Nacional de Desenvolvimento Científico e Tecnológico and the Fundação de Amparo à Pesquisa do Estado do Amazonas. JMQ received a study grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico. Dr. Adrian A. Barnett reviewed the English.

References

Alves Brinn MN, Ivan Rebelo Porto J, Feldberg E (2004) Karyological evidence for interspecific hybridization between *Cichla monoculus* and *C. temensis* (Perciformes, Cichlidae) in the Amazon. Hereditas 141(3): 252–257. https://doi.org/10.1111/j.1601-5223.2004.01830.x

Andrade F, Schneider H, Farias I, Feldberg E, Sampaio I (2001) Análise filogenética de duas espécies simpátricas de tucunaré (*Cichla*, Perciformes), com registro de hibridização em diferentes pontos da bacia Amazônica. Revista Virtual de Iniciação Acadêmica da UFPA 1: 1–11.

Bellafronte E, Margarido VP, Moreira-Filho O (2005) Cytotaxonomy of *Parodon nasus* and *Parodon tortuosus* (Pisces, Characiformes): A case of synonymy confirmed by cytogenetic analyses. Genetics and Molecular Biology 28: 710–716. https://doi.org/10.1590/S1415-47572005000500010

Benzaquem DC, Feldberg E, Porto JIR, Gross MC, Zuanon JAS (2008) Cytotaxonomy and karyoevolution of the genus *Crenicichla* (Perciformes, Cichlidae). Genetics and Molecular Biology 31: 250–255. https://doi.org/10.1590/S1415-47572008000200016

Bühler M (2009) RNA turnover and chromatin-dependent gene silencing. Chromosoma 118: 141–151. https://doi.org/10.1007/s00412-008-0195-z

da Costa GVWF, Cioffi MB, Liehr T, Feldberg E, Bertollo LAC, Molina WF (2019) Extensive Chromosomal Reorganization in *Apistogramma* Fishes (Cichlidae, Cichlinae) Fits the Complex Evolutionary Diversification of the Genus. International Journal of Molecular Sciences 20: 1–4077. https://doi.org/10.3390/ijms20174077

Diamante NA, de Oliveira AV, Petry AC, Catelani PA, Pelicice FM, Prioli SMAP, Prioli AJ (2017) Molecular analysis of invasive *Cichla* (Perciformes: Cichlidae) populations from neotropical ecosystems. Biochemical Systematics and Ecology 72: 15–22. https://doi.org/10.1016/j.bse.2017.03.004

dos Santos LN, Salgueiro F, Sampaio Franco AC, Marques ACPB (2016) First record of the invasive blue peacock cichlid *Cichla piquiti* Kullander and Ferreira 2006 (Cichliformes: Cichlidae) in the Paraíba do Sul River basin, South eastern Brazil. BioInvasions 5(4): 267–275. https://doi.org/10.3391/bir.2016.5.4.12

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 and Oxford, New Dehli and New York, 285–308.

Ferreira M, Garcia C, Matoso DA, Jesus IS, Feldberg E (2016) A new multiple sex chromosome system $X_1X_1X_2X_2/X_1Y_1X_2Y_2$ in Siluriformes: cytogenetic characterization of *Bunocephalus coracoideus* (Aspredinidae). Genetica 144: 591–599. https://doi.org/10.1007/s10709-016-9927-9

Gold JR, Li YC, Shipley NS, Powers PK (1990) Improved methods for working with fish chromosomes with a review of metaphase chromosome banding. Journal of Fish Biology 37: 563–575. https://doi.org/10.1111/j.1095-8649.1990.tb05889.x

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

Gross MC, Feldberg E, Cella DM, Schneider MC, Schneider CH, Porto JIR, Martins C (2009) Intriguing evidence of translocations in Discus fish (*Symphysodon*, Cichlidae) and a report of the largest meiotic chromosomal chain observed in vertebrates. Heredity 102: 435–441. https://doi.org/10.1038/hdy.2009.3

