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RESEARCH ARTICLE

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Karyotype, C-banding and AgNORs of two endemic leuciscine fish, *Pseudophoxinus crassus* (Ladiges, 1960) and *P. hittitorum* Freyhof & Özulug, 2010 (Teleostei, Cyprinidae)

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Abstract

The genus *Pseudophoxinus* Bleeker, 1860 is found in a wide range of habitats in central Anatolia, but it is not well known from a cytogenetic aspect. In this study the first karyotypic description of the spring minnows *Pseudophoxinus crassus* (Ladiges, 1960) and *P. hittitorum* Freyhof & Özulug, 2010 by means of conventional methods (Giemsa staining, C-banding, silver nitrate impregnation (Ag-NORs)) was performed. Both species are endemic and have restricted distributions in Central Anatolia. *P. crassus* and *P. hittitorum* have the same diploid chromosome number, 2n = 50, patterns of distribution of constitutive heterochromatin (CH), and localization of nucleolus organizer regions (NORs), but differ in their karyotypic formulae (KFs). The C-banding technique revealed clear pericentromeric blocks of CH in many chromosomes; Ag-NORs treatment revealed consistent positive signals at the end of the short arms of a submetacentric chromosome pair, likely homologous in both species. The karyotypic differences found between these species can be used for their taxonomical study.

Keywords

Karyotype, C-banding, NOR-phenotype, Leuciscinae, cytotaxonomy

Introduction

Spring minnows of the cyprinid genus *Pseudophoxinus* Bleeker, 1860 are distributed from Central Anatolia east to Azerbaijan and South to Israel (Freyhof and Özuluğ 2010). The genus belongs to the subfamily Leuciscinae, the major element of the Anatolia cyprinid fauna. Leuciscinae fishes include 54 species belonging to 17 genera in Anatolia, of which 26 species and subspecies are endemic. With 19 species recognized in Turkey, *Pseudophoxinus* is one of the most species-rich genera with a great number of the endemic species (Bogutskaya 1997, Freyhof and Özuluğ 2006, Bogutskaya et al. 2007, Karasu et al. 2011, Küçük et al. 2012, Küçük and Güçlü 2014). Species of this genus are found in a wide range of habitats in central Anatolia (Hrbek et al. 2004). According to IUCN, a significant point about the herein studied species is the fact that *P. crassus* and *P. hittitorum* are endangered (EN) species and their population trends are decreasing (IUCN 2014a; IUCN 2014b).

Karyotypic data for the genus are available only for *P. antalyae* Bogutskaya, 1992 and *P. firati* Bogutskaya, Küçük & Atalay, 2007 (Table 1). In both species a karyotype with 2n = 50 was revealed, indicating a conserved karyotypic evolution in relation to the diploid number (Ergene et al. 2010, Karasu et al. 2011). Thus, cytogenetic data for *Pseudophoxinus* are insufficient, and further study is needed to evaluate karyological characteristics of the genus, to improve the taxonomic identification of these fish, and to understand the evolutionary trends in this taxon (Yüksel and Gülkaç 1992).

The aim of this study is to describe the karyotypes of *P. crassus* and *P. hittitorum*, including identification of CH blocks and NORs by conventional cytogenetic techniques (Giemsa staining, C-banding, and Ag impregnation).

Material and methods

Specimens were captured by electrofishing in two distinct localities during the summer-autumn, 2012 and spring-summer, 2013. Three males and two females of *P. crassus* were collected in Cihanbeyli-İnsuyu spring (38°42'N, 32°45'E) and four females and four males of *P. hittitorum* in Beyşehir-Eflatunpınarı spring (37°52'N, 31°34'E). Specimens were transported alive to the laboratory and kept in well-aerated aquaria until analysis was performed. Chromosome spreads were obtained using standard kidney protocol (Collares-Pereira 1992). Chromosomes were stained with 4% Giemsa solution (pH = 6.8). C-bands were obtained according to Sumner technique (Sumner 1972). Silver impregnation to detect NORs followed the method of Howell and Black (1980).

The chromosome slides were observed by 100× objective with immersion oil and photographed using a Leica DM 3000 research microscope. AKAS software was used to take pictures of the metaphase plates. Measurements of chromosomes were performed by digital caliper from each individual and karyotypes were prepared manually. Chromosomes were arranged in decreasing size order and classified according to their arm ratios (Levan et al. 1964) in three categories: metacentric (M), submetacentric

Table 1. Cytogenetic data available for the genus *Pseudophoxinus*.

Species	Locality	2n	Karyotypic formula	FN	NOR	C-band	Reference
P. antalyae	Berdan River	50	16M+14SM+12ST+8A	92	1 pair *st.* *p* terminal	several	Ergene et al. 2010
P. firati	Tohma Creek	50	38M-SM+12ST	88	2 pairs *sm-st.* *p* terminal	6 pairs	Karasu et. al. 2011
P. crassus	İnsuyu Spring	50	12M+30SM+8ST-A	92	1 pair *sm* *p* terminal	several	Present study
P. hittitorum	Beyşehir Spring	50	14M+26SM+10ST-A	90	1 pair *sm* *p* terminal	several	Present study

2n: diploid number; FN: fundamental number; NOR: nucleolus organizer regions type; M: metacentric; SM: submetacentric; ST: subtelocentric; A: acrocentric; *p* short arm.

(SM) and subtelocentric to acrocentric (ST-A). To determine the fundamental number (FN), M and SM chromosomes were considered as bi-armed whereas those of group ST/A as uni-armed.

Results

243 metaphase plates were examined for *P. crassus* and 266 metaphase plates – for *P. hittitorum*. For *P. crassus* the percentage of the finding of 50 chromosomes was 81.50%. Other percentages were: for 49 chromosomes – 14.45%, for 48 chromosomes – 2.70%, for 47 chromosomes – 1.35%. For *P. hittitorum* the percentage of the finding of 50 chromosomes was 80.00%. Other percentages were: for 49 chromosomes – 13.50%, for 48 chromosomes – 3.00%, for 47 chromosomes – 2.30% and for 46 chromosomes – 1.20%. Therefore it was considered that the analyzed individuals of *P. crassus* and *P. hittitorum* had the same diploid numbers 2n = 50, but differed in their karyotypic formulas (KFs), which were 12 M + 30 SM + 8 ST-A (FN = 92) for *P. crassus* and 14 M + 26 SM + 10 ST-A (FN = 90) for *P. hittitorum*, respectively (Fig. 1). No sex chromosomes were identified for either species.

C-banding revealed the presence of the blocks of constitutive heterochromatin at the pericentromeric regions of many chromosome pairs in both species (Fig. 2).

The NORs were localized near to the secondary constriction on the short arm of a SM chromosome pair in both species (Fig. 3).

Discussion

P. crassus and *P. hittitorum* karyotypes demonstrated the general pattern described for most Leuciscinae that have the chromosome number (2n = 50), but their KFs differed. This is consistent with most other species of the genus *Pseudophoxinus*, which share 2n = 50 and differ in their KFs (Ergene et al. 2010, Karasu et al. 2011). The chromosome

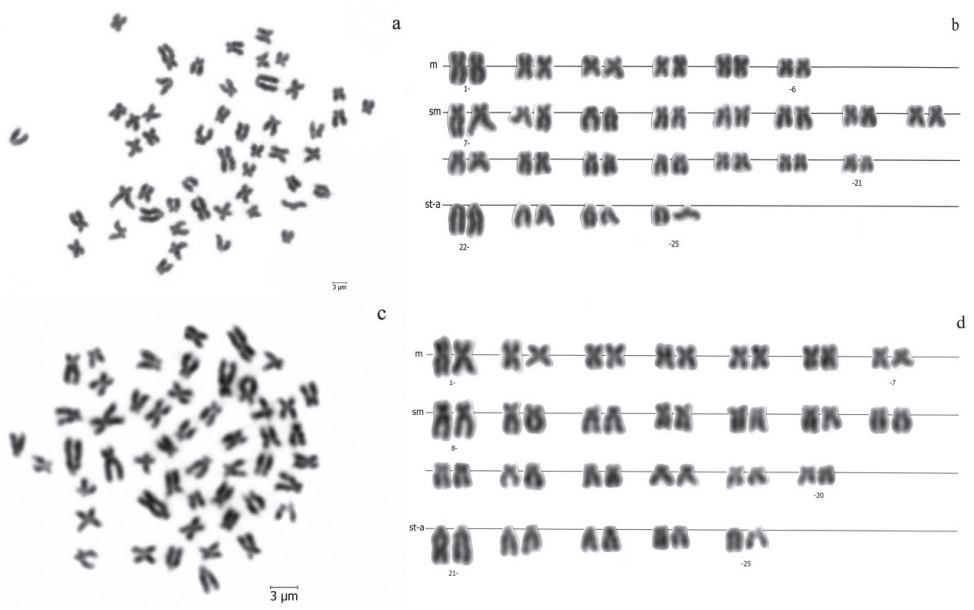

Figure 1. a Giemsa stained metaphase and **b** corresponding karyotype of *P. crassus* from Cihanbeyli stream **c** Giemsa stained metaphase and **d** karyotype of *P. hittitorum* from Beyşehir drainage. Scale bar = 3 µm.

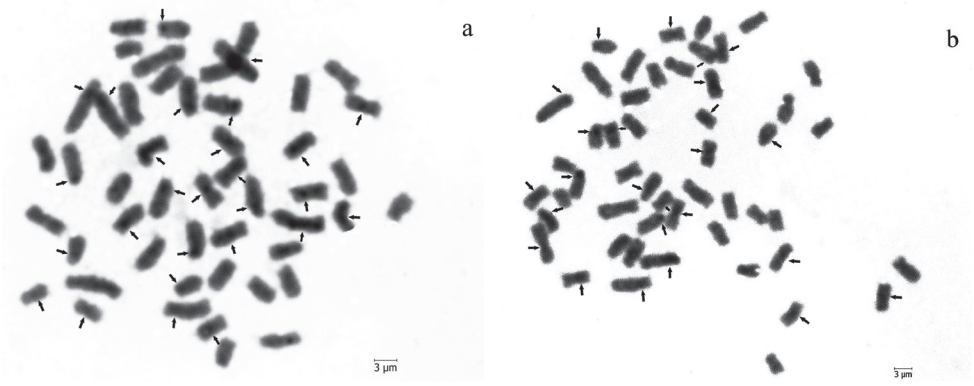

Figure 2. Metaphase spreads of (**a**) *P. crassus* and (**b**) *P. hittitorum* with C-banding. Arrows show CH regions. Scale bar = 3 µm.

sets of leuciscine cyprinids are characterized mainly by bi-armed (meta- and submetacentric) compared to the uni-armed (subtelo- and acrocentric) elements as observed in *P. crassus* and *P. hittitorum*. A large subtelocentric/acrocentric chromosome pair is considered as a cytotaxonomic marker for the subfamily Leuciscinae (Rab and Collares-Pereira 1995, Rab et al. 2008) and it is also present in both analysed species. However, cyprinid sex chromosomes appear to have remained morphologically undifferentiated (Sola and Gornung 2001). *P. crassus* and *P. hittitorum* also display the cyprinid characteristics mentioned above.

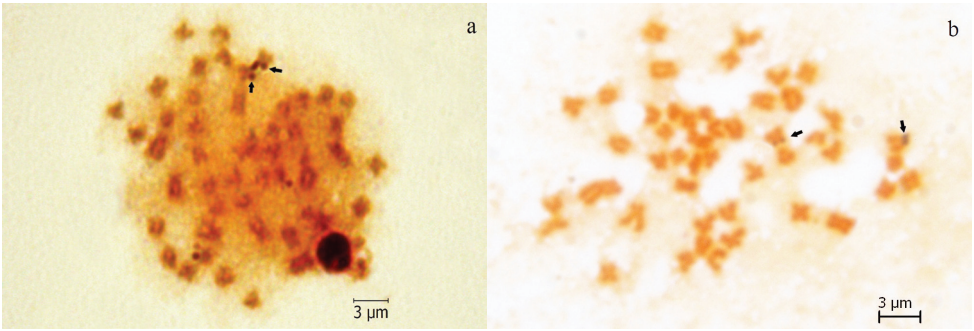

Figure 3. Metaphase spreads of (**a**) *P. crassus* and (**b**) *P. hittitorum* with Ag-NOR treatmets. Arrows show NORs. Scale bar = 3 µm.

C-bands identify regions of constitutive heterochromatin, which contain transcriptionally inactive highly repetitive DNA sequences (Gold et al. 1990). The difference in heterochromatin localization can be used as cytogenetic marker for the differentiation of species and for the reconstruction of chromosome evolution in the taxa (Gaffaroğlu and Yüksel 2009). In *P. crassus* and *P. hittitorum* C-positive blocks were pericentromeric, as in the *P. antalyae* (Ergene et al. 2010) and *P. firati* (Karasu et al. 2011). It was shown, that other studied Leuciscinae species as *Acanthobrama marmid* Heckel, 1843 (Gaffaroğlu and Yüksel 2009), *Squalius anatolicus* (Bogutskaya, 1997) (Ünal 2011) and *S. lucumonis* (Bianco, 1983) (Rossi et al. 2012) also have CH blocks on the pericentromeric regions. This pattern is conserved in Neotelostei as a whole, and also in all the Leuciscine genera examined to date (Collares-Pereira and Rab 1999, Boron et al. 2009, Rossi et al. 2012).

The number and location of NORs have been used as chromosome markers in fish cytotaxonomy (Pereira et al. 2012, Rossi et al. 2012, Nabais et al. 2013). The NORs located on a medium-sized SM chromosome pair corresponds to those observed in many of the leuciscines analyzed (Bianco et al. 2004). In spite of the many exceptions reported in Leuciscinae species from both Eurasia and North America (Pereira et al. 2009, Rossi et al. 2012), a single pair of NOR-carrying chromosome is considered as an ancestral character in this lineage (Rab and Collares-Pereira 1995, Rab et al. 2007). Within the genus *Pseudophoxinus*, a single NOR-bearing chromosome pair as in *P. crassus* and *P. hittitorum*, was observed in *P. antalyae* (Ergene et al. 2010) whereas multiple NOR-carrying chromosomes were detected in *P. firati* (Karasu et al. 2011). Although NORs are usually located on the short arms of chromosomes, sometimes they can be seen on the long arms of metacentric and acrocentric chromosomes (Rab and Collares-Pereira 1995, Rab et al. 1996). Furthermore, NORs can be seen between telomeres and centromeres (Amemiya and Gold 1988). Generally, the NOR-phenotype is observed at the terminal on short arms of mid-sized A-ST chromosomes (Takai and Ojima 1992), and rarely at the terminal on short arms of mid-sized SM chromosomes (Gold et al. 1988, Magtoon and Arai 1993) like in *P. crassus* and *P. hittitorum*. Conversely to what was reported for some others leuciscin cyprinids (Ünal

2011), no NOR polymorphism was observed in the specimens from our study. Further, there is no report of any variation in NORs' phenotype in all analyzed individuals of the genus *Pseudophoxinus* (Ergene et al. 2010, Karasu et al. 2011). Thus the karyotypes of these species conserved plesiomorphic condition that is confirmed by present study.

In conclusion, the karyotypic differences and CH and NOR localizations found in the two *Pseudophoxinus* species studied herein can be used as a cytogenetic comparison data.

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Karyotype rearrangements and telomere analysis in *Myzus persicae* (Hemiptera, Aphididae) strains collected on *Lavandula* sp. plants

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Abstract

Karyotype analysis of nine strains of the peach-potato aphid *Myzus persicae* (Sulzer, 1776), collected on *Lavandula* sp. plants, evidenced showed that five of them had a standard 2n = 12 karyotype, one possessed a fragmentation of the X chromosome occurring at the telomere opposite to the NOR-bearing one and three strains had a chromosome number 2n = 11 due to a non-reciprocal translocation of an autosome A3 onto an A1 chromosome. Interestingly, the terminal portion of the autosome A1 involved in the translocation was the same in all the three strains, as evidenced by FISH with the histone cluster as a probe. The study of telomeres in the *M. persicae* strain with the X fission evidenced that telomerase synthesised *de novo* telomeres at the breakpoints resulting in the stabilization of the chromosomal fragments. Lastly, despite the presence of a conserved telomerase, aphid genome is devoid of genes coding for shelterin, a complex of proteins involved in telomere functioning frequently reported as conserved in eukaryotes. The absence of this complex, also confirmed in the genome of other arthropods, suggests that the shift in the sequence of the telomeric repeats has been accompanied by other changes in the telomere components in arthropods in respect to other metazoans.

Keywords

Aphids, *Myzus persicae*, holocentric chromosomes, karyotype rearrangements, *de novo* telomeres, shelterin

Introduction

Karyotype features are usually stable within species, and chromosomal changes, if they occur, contribute to the formation of a post-zygotic barrier between biological populations causing the establishment of reproductive isolation and speciation as a possible consequence (Noor et al. 2001, Delneri et al. 2003, Kandul et al. 2007). Indeed, mating between individuals with different karyotypes frequently produces hybrids with a reduced fertility due to mis-segregation of homologous chromosomes during the first meiotic divisions (e.g. Kandul et al. 2007).

Despite these general rules, the speciation models were still problematic since numerous cases of intraspecific karyotype instability have been described in literature and at present the most extreme case was published by Lukhtanov et al. (2011) reporting in the butterfly *Leptidea sinapis* (Linnaeus, 1758) the first clearly documented example of explosive chromosome number evolution through intraspecific and intrapopulation accumulation of multiple chromosomal changes. At the same time, the hybrid-sterility model is controversial in some taxa (as revised by Faria and Navarro 2010) so that its true plausibility is difficult to evaluate.

Aside from special cases, such as polyploidy, chromosomal speciation remained a controversial mechanism, especially in animals other than mammals (e.g., Coyne and Orr 2004), since up till now few studies have systematically analyzed the number of chromosomal rearrangements between taxa as a function of the divergence time measured molecularly (Coyne and Orr 2004). An intriguing exception is represented by the large genus *Agrodiaetus* (Hübner, 1822) (Lepidoptera: Lycaenidae), which exhibits an unusual interspecific diversity in chromosome number, from n = 10 to 134, allowing to assess that a rapid karyotypic diversification is likely to have contributed to this explosive speciation rate (Kandul et al. 2007).

The peach potato aphid *Myzus persicae* (Sulzer, 1776) is a good experimental model for the study of chromosome rearrangements since numerous variations regarding both chromosome number and structure have been reported (Blackman 1980, Lauritzen 1982, Rivi et al. 2012; Monti et al. 2011, Monti et al. 2012a, Monti et al. 2012b, Kati et al. 2014). Several populations of *M. persicae* were, for example, heterozygous for a translocation between autosomes 1 and 3 and this rearrangement is involved in the resistance to organophosphate and carbamate insecticides (Spence and Blackman 1998). *M. persicae* populations with 13 chromosomes have also been identified in various countries as the result of independent and diverse fragmentations of the autosome (A) 3 suggesting that different naturally occurring rearrangements of the same chromosomes may be observed in the aphid karyotype (Blackman 1980, Lauritzen 1982; Monti et al. 2012a, Monti et al. 2012b, Rivi et al. 2012). Lastly, some *M. persicae* clones possessed an intra-individual mosaicism, mainly involving fissions of chromosomes A1, A3 and X (Monti et al. 2012a, Kati et al. 2014).

The evolutionary history of the *M. persicae* group is marked with speciation events (for a review see Blackman and Eastop 2007) and the tobacco specialist subspecies *M. persicae nicotianae*, known as the tobacco aphid, is a notable example since it preserved its genomic integrity through time and across a wide geographical scale by investing in

asexual life cycle in most parts of the world (Blackman 1987, Margaritopoulos et al. 2007a, 2007b, 2009).

The frequent occurrence of different chromosome numbers and the inheritance of chromosomal fragments have been related to the holocentric structure of aphid chromosomes (Mandrioli and Manicardi 2012, Manicardi et al. 2014), since chromosomal fragments can contact the microtubules and move properly in the daughter cells during cell division so that they are mitotically stable (Blackman 1980). However, the molecular machinery involved in such rearrangements is still not clarified and the holocentric nature of chromosomes may explain the inheritance of rearranged chromosomes, but not their origin.

The spread of chromosomal rearrangements has also been favoured in *M. persicae* by the continuous expression of the telomerase gene, which allows a *de novo* synthesis of new telomeres at the chromosomal breakage sites (Monti et al. 2011) and by the fast aphid reproduction based on apomictic parthenogenesis (Manicardi et al. 2014). This aspect is particularly intriguing considering that parthenogenesis has been described in bdelloid rotifers as a mechanism favouring speciation since it forces the reproductive isolation (D. Fontaneto, personal communication).

As Loxdale et al. (2011) mentioned in their review about specialization in animals, *M. persicae* could be an ideal experimental model to analyze rapid evolution, i.e. measured in perceptible time scale, since the agricultural practices could act as a strong selection pressure favouring evolutionary changes over short periods.

In the present paper we analysed the presence of karyotype variants in nine *M. persicae* strains collected on *Lavandula* sp. plants. Moreover we verified if the synthesis of *de novo* telomeres is common in *M. persicae* populations with fragmented chromosomes and analysed the evolutionary conservation of the shelterin complex, a group of proteins generally associated with telomere functioning.

Material and methods

Specimens of *M. persicae* were obtained from 9 different aphid populations collected on *Lavandula* sp. plants. In particular, the strains labelled as Mo1, Mo2, Mo3 and Mo4 have been collected in Modena (Italy), whereas the strains Re1, Re2a, Re2b, Re3 and Re4 have been collected in Correggio (Reggio Emilia, Italy). Each population was established as a clone from a single female aphid originally collected from the field and thereafter maintained as a colony of parthenogenetic females on pea (*Pisum sativum*, Linnaeus, 1758) plants at 19 °C with a light-dark regime of 16 hours light and 8 hours darkness.

Chromosome preparations were obtained from parthenogenetic females by spreading embryo cells, as reported by Mandrioli et al. (1999) In order to analyse chromosome number, slides were stained with a 100 ng/ml propidium iodide solution in phosphate buffer for 15 minutes at room temperature. For each different karyotype, measurements of chromosome length were performed on 50 metaphases using the software MicroMeasure, available at the Biology Department at Colorado State University website (http://rydberg.biology.colostate.edu/MicroMeasure).

Table 1. GenBank sequences used for bioinformatic comparative analyses.

Telomere-associated proteins	Orthologous proteins in GenBank
POT1	*Homo sapiens* (AAH02923), *Schizosaccharomyces pombe* (CAB16192)
TRF1	*H. sapiens* (NP_059523), *S. pombe* (NP_595979)
TRF2	*H. sapiens* (NP_005643)
RAP1	*H. sapiens* (ABA64473), *S. pombe* (BAB70735)
TPP1/ TEBPα	*Danio rerio* (NP_001124265), *Stylonychia lemnae* (AAU95535)
TIN2	*H. sapiens* (AF195512)

DNA extraction, following a standard phenol-chloroform protocol, and fluorescent *in situ* hybridization (FISH) have been described in Mandrioli et al. (1999).

The 28S rDNA genes have been amplified using the primers F (5'-AACAAACAAC-CGATACGTTCCG) and R (5'-CTCTGTCCGTT TACAACCGAGC), designed according to the insect 28S rDNA sequences available in GenBank. Amplification was performed using a Hybaid thermal-cycler at an annealing temperature of 60 °C for 1 minute (min) with an extension time of 1 min at 72 °C.