Gross MC, Schneider CH, Valente GT, Martins C, Feldberg E (2010) Variability of 18S rDNA locus among *Symphysodon* fishes: chromosomal rearrangements. Journal of Fish Biology 76(5): 1117–1127. https://doi.org/10.1111/j.1095-8649.2010.02550.x

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

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: 1–4780. https://doi.org/10.1093/nar/19.17.4780

Kullander SO, Ferreira EJG (2006) A review of the South American cichlid genus *Cichla*, with descriptions of nine new species (Teleostei: Cichlidae). Ichthyological Exploration of Freshwaters 17: 289–398.

Leo Smith W, Chakrabarty P, Sparks JS (2008) Phylogeny, taxonomy, and evolution of Neotropical cichlids (Teleostei: Cichlidae: Cichlinae). Cladistics 24: 625–641. https://doi.org/10.1111/j.1096-0031.2008.00210.x

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, Galetti PM (1999) Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Research 7: 363–367. https://doi.org/10.1023/A:1009216030316.

Martins C, Oliveira C, Wasko AP, Wright JM (2004) Physical mapping of the Nile tilapia (*Oreochromis niloticus*) genome by fluorescent in situ hybridization of repetitive DNAs to metaphase chromosomes – a review. Aquaculture 231: 37–49. https://doi.org/10.1016/j.aquaculture.2003.08.017

Mourão AAF, Freitas-Souza D, Hashimoto DT, Ferreira DC, Prado FD, Silveira RV, Foresti F, Porto-Foresti F (2017) Molecular and morphological approaches for species delimitation and hybridization investigations of two *Cichla* species. Iheringia, Série Zoologia 107: e2017016. https://doi.org/10.1590/1678-4766e2017016

Nascimento FL, Catella AC, Moraes AS (2001) Distribuição espacial do tucunaré, *Cichla* sp. (Pisces, cichlidae), peixe amazônico introduzido no Pantanal, Mato Grosso do Sul, Brasil. Embrapa Pantanal Boletim Pesquisa e Desenvolvimento 24: 1–15.

Oliveira AV, Prioli AJ, Prioli S, Bignotto TS, Júlio Jr HF, Carrerk H, Agostinho CS, Prioli LM (2006) Genetic diversity of invasive and native *Cichla* (Pisces: Perciformes) populations in Brazil with evidence of interspecific hybridization. Journal of Fish Biology 69: 260–277. https://doi.org/10.1111/j.1095-8649.2006.01291.x

Oliveira C, Toledo LFA, Foresti F, Toledo FSA (1988) Supernumerary chromosomes, robertsonian rearrangement and multiple NORs in *Corydoras aeneus* (Pisces, Siluriformes, Callichthyidae). Caryologia 41: 227–236. https://doi.org/10.1080/00087114.1988.10797863

Perazzo G, Noleto RB, Vicari MR, Machado PC, Gava A, Cestari MM (2011) Chromosomal studies in *Crenicichla lepidota* and *Australoheros facetus* (Cichlidae, Perciformes) from extreme Southern Brazil. Reviews in Fish Biology and Fisheries 21: 509–515. https://doi.org/10.1007/s11160-010-9170-x

Poletto AB, Ferreira IA, Cabral-de-Mello DC, Nakajima RT, Mazzuchelli J, Ribeiro HB, Venere PC, Nirchio M, Kocher TD, Martins C (2010) Chromosome differentiation patterns during cichlid fish evolution. BMC Genetics 11: 1–50. https://doi.org/10.1186/1471-2156-11-50

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: 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Ribeiro LB, Moraes Neto A, Artoni RF, Matoso DA, Feldberg E (2017) Chromosomal mapping of repetitive sequences (*Rex3*, *Rex6*, and rDNA Genes) in hybrids between *Colossoma macropomum* (Cuvier, 1818) and *Piaractus mesopotamicus* (Holmberg, 1887). Zebrafish 14: 155–160. https://doi.org/10.1089/zeb.2016.1378