In order to amplify a DNA sequence containing the complete aphid histone gene cluster, the primers HIS-CLUST-F (5'-CGAAACCGTAAAGGGTACGA) and HIS-CLUST-R (5'-GGCGGCTTTGACTTTATTGA) have been designed on the basis of the *Acyrthosiphon pisum* genomic scaffold 368 (NW_003383857.1, from base 259987 to 272662). The amplification of a 7379 bp fragment was carried out by an Hybaid thermal-cycler using the Fermentas Long PCR Enzyme Mix making annealing and extension at 68 °C for 8 min for 25 cycles, according to the manufacturer's instructions.

PCR digoxigenin labelling of the subtelomeric repeat was performed with a PCR DIG labelling kit according to the Roche protocol using the specific oligonucleotide primers MpR-F (5'–TCAAAGTTCTCGTTCTCC–3') and MpR-R (5'–GTTT-TAACAGAGTGCTGG–3'), designed according to the subtelomeric repeat sequence available in the literature (Spence et al. 1998). The reaction conditions were 94 °C for 90 sec (denaturation), a total of 25 cycles of 94 °C for 30 s, 51 °C for 30 sec (annealing) and 72 °C for 30 sec (extension), and with a final extension step at 72 °C for 7 min.

In order to localize the telomeric $(TTAGG)_n$ repeats, a probe was obtained by PCR amplification using the two primers F $(TTAGG)_5$ and R $(CCTAA)_5$ in the absence of template, as described by Ijdo et al. (1991).

Random priming probe biotin-labelling was performed with the Biotin High Prime (Roche), whereas the PCR digoxigenin labelling were performed using the Dig High Prime (Roche). Both labelling were done according to the Roche protocols.

Propidium-stained and FISH slides were observed using a Zeiss Axioplan epifluorescence microscope. Photographs of the fluorescent images were taken using a CCD camera (Spot, Digital Instrument, Madison, USA) and the Spot software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Bioinformatic analyses for homologous genes coding for the proteins POT1, TRF1, TRF2, RAP1, TPP1 and TIN2 in aphids and other arthropods have been performed by BLAST alignments in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) both at DNA

and protein levels using different homologous genes as reference sequences (Table 1). A further search has been performed by BLAST alignments in aphids at AphidBase (http://www.aphidbase.com).

Results

The standard karyotype of *M. persicae* parthenogenetic females consists of 12 chromosomes (five pairs of autosomes and two X chromosomes) (Mandrioli et al. 1999). The analysis of the strains collected on *Lavandula* plants showed chromosome numbers ranging from 2n = 11 to 2n = 13. In particular, Mo1 (Fig. 1a, e), Mo2 (Fig. 1b, f), Re2a (Fig. 2c, h), Re3 (Fig. 2e, j) and Re4 (Fig. 2f, k) showed a standard 2n = 12 karyotype, whereas Mo3 (Fig. 1c, g), Mo4 (Fig. 1d, h) and Re2b (Fig. 2d, i) have a chromosome number 2n = 11 due to the non-reciprocal translocation of an autosome A3 onto an A1 chromosome.

Previous study showed that the histone cluster map eccentrically on the autosome 1 (Mandrioli and Manicardi 2013), so that double *in situ* hybridization with the subtelomeric DNA repeat and the histone cluster as probes indicated that the non-reciprocal translocation observed in Mo3 (Fig. 1i), Mo4 (Fig. 1j) and Re2b (Fig 2l) strains occurred at the same telomere of the autosome 1. Furthermore, propidium iodide staining revealed that the strain Re1 (Fig. 2a, g) has 2n = 13, as a consequence of a fragmentation of a single X chromosome involving the telomere opposite to the NOR-bearing one, as evident after FISH with the 28S probe (Fig. 2b).

Interestingly, in the clone Re1 all telomeres resulted labelled by the $(TTAGG)_n$ telomeric probe including the X chromosome (and its fragment) involved in the fission suggesting that a *de novo* synthesis of telomeres occurred in this clone (Fig. 3c). No interstitial telomeric signals have been observed in clones Mo3, Mo4 and Re2b possessing a fusion between a copy of autosomes A1-A3 (Fig. 3a, b, d). This result indicated that the A1-3 translocation also involved the loss of both the telomeric and subtelomeric sequences originally present at the chromosomal termini involved in the translocation site, as highlighted in the karyogram drawn in Fig. 4.

Taking into account that the unique aphid protein studied regarding the telomere functioning has been the telomerase (Monti et al. 2011), a survey for orthologues of the proteins consituting the shelterin complex has been performed in the genomes of the aphids *Acyrthosiphon pisum* (Harris, 1776) and *M. persicae* by BLAST alignments both at DNA and protein levels (Table 1), but no orthologues have been found for genes/proteins POT1, TRF1, TRF2, RAP1, TPP1 and TIN2. Similar results have also obtained in the insects *Tribolium castaneum* (Herbst, 1797) (order Coleoptera), *Apis mellifera* (Linnaeus, 1758) (order Hymenoptera), *Anopheles gambiae* (Giles, 1902) (order Diptera) and *Bombyx mori* (Linnaeus, 1758) (order Lepidoptera) and the mites *Tetranychus urticae* (Koch, 1836) and *Varroa destructor* (Anderson & Truman, 2000) assessing that genes coding for the shelterin proteins are not present in all the currently available arthropod genomes.

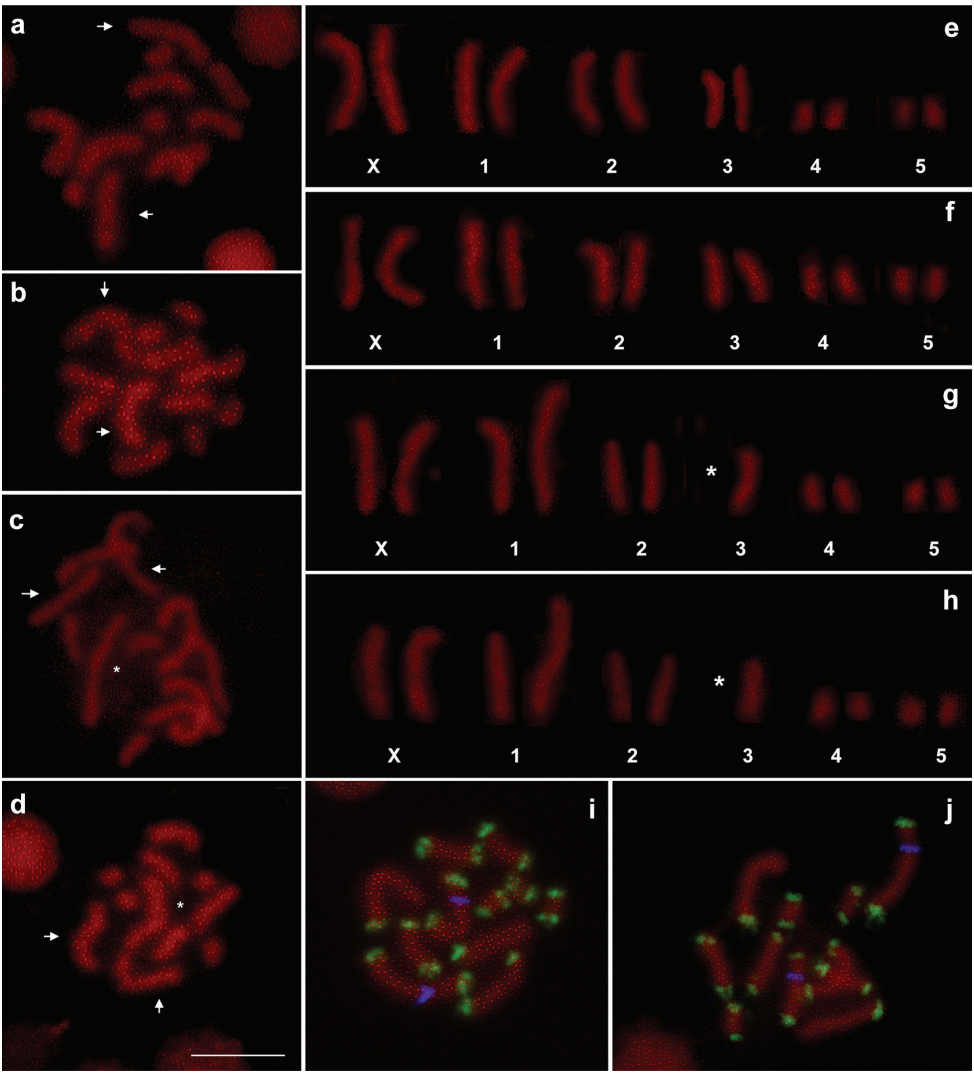

Figure 1. Chromosomal plates (**a–d**) and karyotypes (**e–h**) obtained from specimens belonging to clones Mo1 (**a**, **e**) e Mo2 (**b**, **f**), Mo3 (**c**, **g**) and Mo4 (**d**, **h**). Simultaneous *in situ* hybridization with the histone (in blue) and subtelomeric DNA probes (in green) (**i–j**) revealed in both clones Mo3 (i) and Mo4 (**j**) that the A1–A3 translocation involved the autosome 1 telomere close to the histone probe. Arrows indicate X chromosomes; asterisks indicate rearranged autosomes. Bar = 10 mm.

Discussion

Holocentric chromosomes have been frequently described as a powerful tool to stabilize and inherit chromosomal mutations resulting in karyotype changes (Monti et al. 2012a, 2012b). Even if it is not clear if the observed karyotype variants have phenotypic effects over short temporal and spatial scales on aphid evolution and adaptation, the presence

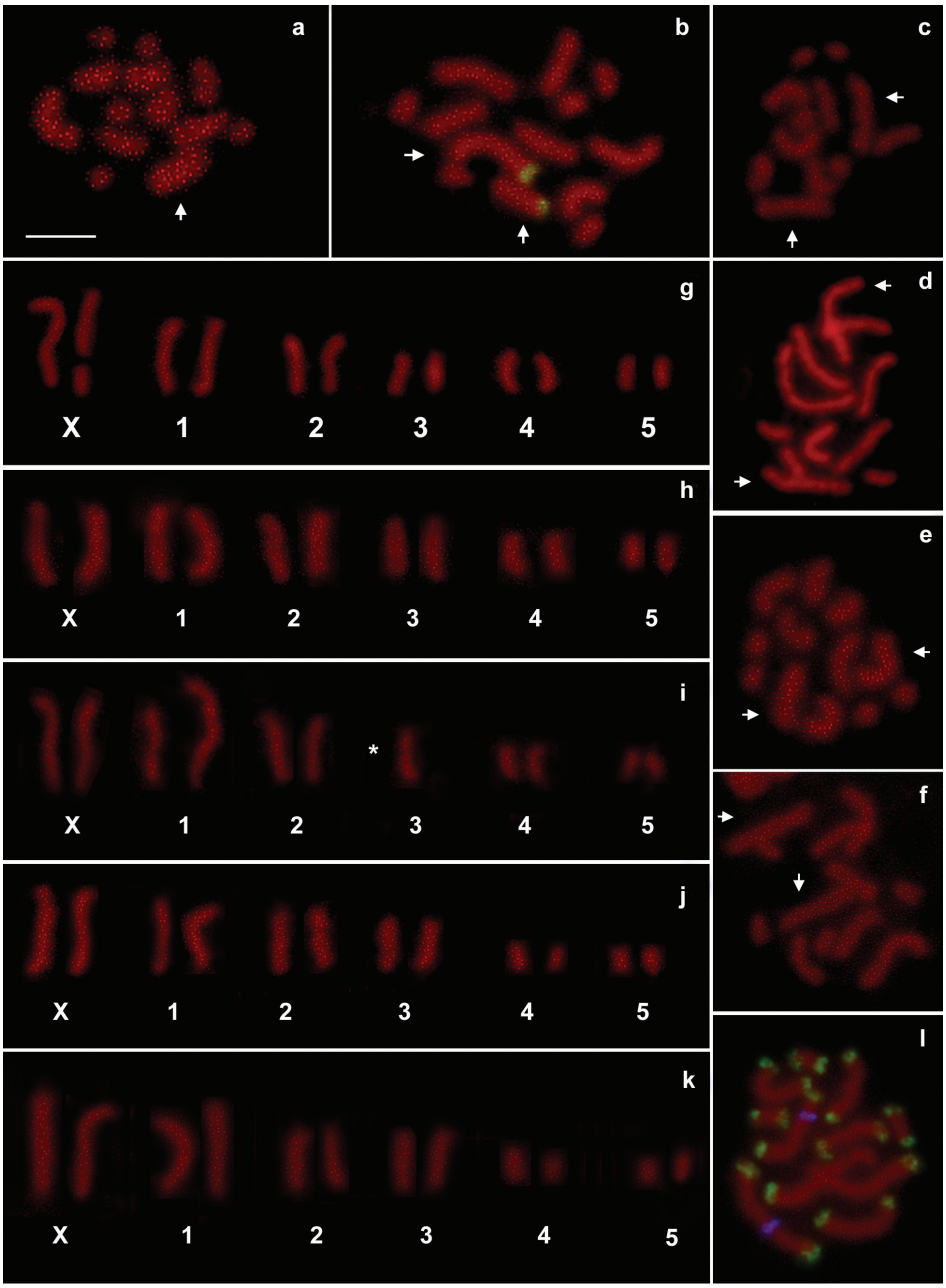

Figure 2. Chromosomal plates (**a**, **c–f**) and karyotypes (**g–k**) obtained from specimens belonging to clones Re1 (**a**, **g, e**) Re2a (**c**, **h**), Re2b (**d**, **i**), Re3 (**e**, **j**) and Re4 (**f**, **k**). Fluorescent *in situ* hybridization with the 28S probe (in green) (**b**) revealed that the fragmentation at the X chromosome in clone Re1 occurred at the telomere opposite to the NOR (**b**). Simultaneous *in situ* hybridization with the histone (in blue) and subtelomeric DNA probes (in green) in clone Re2b (**i**) revealed that the A1–A3 translocation involved the autosome 1 telomere close to the histone cluster. Arrows indicate X chromosomes. Asterisks indicate rearranged autosomes. Bar = 10 mm.

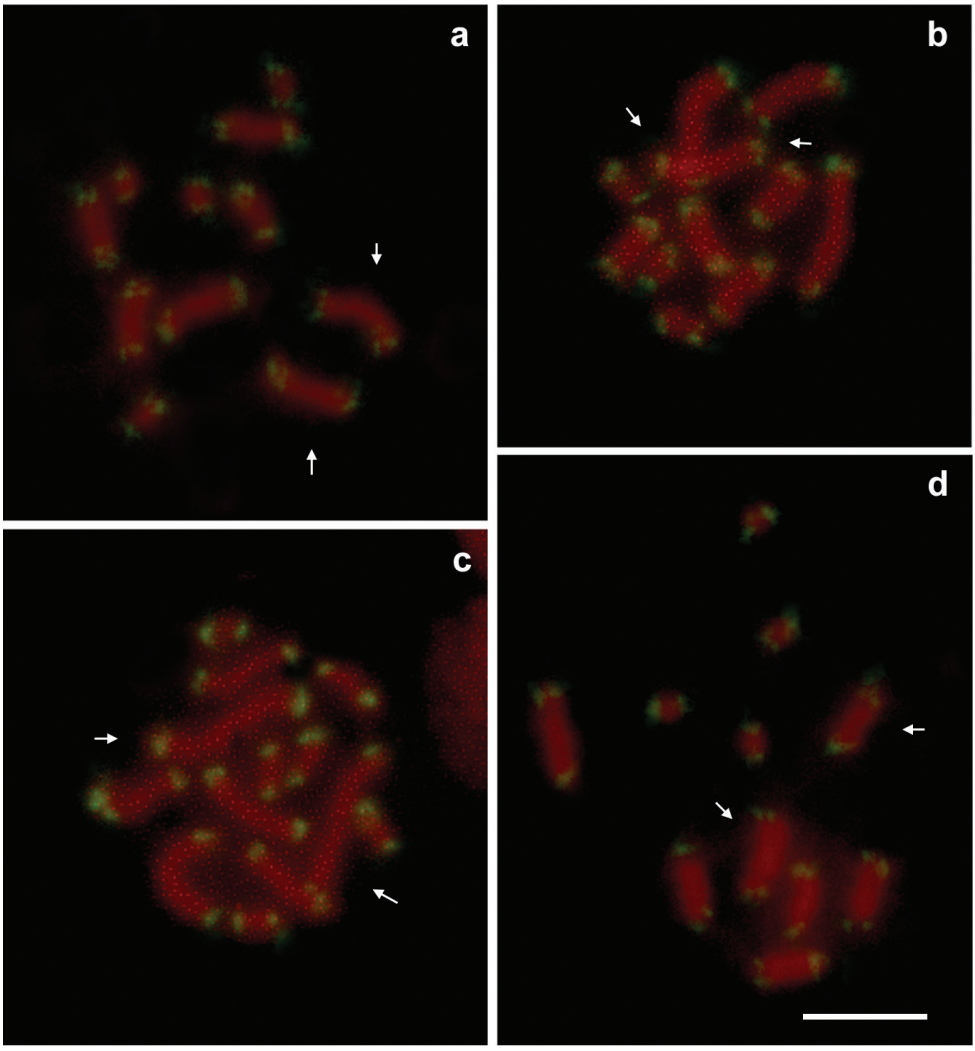

Figure 3. FISH with the telomeric $(TTAGG)_n$ probe showed evident telomeric repeats at each chromosomes in clones Mo3 (**a**), Mo4 (**b**), Re1 (**c**) and Re2b (**d**). No interstitial telomeric signals were present in clones Mo3 (**a**), Mo4 (b) and Re2b (**d**) possessing a chromosomes derived from an autosome A1–A3 fusion. All telomeres resulted labelled by the $(TTAGG)_n$ telomeric probe in clone Re1 including the X chromosome involved in the fission suggesting that a *de novo* telomere synthesis occurred in this clone (**c**). Arrows indicate X chromosomes. Bar = 10 mm.

of chromosomal fissions and fusions (together with holocentrism and a constitutive expression of telomerase) could allow a rapid karyotype evolution at fine geographic scales so that aphid species could be the sum of populations that can have different karyotypes that in turn can give diverse genetic/ecological/evolutionary responses in relation to imposed selective environmental forces (Monti et al. 2012a, 2012b). Indeed the fine-scale patchwork of chromosome rearrangements observed in aphids

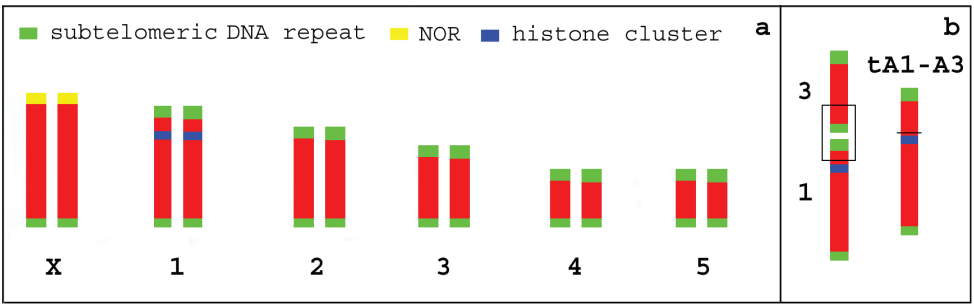

Figure 4. According to previous studies (Spence et al. 1998, Mandrioli and Manicardi 2012), the standard karyotype of *M. persicae* females consists of five couples of autosomes and two X chromosomes, whose rearrangements can be studied using different chromosomal markers (the subtelomeric DNA repeat, the NOR regions, and the histone cluster) (**a**). The analysis of karyotypes of clone Mo3, Mo4 and Re1 indicated that the observed A1-A3 translocation involved the A1 telomere near to the histone cluster an resulted in the loss of both the subtelomeric and the telomeric sequences (**b**).

could increase their potential for local adaptation and differentiation for instance on different host plants that could also explain the success of *M. persicae* as a polyphagous pest crop species.

The identification of several *M. persicae* populations bearing rearranged karyotypes made this species a complex, but intriguing, model for the study of aphid cytogenetics (Lauritzen 1982, Blackman 1987, Fenton et al. 1998, Spence and Blackman 1998, Loxdale 2007, Monti et al. 2012a, 2012b, Manicardi et al. 2014).

In this paper we report the presence of rearranged karyotypes, including fissions and translocations, in *M. persicae* strains collected on *Lavandula* plants. The analysis of their karyotypes confirmed that autosomes 3 and 1 are the chromosomes mostly involved in changes in the *M. persicae* complement (Rivi et al. 2012, Monti et al. 2012a, Kati et al. 2014) and supported previous results suggesting that also the X chromosome can be fragmented (Monti et al. 2012a, 2012b).

Previous literature data (Monti et al. 2012a, 2012b, Kati et al. 2014) highlighted that most of the rearranged karyotypes has been observed in aphid clones collected on tobacco plants, where the stability of the karyotype can be influenced by the clastogenic effects of nicotine (Trivedi et al. 1990, 1993, Sen et al. 1991, Arabi 2004, Sassen et al. 2005). Similarity to nicotine, also the linalyl acetate (one of the major components of the lavender oil) has a genotoxic effect in mammalian cells, where it induced the formation of micronuclei (Di Sotto et al. 2011) so that we could hypothesize that this compound could be at the basis of the chromosomal fragmentations described in this paper. Interestingly, not all the aphid strains collected on *Lavandula* plants showed rearranged karyotypes suggesting that *M. persicae* populations on *Lavandula* plants could consist of strains with a different capacity to metabolize the linalyl acetate in other compounds (such as the linalool) without any genotoxic activity (Di Sotto et al. 2011).

The fission of chromosomes by tobacco and lavender oil mutagens may be lethal in organisms with monocentric chromosomes (possessing a localized centromeres), since

chromosomal fragments tend to be lost during mitosis and meiosis. By contrast, aphids can cope with this due to the holocentric nature of their chromosomes. As a consequence, chromosome fragments can move to the daughter cells at successive cell divisions.

Our results confirmed that some portions of the aphid chromosomes seem to be more prone to fragmentation than others in presence of potential genotoxic compounds. Indeed, a fragmentation of the X chromosome similar to that reported in the present paper has been described in other *M. persicae* strains and it was localized near (or within) the heterochromatic band enriched in satellite DNAs (Monti et al. 2012a, 2012b). The presence of chromosome breakpoints occurring within constitutive heterochromatin is well established in the scientific literature and, for instance, much of the evolution of mammals and some insects (such as grasshoppers) involved pericentromeric heterochromatin that is known to be particularly variable (John 1983, Blackman et al. 2000). *M. persicae* autosome A3 is involved in a heterozygous translocation on an autosome A1 in three *Lavandula* strains further supporting the suggestion that translocations between these autosomes are frequent. Indeed, the same translocation has been previously found in two Greek clones collected on tobacco plants (Kati et al. 2014) and a variant consisting in a partial reciprocal translocation between the A1 and A3 has been reported to have a worldwide distribution (Blackman 1980, Blackman et al. 2007).

Our data showed that the A1-A3 fusion seems to involve always the same terminal end of the autosome 1. Previous experiments (Mandrioli et al. 2014) reported that the terminal portions of autosomes 1 and 3 are in tight proximity in *M. persicae* interphase nuclei suggesting that their proximity could favour their fusion resulting in reciprocal and/or non-reciprocal translocations. The presence of recurrent chromosomal rearrangements in *M. persicae* could therefore be related to the specific architecture of the aphid interphase nucleus.

From a chromosomal point of view, the species *M. persicae* is the sum of populations that have different karyotypes. Interestingly, similar karyotypic variants have been identified on different host plants (Monti et al. 2012a, 2012b, Rivi et al. 2012, 2013, Kati et al. 2014) suggesting that no host-specific karyotype are present in this species with the exception of *M. persicae nicotianae* on tobacco (Blackman 1987).