Richards CL, Bossdorf O, Pigliucci M (2010) What role does heritable epigenetic variation play in phenotypic evolution? Bioscience 60: 232–237. https://doi.org/10.1525/bio.2010.60.3.9

Sabaj MH, López-Fernández H, Willis SC, Hemraj DD, Taphorn DC, Winemiller KO (2020) *Cichla cataractae* (Cichliformes: Cichlidae), new species of peacock bass from the Essequibo Basin, Guyana and Venezuela. Proceedings of the Academy of Natural Sciences of Philadelphia 167(1): 69–86. https://doi.org/10.1635/053.167.0106

Schneider CH, Gross MC, Terencio ML, Artoni RF, Vicari MR, Martins C, Feldberg E (2013) Chromosomal evolution of neotropical cichlids: the role of repetitive DNA sequences in the organization and structure of karyotype. Reviews in Fish Biology and Fisheries 23: 201–214. https://doi.org/10.1007/s11160-012-9285-3

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

Teixeira WG, Ferreira IA, Cabral-de-Mello DC, Mazzuchelli J, Valente GT, Pinhal D, Poletto AB, Venere PC, Martins C (2009) Organization of repeated DNA elements in the genome of the cichlid fish *Cichla kelberi* and its contributions to the knowledge of fish genomes. Cytogenetic and Genome Research 125: 224–234. https://doi.org/10.1159/000230006

Teixeira AS, Oliveira SS (2005) Evidence for a natural hybrid of peacock bass (*Cichla monoculus* vs *Cichla temensis*) based on esterase electrophoretic patterns. Genetics and Molecular Research 4(1): 74–83.

Terencio ML, Schneider CH, Gross MC, Nogaroto V, Almeida MC, Artoni RF, Vicari MR, Feldberg E (2012) Repetitive sequences associated with differentiation of W chromosome in *Semaprochilodus taeniurus*. Genetica 140: 505–512. https://doi.org/10.1007/s10709-013-9699-4

Thompson KW (1979) Cytotaxonomy of 41 species of Neotropical Cichlidae. Copeia 1979: 679–691. https://doi.org/10.2307/1443877

Varriale A, Torelli G, Bernardi G (2008) Compositional properties and thermal adaptation of 18S rRNA in vertebrates. RNA 14: 1492–1500. https://doi.org/10.1261/rna.957108

Viana Ferreira AM, Marajó L, Matoso DA, Ribeiro LB, Feldberg E (2019) Chromosomal mapping of rex retrotransposons in tambaqui (*Colossoma macropomum* Cuvier, 1818) exposed to three climate change scenarios. Cytogenetics and Genome Research 159(1): 39–47. https://doi.org/10.1159/000502926

Vicari MR, Moreira-Filho O, Artoni RF, Bertollo LAC (2006) ZZ/ZW sex chromosome system in an undescribed species of the genus *Apareiodon* (Characiformes, Parodontidae). Cytogenetic and Genome Research 114: 163–168. https://doi.org/10.1159/000093333

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

Willis SC (2017) One species or four? yes!... and, no. Or, arbitrary assignment of lineages to species obscures the diversification processes of Neotropical fishes. PLoS ONE 12(2): e0172349. https://doi.org/10.1371/journal.pone.0172349

Willis SC, Macrander J, Farias IP, Ortí G (2012) Simultaneous delimitation of species and quantification of interspecific hybridization in Amazonian peacock cichlids (genus *Cichla*) using multilocus data. BMC Evolutionary Biology 12: 1–96. https://doi.org/10.1186/1471-2148-12-96

Willis SC, Nunes M, Montana CG, Farias IP, Ortí G, Lovejoy NR (2010) The Casiquiare River acts as a corridor between the Amazonas and Orinoco rivers basins: biogeographic analysis of the genus *Cichla*. Molecular Ecology 19: 1014–1030. https://doi.org/10.1111/j.1365-294X.2010.04540.x