A further element of interest in the *Lavandula* clones is related to their ability to synthesize new telomeres after chromosomal breakages. In aphids, telomeres consist of stretches of the $(TTAGG)_n$ repeat. This simple sequence has been reported also in the majority of insects (Sahara et al. 1999, Mandrioli 2002, Frydrychová et al. 2004, Vitkova et al. 2005, Monti et al. 2011, Kuznetsova et al. 2012) and in other arthropod groups (sea spiders, chelicerates, myriapods, and crustaceans) (Traut et al. 2007), and seems to be ancestral for the phylum Arthropoda (Lukhtanov and Kuznetsova 2010). However, the ancestral $(TTAGG)_n$ telomeric motif has been repeatedly lost or replaced with other sequences during insect evolution (Vitkova et al. 2005, Mravinac et al. 2011, Mandrioli et al. 2012, Gokhman et al. 2014). For example, in the clade Antliophora (Diptera, Siphonaptera and Mecoptera) the canonical telomeres have been replaced by long repeated sequences, as reported in the non-biting midge

Chironomus pallidivittatus (Malloch, 1915) (Zhang et al. 1994), or by the HetA and TART retrotransposons, as occurred in the fruit fly *Drosophila melanogaster* (Meigen, 1830) (Pardue and DeBaryshe 2003).

Differently from the extensive study of the telomere composition, few papers have been focussed on the proteins associated to the telomere functioning in insects, with the exception of *D. melanogaster*, where telomeres are capped by a complex of fast-evolving proteins, called terminin (Raffa et al. 2011). However, none of the terminin proteins is evolutionarily conserved outside the *Drosophila* genus suggesting that flies rapidly evolved terminin to bind chromosome ends in a sequence-independent fashion probably slightly before the loss of the canonical insect telomeres (Raffa et al. 2011).

In mammals, telomeres are capped by different proteins that play vital roles in telomere length regulation and chromosomal end protection (Giannone et al. 2010). In particular, a relevant role in the mammalian telomeres is played by shelterin, a six subunit complex composed of the telomere repeat binding proteins POT1, TRF1 and TRF2, and their associated proteins TIN2, RAP1 and TPP1 (Liu et al. 2004, Palm and de Lange 2008, Xin et al. 2008, Giannone et al. 2010).

According to literature data, shelterin complex is essential in telomere capping so that telomeres that are severely or completely devoid of telomeric proteins are more prone to damages and/or become the target of frequent recombination (Baumann and Cech 2001, de Lange 2005, Shakirov et al. 2005, 2009, Xin et al. 2008, Palm and de Lange 2008, Giannone et al. 2010). At the same time, shelterin regulates telomere transcription, telomere silencing and telomere sister cohesion through the association of shelterin with other proteins or protein complexes (Giannone et al. 2010).

Due to the importance of the shelterin complex in the telomere functioning, it is very intriguing that this important set of proteins is absent in the studied arthropod genomes, including the aphid one. According to different essays performed both in animal and plants, shelterin complex has a exquisite specificity for the telomeric TTAGGG repeats due to the presence of multiple TTAGGG recognition folds in the complex (de Lange 2005). The TTAGGG motif prevails in all multicellular animals, except round worms and arthropods, and is probably ancestral for all Metazoa (Traut et al. 2007). In arthrodopds the derived TTAGG motif has been evolved (Traut et al. 2007, Mandrioli et al. 2012). As a whole, a plausible scenario is that the shift to the TTAGG telomeric sequence negatively affected the binding of shelterin proteins to the single-strand G-rich telomeric DNA bringing to the loss of the shelterin genes in arthropods.

Exceptions to the presence of all the shelterin proteins have been already reported in literature since, for instance, the subunit TIN2 and TPP1 have been so far only found in vertebrates (de Lange 2005). At the same time the yeast *Saccharomyces cerevisiae* Meyen, 1883 lacks the TRF-like protein and uses instead a highly diverged Rap1 orthologue that binds double-stranded telomeric DNA (de Lange 2005). Conversely, yeast telomeres contain Rif1, a conserved protein that has no known role at mammalian telomeres and instead functions in the intra-S-phase checkpoint (Silverman et al. 2004).

The absence of the whole shelterin complex is extremely interesting from a functional point of view since it is generally implicated in the generation of the t-loop

and in the control of the synthesis of telomeric DNA by telomerase (de Lange 2005). According to the reported role for the shelterin complex, it could be interesting to better understand how t-loops can be generated in the absence of TRF1 and POT1. Interestingly, the availability of antibodies against G-quadruplex DNA (Schaffitzel et al. 2010) could allow to use them as a specific probe to identify and study the interaction of the telomere end-binding proteins with the G-quadruplex in different arthropods (including aphids) making possible to go in depth in the study of arthropod telomere functioning.

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Chromosome number evolution in skippers (Lepidoptera, Hesperiidae)

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Abstract

Lepidoptera (butterflies and moths), as many other groups of animals and plants, simultaneously represent preservation of ancestral karyotype in the majority of families with a high degree of chromosome number instability in numerous independently evolved phylogenetic lineages. However, the pattern and trends of karyotype evolution in some Lepidoptera families are poorly studied. Here I provide a survey of chromosome numbers in skippers (family Hesperiidae) based on intensive search and analysis of published data. I demonstrate that the majority of skippers preserve the haploid chromosome number n=31 that seems to be an ancestral number for the Hesperiidae and the order Lepidoptera at whole. However, in the tribe Baorini the derived number n=16 is the most typical state which can be used as a (syn)apomorphic character in further phylogenetic investigations. Several groups of skippers display extreme chromosome number variations on within-species (e.g. the representatives of the genus *Carcharodus* Hübner, [1819]) and between-species (e.g. the genus *Agathymus* Freeman, 1959) levels. Thus, these groups can be used as model systems for future analysis of the phenomenon of chromosome instability. Interspecific chromosomal differences are also shown to be useful for discovering and describing new cryptic species of Hesperiidae representing in such a way a powerful tool in biodiversity research. Generally, the skipper butterflies promise to be an exciting group that will significantly contribute to the growing knowledge of patterns and processes of chromosome evolution.

Keywords

Lepidoptera, Hesperiidae, karyotype evolution, chromosome number, cryptic species, phylogeny, chromosomal conservatism, chromosomal instability

Introduction

The main karyotypic features of organisms, particularly the number of chromosomes, tend to be stable within species (White 1973, King 1993). New chromosomal rearrangements usually originate as heterozygotes and are often – although not always (Lukhtanov et al. 2011) – associated with heterozygote disadvantage. The spread of such rearrangements to fixation within a large population has low probability (King 1993). Therefore, many organisms are characterized by chromosomal conservatism, a situation in which all closely related taxa demonstrate the same chromosome number.

In contrast to chromosomal conservatism, chromosomal instability characterizes situations where multiple closely related taxa (populations, subspecies and/or species) belonging to a single phylogenetic lineage differ drastically from each other by major chromosomal rearrangements, sometimes resulting in high variability in chromosome number.

Both phenomena - chromosomal conservatism and chromosomal instability - are clearly expressed in insects of the order Lepidoptera (butterflies and moths). The modal haploid number of chromosomes (n) of n = 31 or n = 30 (Suomalainen 1969, Lukhtanov 2000) is preserved in the majority of lepidopteran families (Robinson 1971). At the same time, numerous cases of chromosomal instability have been discovered in the butterfly families, e.g. in Papilionidae (Emmel et al. 1995), Pieridae (Lukhtanov 1991, Lukhtanov et al. 2011, Dinca et al. 2011), Nymphalidae (Brown et al. 1992, 2004, 2007a, 2007b) and Riodinidae (Brown et al. 2012). This phenomenon was analyzed in more detail in the family Lycaenidae (Kandul et al. 2004, 2007, Lukhtanov et al. 2005, 2006, 2008, Vershinina and Lukhtanov 2010, 2013, Vila et al. 2010, Talavera et al. 2013, Przybyłowicz et al. 2014).

Skippers (the family Hesperiidae) are studied to a lesser extent with the respect of karyotype evolution than the other butterfly families mentioned above (but see: Emmel and Trew 1973, Saura et al. 2013). This family includes about 4000 species under 567 genera and is a globally distributed group found in all continents except Antarctica (Warren et al. 2008). The tribal level classification of skippers, based on combined analysis of molecular and morphological data, was recently elaborated by Warren and colleagues (Warren et al. 2008, 2009).

Here I provide a first world-wide survey of chromosome numbers in skippers based on intensive search and analysis of published data.

Results

The results of literature search are presented in the Table below. It includes all the discovered chromosome counts except n=13 for *Ochlodes venatus* (Bremer et Grey, 1853), noted by Bigger (1960) as "*Augiades venata*". The name *Ochlodes venatus* was long used for the *Ochlodes* species of Europe, but it actually refers to its Far Eastern sister species, and the European taxon is now called *O. sylvanus* (Esper, 1777) (ICZN 2000). Both European and Far Eastern species have the same chromosome number n=29 (Federley

1938, Lorković 1941, Abe et al. 2006), not n=13 as indicated by Bigger (1960). Thus, the species name used by Bigger (1960) was probably misidentification.

The classification of skippers accepted in this paper follows Warren and colleagues (Warren et al. 2008, 2009).

Discussion

Modal chromosomal numbers

The table gives the chromosome numbers of 205 species of skippers, i.e. about 5% of the species of the world fauna. This number is not enough to infer any final statements about peculiarities of chromosome numbers distribution within the Hesperiidae. However, several tentative conclusions can be made. The haploid chromosome number n=31 was found in 50 studied species of skippers and, thus, it is a clear modal number for the family at whole. Interestingly, n=31 was found in representatives of all investigated subfamilies, except for Heteropterinae. However, in the last subfamily only one species was karyologically studied until now, and discovery of n=31 in Heteropterinae is not excluded in future. The next most common numbers are n=29 (43 species), n=30 (33 species) and n=28 (13 species).

Subfamilies Coeliadinae and Eudaminae have a sharp peak at n=31. In the subfamily Trapezitinae n=31 was also found (only one species studied).

Within the subfamily Pyrginae, the modal number n=31 is found in the tribe Erynnini. The tribe Pyrrhopygini is characterized by the most common n=28. The modal number in the tribe Tagiadini is n=30. The tribe Carcharodini has peaks at n=30 and n=31. In the tribe Pyrgini, n=29, n=30 and n=31 were found as the most common numbers.

In the family Heteropterinae n=29 was found (only one species studied).

Within the subfamily Hesperiinae, the tribes Taractrocerini, Thymelicini, Calpodini, Moncini and Hesperiini are characterized by the most common n=29. Very variable chromosome numbers (from n=5 to n=50) were found in the tribe Aeromachini. It is difficult to infer the modal number for the last tribe. However, it should be noted that one species, *Thoressa varia*, has n=31 as the majority of other skippers. The tribe Baorini (subfamily Hesperiinae) has a clear peak at n=16, so it is exceptional with respect to the modal number of chromosomes.

The overall evidence indicates that chromosome numbers of Coeliadinae, Eudaminae, Trapezitinae, Pyrginae and Hesperiinae conform to the lepidopteran modal of n=31 (Robinson 1971). This number seems to be an ancestral one for the Hesperiidae as for the order Lepidoptera at whole (Suomalainen 1969, Lukhtanov 2000). This modal number (or its deviation to n=30, n=29 and 28) were preserved in the majority of skippers. However, in the tribe Baorini the number n=16 was evolved and, thus, represents a derived trait which can be used as a (syn)apomorphic character in further phylogenetic studies of the family Hesperiidae.

Table 1. Chromosome number of skippers (Lepidoptera, Hesperiidae) of the world fauna (Us are univalents; 2n is diploid chromosome number).
Years of the species descriptions are given square brackets in cases where they were not stated in the original sources but were inferred from reliable external evidence.

#	Species	Haploid chromosome number	Country	Reference
Subfamily Coeliadinae				
1	*Bibasis aquilina* (Speyer, 1879)	29	Japan	Maeki 1953
	B. a. chrysaeglia (Butler, 1881)	31 (2n=62)	Japan	Abe et al. 2006
2	*B. jaina formosana* Fruhstorfer, 1911	31	Taiwan	Maeki and Ae 1968b
3	*Choaspes benjaminii* (Guérin-Méneville, 1843)	31	Japan	Maeki 1953
	Ch. b. japonica (Murray, 1875)	31	Japan	Saitoh et al. 1978
4	*Coeliades anchises jucunda* (Butler, 1881)	30	Oman	Saitoh 1982
5	*C. ernesti* (Grandidier, 1867)	31	Madagascar	de Lesse 1972
6	*C. fervida* (Butler, 1880)	23	Madagascar	de Lesse 1972
7	*C. forestan arbogastes* (Guenee, 1863)	31	Madagascar	de Lesse 1972
8	*C. ramanatek* (Boisduval, 1833)	31	Madagascar	de Lesse 1972
Subfamily Euschemoninae no chromosomal data available				
Subfamily Eudaminae				
9	*Achalarus casica* (Herrich-Schäffer, 1869)	29	USA (Texas)	Emmel and Trew 1973
10	*A. lyciades* (Geyer, 1832)	31	USA (Connecticut)	Maeki 1961
11	*A. toxeus* (Plötz, 1882)	16	Mexico	Maeki and Remington 1960
12	*Astraptes anaphus* (Godman et Salvin, 1896)	31	Bolivia	de Lesse 1967a
13	*A. fulgerator* (Walch, 1775)	31	Peru	Kumagai et al. 2010
14	*A. naxos* (Hewitson, 1867)	31	Brazil	Saura et al. 2013
15	*A. phalaecus* (Godman et Salvin, 1893)	25	Guatemala	de Lesse 1967a
16	*A. longipennis* (Plötz, 1882)	31	Costa Rica	Kumagai et al. 2010
		31	Peru	Kumagai et al. 2010
		31	Brazil	Kumagai et al. 2010
17	*Autochton* sp.	20, 21	Brazil	Kumagai et al. 2010
18	*Chioides albofasciatus* (Hewitson, 1867)	31	Mexico	de Lesse 1970a
	Ch. albofasciatus (Hewitson, 1867) (as *Ch. catillus*)	31	Mexico	Maeki and Remington 1960
	Ch. albofasciatus (Hewitson, 1867)	31	USA (Texas)	Emmel and Trew 1973
19	*Entheus priassus pralina* Evans, 1952	22	Brazil	Saura et al. 2013
20	*Epargyreus barisses* (Hewitson, 1874)	31	Argentina	de Lesse 1967
21	*E. clarus* (Cramer, 1775)	31	USA (Florida)	Maeki 1961
22	*E. clavicornis tenda* Evans, 1955	ca 29–30	Guatemala	de Lesse 1970a
23	*Oechydrus chersis* (Herrich-Schäffer, 1869)	31	Bolivia	de Lesse 1967a

#	Species	Haploid chromosome number	Country	Reference
24	*Phocides polybius phanias* (Burmeister, 1880)	16	Brazil	Saura et al. 2013
25	*Tarsoctenus praecia plutia* (Hewitson, 1857)	15	Brazil	Saura et al. 2013
26	*Thorybes pylades pylades* (Scudder, 1870)	31	USA (Connecticut)	Maeki 1961
27	*Udranomia spitzi* (Hayward, 1942)	29	Brazil	de Lesse and Brown 1971
28	*Urbanus dorantes dorantes* (Stoll, 1790)	31	Mexico	de Lesse 1970a
29	*U. doryssus doryssus* (Swainson, 1831)	14	Costa Rica	Kumagai et al. 2010
30	*Urbanus proteus* (Linnaeus, 1758)	31	Bolivia	de Lesse 1967a
		31	Mexico	de Lesse 1970a
		31	USA (Florida)	Maeki 1961
31	*U. simplicius* (Stoll, 1790)	31	Argentina	de Lesse 1967a
32	*U. teleus* (Hübner, 1821)	31	Argentina	de Lesse 1967a
Subfamily Pyrginae				
Tribe Pyrrhopygini				
33	*Elbella lamprus* (Hopffer, 1874)	40	Brazil	de Lesse 1970a
34	(?) *Jemadia* sp.	32(?)	Brazil	Saura et al. 2013
35	*Mimoniades montana* J. Zikán, 1938	27	Brazil	Saura et al. 2013
36	*M. nurscia* (Swainson, 1821)	28	Ecuador	de Lesse 1967a
	M. n. malis (Godman et Salvin, 1879)	28	Colombia	Saura et al. 2013
37	*Mimoniades* sp.	21	Colombia	Saura et al. 2013
38	*Mimoniades* sp.	28	Colombia	Saura et al. 2013
39	*M. versicolor* (Latreille, [1824])	28	Brazil	de Lesse and Brown 1971
40	*Pyrrhopyge charybdis* Westwood, 1852	14(?)	Brazil	Saura et al. 2013
41	*P. pelota* Plötz, 1879	28	Argentina	de Lesse 1967a
42	*Pyrrhopyge* sp.	15	Brazil	Saura et al. 2013
43	*Sarbia* sp.	30	Brazil	Saura et al. 2013
Tribe Tagiadini				
44	*Daimio tethys* (Ménétriés, 1857)	30	Japan	Maeki 1953, Maeki and Makino 1953
45	*D. t. moorei* Mabille, 1876	30	Taiwan	Maeki and Ae 1968b
46	*Eagris lucetia* (Hewitson, 1876)	30	Uganda	de Lesse 1968
47	*E. sabadius astoria* Holland, 1896	30	Kenya	de Lesse 1968
48	*Eretis lugens* (Rogenhofer, 1891	28	Kenya	de Lesse 1968
Tribe Celaenorrhinini				
49	*Sarangesa phidyle* (Walker, 1870)	29	Senegal	de Lesse and Condamin 1962
Tribe Carcharodini				
50	*Carcharodus alceae* (Esper, [1780])	31	Croatia	Lorkovic 1941
51	*C. boeticus* Reverdin, 1913	43–47	Spain	de Lesse 1960
	C. boeticus Reverdin, 1913	40–52	France	de Lesse 1960
	C. boeticus Reverdin, 1913	38–46	Italy	de Lesse 1960

#	Species	Haploid chromosome number	Country	Reference
52	*C. dravira* (Moore, 1874)	37–48 (with Us)	Iran	de Lesse 1960
53	*C. flocciferus* (Zeller, 1847)	32–41 (with Us)	France (Cauterets)	de Lesse 1960
54	*C. flocciferus* (Zeller, 1847)	42–58 (with Us)	Italy	de Lesse 1960
55	*C. lavatherae* (Esper, [1783])	30	France (Salau, Ariege)	de Lesse 1960
56	*C. orientalis* Reverdin, 1913	31–32	Lebanon	de Lesse 1960
		30	Turkey (Van)	de Lesse 1960
		30–37 (with Us)	Turkey (Amasya)	de Lesse 1960
57	*C. stauderi ambiguus* Verity, 1925	30	Lebanon	de Lesse 1960
		30	Turkey	de Lesse 1960
58	*Hesperopsis alpheus* (W. H. Edwards, 1876) (as *Pholisora*)	34	USA (Texas)	Emmel and Trew 1973
59	*Muschampia nomas* (Lederer, 1855)	30	Lebanon	de Lesse 1960
60	*M. proteides* (Wagner, 1929)	30	Lebanon	Larsen 1975
61	*M. proto* (Ochsenheimer, 1808)	30	Spain	de Lesse 1960
		30	Lebanon	Larsen 1975
62	*Pholisora catullus* (Fabricius, 1793)	29	?USA	Lorkovic in Robinson 1971
63	*Spialia orbifer* (Hübner, [1823])	30	Croatia	Lorkovic 1941
		31	Turkey	de Lesse 1960
64	*S. phlomidis* (Herrich-Schäffer, [1845])	31	Turkey	de Lesse 1960
65	*S. sertorius* (Hoffmannsegg, 1804)	31	Slovenia	Lorkovic 1941
Tribe Erynnini				
66	*Chiomara asychis georgina* (Reakirt, 1868)	31	Mexico	de Lesse 1970a
	Ch. asychis georgina (Reakirt, 1868)	32	USA (Texas)	Emmel and Trew 1973
67	*Chiomara* sp.	31	Trinidad	Wesley and Emmel 1975
68	*Ebrietas anacreon* (Staudinger, 1876)	31	Argentina	de Lesse 1967a
69	*E. osyris* (Staudinger, 1876)	31	Argentina	de Lesse 1967a
70	*Erynnis baptisiae* (W. Forbes, 1936)	31	USA (Connecticut)	Maeki 1961
71	*E. funeralis* (Scudder et Burgess, 1870)	31	Argentina	de Lesse 1967a
72	*E. horatius* (Scudder et Burgess, 1870)	31	USA (Florida)	Maeki 1961
73	*E. icelus* (Scudder et Burgess, 1870)	30	USA (Connecticut)	Maeki 1961
74	*E. juvenalis juvenalis* (Fabricius, 1793)	30	USA (Connecticut)	Maeki 1961
75	*E. lucilius* (Scudder et Burgess, 1870)	31	USA (Connecticut)	Maeki and Remington 1960a
76	*E. marloyi* (Boisduval, [1834])	31	Lebanon	de Lesse 1960
77	*E. montanus* (Bremer, 1861)	31 (2n=62)	Japan	Abe et al. 2006
	E. montanus (Bremer, 1861)	31	Japan	Maeki 1953

#	Species	Haploid chromosome number	Country	Reference
78	E. *persius* (Scudder, 1863)	31	USA (Connecticut)	Maeki 1961
79	*E. tages* (Linnaeus, 1758)	31	Croatia	Lorkovic 1941
		31	France	de Lesse 1960
		31	England	Bigger 1960
80	*E. tristis tatius* (W. H. Edwards, 1883)	31	USA (Texas)	Emmel and Trew 1973
81	*Gesta gesta* (Herrich-Schäffer, 1863)	32	Tobago	Wesley and Emmel 1975
82	*Grais stigmaticus* (Mabille, 1883)	31	Mexico	Maeki and Remington 1960a
83	*Theagenes albiplaga* (C. Felder et R. Felder, 1867)	31	Bolivia	de Lesse 1967a
Tribe Achlyodidini				
84	*Achlyodes pallida* (R. Felder, 1869) (as *A. selva*)	15	Bolivia	de Lesse 1967a
		15	Mexico	de Lesse 1970a
85	*Zera zera zera* (Butler, 1870)	34	Brazil	de Lesse and Brown 1971
Tribe Pyrgini				
86	*Anisochoria sublimbata* Mabille, 1883	31	Argentina	de Lesse 1967a
87	*Antigonus erosus* (Hübner, [1812])	31	Mexico	de Lesse 1970a
88	*A. liborius* Plötz, 1884	31	Argentina	de Lesse 1967a
89	*Celotes nessus* (W. H. Edwards, 1877)	14, 13	USA (Texas)	Emmel and Trew 1973
90	*Heliopetes arsalte* (Linnaeus, 1758)	30	Bolivia	de Lesse 1967a
	H. arsalte (Linnaeus, 1758)	30	Mexico	de Lesse 1970a
91	*H. laviana* (Hewitson, 1868)	29	USA (Texas)	Emmel and Trew 1973
92	*H. macaira* (Reakirt, [1867])	29	USA (Texas)	Emmel and Trew 1973
93	*H. omrina* (Butler, 1870)	30	Argentina	de Lesse 1967a
94	*Heliopyrgus americanus* (Blanchard, 1852)	30	Chile	de Lesse 1967a
95	*Paches loxus* (Westwood, [1852])	31	Guatemala	de Lesse 1970a
96	*Pyrgus aladaghensis* De Prins et van der Poorten, 1995	ca 18–21	Turkey	Lukhtanov and Kandul 1995 (in Hesselbarth et al. 1995)
97	*P. albescens* Plötz, 1884	30 (2n=60)	USA (Texas)	Goodpasture 1976
	P. albescens Plötz, 1884	28	USA (Texas)	Emmel and Trew 1973
98	*P. alveus* (Hübner, [1803])	24	Finland	Federley 1938
		24	Croatia	Lorkovic 1941
		24	Turkey	Lukhtanov and Kandul 1995 (in Hesselbarth et al. 1995)
99	*P. bellieri* (Oberthür, 1910)	27	France	de Lesse 1960
100	*P. bocchoris* (Hewitson, 1874)	30	Argentina	de Lesse 1967a
101	*P. bolkariensis* De Prins et van der Poorten, 1995	30	Turkey	Lukhtanov and Kandul 1995 (in Hesselbarth et al. 1995)
102	*P. cacaliae* (Rambur, 1839)	30	Italy	de Lesse 1960
103	*P. carlinae* (Rambur, [1839])	30	Italy	de Lesse 1960
104	*P. carthami* (Hübner, [1813])	29	Italy	de Lesse 1960

#	Species	Haploid chromosome number	Country	Reference
105	*P. cirsii* (Rambur, [1839])	30	France (Peyreleau, Aveyron)	de Lesse 1960
106	*P. fides* Hayward, 1940	30	Chile	de Lesse 1967a
107	*P. maculates* (Bremer et Grey, 1852)	31 (2n=62)	Japan	Abe et al. 2006
108	*P. malvae* (Linnaeus, 1758)	31	Finland	Federley 1938
		33	England	Bigger 1960
109	*P. oileus* (Linnaeus, 1767)	30 (2n=60)	USA (Texas)	Goodpasture 1976
		32	USA (Texas)	Emmel and Trew 1973
110	*P. onopordi* (Rambur, [1839])	30	France	Lorkovic 1941
111	*P. serratulae* (Rambur, [1839])	30	France	Lorkovic 1941
112	*Trina geometrina geometrina* (C. Felder et R. Felder, 1867)	31	Brazil	de Lesse and Brown 1971
Subfamily Heteropterinae				
113	*Butleria quilla* Evans, 1939	29	Chile	de Lesse 1967a
Subfamily Trapezitinae				
114	*Trapezites eliena* Hewitson, 1868	31	Australia	Maeki and Ogata 1971
Subfamily Hesperiinae				
Tribe Aeromachini				
115	*Aegiale hesperiaris* (Walker, 1856)	24	Mexico	Freeman 1969
116	*Agathymus alliae* (Stallings et Turner, 1957)	38	USA (Arizona)	Freeman 1969
117	*A. aryxna* (Dyar, 1905)	5	Mexico	Freeman 1969
118	*A. baueri* (Stallings et Turner, 1954)	15	USA (Arizona)	Freeman 1969
119	*A. chisosensis* (Freeman, 1952)	18	USA (Texas)	Freeman 1969
120	*A. estelleae valverdiensis* Freeman, 1966	9	USA (Texas)	Freeman 1969
	A. e. estelleae (Stallings et Turner, 1958)	9	Mexico	Freeman 1969
121	*A. freemani* Stallings, Turner et Stallings, 1960	15	USA (Arizona)	Freeman 1969
122	*A. gilberti* Freeman, 1964	21	USA (Texas)	Freeman 1969
123	*A. mariae chinatiensis* Freeman, 1964	22	USA (Texas)	Freeman 1969
	A. mariae lajitaensis Freeman, 1964	22	USA (Texas)	Freeman 1969
	A. mariae mariae (Barnes et Benjamin, 1924)	22	USA or Mexico	Freeman 1969
	A. mariae rindgei Freeman, 1964	22	USA (Texas)	Freeman 1969
124	*A. micheneri* Stallings, Turner et Stallings, 1961	20	Mexico	Freeman 1969
125	*A. neumoegeni florenceae* (Stallings et Turner, 1957)	10	USA (Texas)	Freeman 1969
	A. neumoegeni macalpinei (Freeman, 1955)	10	USA (Texas)	Freeman 1969
126	*A. polingi* (Skinner, 1905)	10	USA (Arizona)	Freeman 1969
127	*A. remingtoni* (Stallings et Turner, 1958)	9	Mexico	Freeman 1969

#	Species	Haploid chromosome number	Country	Reference
128	*Alera vulpina* (C. Felder et R. Felder, 1867)	ca27	Ecuador	de Lesse 1967a
129	*Ankola fan* (Holland, 1844)	10	Uganda	De Lesse 1968
130	*Arotis derasa* (Herrich-Schäffer, 1870) (as *Euphyes*)	28	Brazil	de Lesse and Brown 1971
131	*Erionota thrax thrax* (Linnaeus, 1767)	29	Malaysia	Saitoh and Kumagai 1974
132	*Euphyes leptosema* Mabille, 1891	ca28	Argentina	de Lesse 1967a
133	*Megathymus coloradensis coloradensis* Riley, 1877	27	USA	Freeman 1969
134	*M. coloradensis kendalli* Freeman, 1965	27	USA (South central Texas)	Freeman 1969
	M. coloradensis louiseae Freeman, 1963	27	USA (Western Texas)	Freeman 1969
	M. coloradensis navajo Skinner, 1911	27	USA	Freeman 1969
	M. coloradensis reinthali Freeman, 1963	27	USA (Texas)	Freeman 1969
	M. coloradensis reubeni Stallings, Turner et Stallings, 1963	27	USA (Texas)	Freeman 1969
	M. coloradensis stallingsi Freeman, 1943	27	USA	Freeman 1969
	M. coloradensis wilsonorum Stallings et Turner, 1958	27	?Mexico	Freeman 1969
135	*M. violae* Stallings et Turner, 1956	27	USA	Maeki 1961, Freeman 1969
136	*M. yuccae buchholzi* Freeman, 1952	26	USA (Florida)	Freeman 1969
137	*Pardaleodes incerta* (Snellen, 1872)	17	Uganda	de Lesse 1968
138	*Stallingsia maculosus* (Freeman, 1955)	50	USA (Texas)	Maeki 1961, Freeman 1969
139	*Suastus gremius* (Fabricius, 1798)	23	Taiwan	Maeki and Ae 1968b
140	*Thoressa varia* (Murray, 1875)	31 (2n=62)	Japan	Abe et al. 2006
141	*T. varia* (Murray, 1875)	31	Japan	Maeki 1953
Tribe Baorini				
142	*Gegenes gambica* (Mabille, 1878)	41	Yemen	Saitoh 1984
		41	Turkey	de Lesse 1960
		41	Lebanon	Larsen 1982
143	*Gegenes nostrodamus* (Fabricius, 1793)	15	Egypt	Larsen 1982
		15	Israel	Saitoh 1979, Larsen 1982
144	*Gegenes pumilio* (Hoffmansegg, 1804)	24	France	de Lesse 1960
		24	Alger	de Lesse 1967b
145	*Parnara guttata* (Bremer et Grey, 1852)	16	Japan	Maeki 1953, Maeki and Makino 1953
		16	China	Saitoh and Abe 1981
146	*Pelopidas conjucta conjucta* (Herrich-Schäffer, 1869)	16	Hong Kong	Maeki and Ae 1968a
147	*P. jansonis* (Butler, 1878)	16 (2n=32)	Japan	Abe et al. 2006

#	Species	Haploid chromosome number	Country	Reference
148	*P. mathias* (Fabricius, 1798)	16	Japan	Maeki and Remington 1960
149	*P. thrax* (Hübner, [1821])	16	Lebanon	Larsen 1975
150	*Polytremis lubricans* (Herrich-Schäffer, 1869)	16	Taiwan	Maeki and Ae 1968b
151	*P. pellucida* (Murray, 1875)	16, 17, 18 (2n=32, 33)	Japan	Abe et al. 2006
		16	Japan	Maeki and Remington 1960
152	*Zenonia zeno* (Trimen, 1864)	16	Uganda	de Lesse 1968
Tribe Taractrocerini				
153	*Ocybadistes walkeri sothis* Waterhouse, 1933	28	Australia	Maeki and Ogata 1971
154	*Potanthus flavus* (Murray, 1875)	29 (2n=58)	Japan	Abe et al. 2006
155	*Telicota ancilla horisha* Evans, 1934	29	Taiwan	Maeki and Ae 1968b
156	*Telicota colon stinga* Evans, 1949	29	Japan (Okinava)	Abe et al. 2006
157	*T. ohara formosana* Fruhstorfer, 1911	29 (2n=58)	Taiwan	Abe et al. 2006
Tribe Thymelicini				
158	*Copaeodes minima* (W.H. Edwards, 1870)	29	USA (Florida)	Maeki 1961
159	*Thymelicus sylvestris* (Poda, 1761)	27	England	Bigger 1960
160	*Th. sylvaticus* (Bremer, 1861)	10 (2n=20)	Japan	Abe et al. 2006
161	*Th. acteon* (Rottemburg, 1775)	28	Spain	de Lesse 1970c
162	*Th. hyrax* (Lederer, 1861)	29	Lebanon	Larsen 1975
163	*Th. leoninus* (Butler, 1878)	9 (2n=18)	Japan	Abe et al. 2006
164	*Th. lineola* (Ochsenheimer, 1808)	29	Finland	Federley 1938
		29	Lebanon	Larsen 1975
Tribe Calpodini				
165	*Ebusus ebusus* (Cramer, [1780])	29	Mexico	de Lesse 1970a
166	*Lychnuchus celsus* (Fabricius, 1793)	30	Brazil	de Lesse and Brown 1971
167	*Panoquina hecebolus* (Scudder, 1872)	29	USA (Texas)	Emmel and Trew 1973
168	*Panoquina ocola* (W. H. Edwards, 1863)	29	USA (Texas)	Emmel and Trew 1973
169	*P. panoquin* (Scudder, 1863)	29	USA (Florida)	Maeki 1961
170	*P. panoquinoides* (Skinner, 1891)	29	USA (Texas)	Emmel and Trew 1973
Tribe Anthoptini no chromosomal data available				
Tribe Moncini				
171	*Amblyscirtes aenus* W.H. Edwards, 1878	28, 29	USA (Texas)	Emmel and Trew 1973
172	*A. cassus* W. H. Edwards, 1883	29	USA (Texas)	Emmel and Trew 1973
173	*A. celia* (Skinner, 1895)	29	USA (Texas)	Emmel and Trew 1973
174	*A. phylace* W.H. Edwards, 1878	29	USA (Texas)	Emmel and Trew 1973
175	*A. texanae* Bell, 1927	29	USA (Texas)	Emmel and Trew 1973
176	*A. vialis* (W. H. Edwards, 1862)	29	USA (Connecticut)	Maeki 1961
177	*Cymaenes* sp.	31	Tobago	Wesley and Emmel 1975

#	Species	Haploid chromosome number	Country	Reference
178	*Enosis immaculata immaculata* (Hewitson, 1868)	29	Ecuador	Kumagai et al. 2010
179	*Lerema accius* (Smith, 1797)	29 (2n=58)	USA (Texas)	Goodpasture 1976
		29	USA (Texas)	Emmel and Trew 1973
180	*Moeris vopiscus* (Herrich-Schäffer, 1869)	27	Peru	Kumagai et al. 2010
181	*Nastra lherminier* (Latreille, [1824])	30	USA (Connecticut)	Maeki 1961
182	*Thargella caura* (Plötz, 1882)	25	Brazil	de Lesse and Brown 1971
183	*Vettius coryna* (Hewitson, [1866])	31, ca32	Ecuador	de Lesse 1967a
184	*V. phyllus prona* Evans, 1955	26	Brazil	de Lesse and Brown 1971
185	*V. triangularis* (Hübner, [1831])	26	Brazil	Kumagai et al. 2010
Tribe Hesperiini				
186	*Asbolis capucinus* (Lucas, 1857)	48	USA (Florida)	Maeki 1961
187	*Cynea iquita* (Bell, 1941)	29	Argentina	de Lesse 1967a
188	*Hesperia comma* (Linnaeus, 1758)	28	Italy	de Lesse 1970c
		28	Lebanon	Larsen 1975
189	*H. florinda* Butler, 1878	28 (2n=56)	Japan	Abe et al. 2006
190	*Hylephila fasciolata* (Blanchard, 1852)	29	Argentina	de Lesse 1967a
191	*H. phyleus* (Drury, 1773)	29	Argentia	de Lesse 1967a
		29	USA (Florida)	Maeki 1961
192	*H. signata* (Blanchard, 1852)	29	Chile	de Lesse 1967a
193	*Ochlodes ochraceus* (Bremer, 1861)	29 (2n=58)	Japan	Abe et al. 2006
		24	Japan	Maeki and Remington 1960
194	*O. sylvanoides* (Boisduval, 1852)	29	USA	Maeki 1961
195	*O. sylvanus* (Esper, 1777)	29	Finland	Federley 1938
		29	Croatia	Lorkovic 1941
196	*O. venatus* (Bremer et Grey, 1853) (as *sylvanus* Esper, 1777)	29 (2n=58)	Japan	Abe et al. 2006
197	*Oligoria maculata* (W. H. Edwards, 1865)	29	USA (Florida)	Maeki 1961
198	*Poanes hobomok hobomok* (Harris, 1862)	29	?USA	Lorkovic in Robinson 1971
199	*P. taxiles* (W. H. Edwards, 1881)	29	USA	Maeki 1961
200	*P. zabulon* (Boisduval et Le Conte, [1837]) (as *Polites zabulon*)	29	USA (Connecticut)	Maeki 1961
201	*Polites themistocles* (Latreille, [1824])	29	USA (Florida)	Maeki 1961
202	*P. vibex catilina* (Plötz, 1886)	29	Argentina	de Lesse 1967a
	P. vibex praeceps (Scudder, 1872)	27	USA (Texas)	Emmel and Trew 1973
	P. vibex vibex (Geyer, 1832)	29	USA (Florida)	Maeki 1961
203	*Wallengrenia egeremet* (Scudder, 1863)	28	USA (Texas)	Emmel and Trew 1973
204	*W. otho curassavica* (Snellen, 1887)	28–30	USA (Texas)	Emmel and Trew 1973
205	*W. premnas* (Wallengren, 1860)	27	Argentina	de Lesse 1967

Between- and within-species variations in chromosome number

Several groups of skippers display extreme chromosome number variations at the within-species level (Table). The most extreme variations in number of chromosome elements were observed in first meiotic metaphase of *Carcharodus boeticus, C. dravira* and *C. flocciferus* (Table, de Lesse 1960). The nature of these variations remains unknown, and there are two plausible explanations for this phenomenon. First, this variation can be explained by presence of so-called B-chromosomes (=additional chromosomes, =supernumerary chromosomes) (de Lesse 1960). B-chromosomes consist mainly of repetitive DNA and can sometimes accumulate through processes of mitotic or meiotic drive (Jones et al. 2008). B-chromosomes can be distinguished from normal A-chromosomes because they are usually smaller and can be seen as additional chromosomes present in only some of the individuals in a population (Camacho et al. 2000, Jones et al. 2008).

Second, this kind of variation can be caused by violations in meiotic chromosome pairing resulting in appearance of univalents (instead of bivalents) in meiotic prophase (Lorković 1990). This type of variation was studied in detail by Maeki and Ae (1979) in butterfly genus *Papilio* and is expected if regular or irregular interspecific mating occurs in nature. Anyway, the nature of intraspecific variations observed in *Carcharodus* is different from that discovered in the Wood White butterfly *Leptidea sinapis* (Linnaeus, 1758) (Pieridae). In the last species the compared range of intraspecific variation in chromosome number (from n=28 to n=53) was caused by multiple chromosome fusions/fissions accumulated within the species (Lukhtanov et al. 2011, Dinca et al. 2011).

Between-species variation exists in numerous genera of skippers (Table 1) and is especially expressed in the Nearctic genus *Agathymus* Freeman, 1959, in which the range of haploid numbers was discovered from n =5 in *A. aryxna* to n=38 in *A. alliae* (Freeman 1969). This range is comparable of even exceeds the range found in chromosomally diverse genera from other butterfly families (Lorković 1990, Lukhtanov et al. 2005, Talavera et al. 2013). Thus, the genera of Hesperiidae can be used as model systems for future analysis of the phenomenon of chromosome instability.

Detecting cryptic species using analysis of chromosomal differences

Recent years karyological data have been widely used in studies of butterfly taxonomy and in biodiversity research as main or additional chracters for detecting cryptic species (e.g. Dinca et al. 2011) and for synonymizing biological entities that were incorrectly described as distinct species (e.g. Vila et al. 2010). The family Hesperiidae is not excluded in this respect. In the genus *Gegenes* Hübner, [1819], two cryptic species *G. pumilio* (n=24) and *G. gambica* (n=41) were discovered through extensive chromosome analysis of different populations (de Lesse 1960, 1967b, Larsen 1982, Saitoh 1984).

In the genus *Pyrgus* Hübner, [1819], our unpublished chromosome data (see Table) were used to recognize and then to describe two morphologically similar species, *P. bolkariensis* and *P. aladaghensis* (De Prins and van der Poorten 1995).

Thus, interspecific chromosomal differences are useful for discovering and describing new cryptic species of Hesperiidae representing in such a way a powerful tool in biodiversity research.

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SHORT COMMUNICATIONS

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First karyotype data on the family Myerslopiidae (Hemiptera, Auchenorrhyncha, Cicadomorpha)

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Abstract

In the first cytogenetic study of the recently proposed family Myerslopiidae the male karyotype of *Mapuchea chilensis* (Nielson, 1996) was analyzed using conventional chromosome staining, AgNOR- and C-bandings, and fluorescence *in situ* hybridization (FISH) with 18S rDNA and $(TTAGG)_n$ telomeric probes. A karyotype of 2n = 16 + XY, NOR on a medium-sized pair of autosomes, subterminal location of C-heterochromatin, and presence of $(TTAGG)_n$ telomeric sequence were determined. Additionally, the male internal reproductive system was studied.

Keywords

Karyotype, NOR, C-heterochromatin, rDNA, TTAGG telomeric sequence, *Mapuchea chilensis*, Myerslopiidae, Hemiptera, Auchenorrhyncha, Cicadomorpha, Membracoidea

Introduction

The family Myerslopiidae includes three recent genera of cicadomorphan Auchenorrhyncha with 19 species in New Zealand and temperate Chile (Szwedo 2004). Myerslopiids are small, heavily sclerotized, flightless insects dwelling in leaf litter. The family status of this group, previously classified as a subfamily within Cicadellidae

or a tribe within the cicadellid subfamily Ulopinae, was proposed by Hamilton (1999), who argued from morphological evidence that it represents the basal branch of the superfamily Membracoidea (leafhoppers and treehoppers) and shares multiple plesiomorphic characters with Cicadoidea (cicadas), Cercopoidea (froghoppers), or both. This hypothesis received some support from molecular phylogenetic analyses, which recovered myerslopiids outside the rest of Membracoidea (Dietrich et al. 2001, Cryan 2005). Therefore, additional data on these poorly known insects are of considerable interest. We describe here the karyotype of *Mapuchea chilensis* (Nielson, 1996), the data representing the first cytogenetic report on the family Myerslopiidae.

Material and methods

Four adult males of *M. chilensis* were collected by the third author in Chile, P.N. Puyehue, Anticura (40.6667°S, 72.1742°W) on 15–17 January 2014 from leaf litter between creeping stems of *Hydrangea serratifolia* (Hooker & Arnott, 1833). Specimens were fixed in 3:1 fixative (96% ethanol: glacial acetic acid) and stored at +4°C. Testes were dissected in a drop of 45% acetic acid and squashed. The cover slip was removed using dry ice. Chromosome staining techniques used were as follows: the Feulgen-Giemsa method (Grozeva and Nokkala 1996) for visualization of standard karyotype; Ag-NOR banding (Howell and Black 1980) for visualization of nucleolus organizing regions, NORs; C-banding (Sumner 1972) for revealing constitutive heterochromatin; and fluorescence *in situ* hybridization (FISH) with 18S rDNA and $(TTAGG)_n$ telomeric probes for detecting the telomeric sequence and the number and chromosomal location of rRNA gene sites (Schwarzacher and Heslop-Harrison 2000). Chromosome slides were analyzed under a Leica DM 6000 B microscope; images were taken with a Leica DFC 345 FX camera using Leica Application Suite 3.7 software with an Image Overlay module.

The classification of cicadomorphan Auchenorrhyncha accepted in this paper follows Dietrich (2005).

Results

Reproductive system

In adult *M. chilensis* males, the reproductive system consisted of a pair of testes, pair of seminal vesicles, and pair of accessory glands (Fig. 1). In two males, the number of follicles was the same in both testes, 6+6, but in two other males it was 6+5 and 6+4 respectively. The seminal vesicles were cylindrical in shape, fused almost throughout their entire lengths. The accessory glands were oval in shape and narrowed apically.

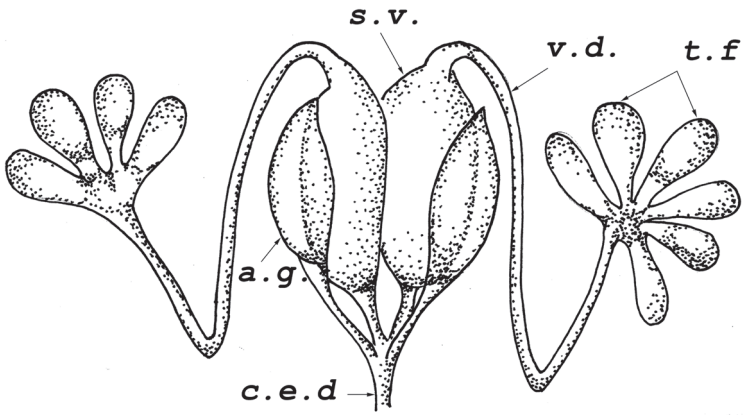

Figure 1. Male reproductive system of *Mapuchea chilensis*. **t.f.** testicular follicles (4 and 6 in different testes); **v.d.** vasa differentia **s.v.** seminal vesicle; **a.g.** accessory gland; **c.d.e.** common ejaculatory duct.

Standard karyotype

M. chilensis showed a karyotype of 2n = 16 + XY. At MI, 8 bivalents of autosomes and an XY-pair were present (Fig. 2a). One of the bivalents was very large and the others gradually decreased in size. The autosomal bivalents formed one or two subterminal or occasionally interstitial chiasmata (Fig. 2b). In some nuclei, almost all bivalents appeared as rings, evidencing the presence of two subterminal chiasmata (Fig. 2c). At MII, the chromosomes tended to form a ring with the largest bivalent at its center (Fig. 2d). In some cells, non-homological chromosomal associations (Fig. 2d, e) and lagging chromosomes (Fig. 2f) were observed.

C- and AgNOR-bandings and FISH

After C-banding, the majority of bivalents showed C-blocks at the ends of chromosomes (Fig. 2g). In early prophase cells, a large Ag-positive mass connected with autosomes was identified; in some cases, nucleolar material was present as multiple argyrophilic bodies (Fig. 2h). The 18S rDNA FISH probe localized ribosomal clusters near the ends of one of the medium-sized bivalents (Fig. 2i, j). The $(TTAGG)_n$ telomeric FISH probe produced bright fluorescent signals at the ends of chromosomes (Fig. 2i, j, k).

Discussion

The number of testicular follicles is generally characteristic of an insect species, although variation between the two testes of the same male has occasionally been reported

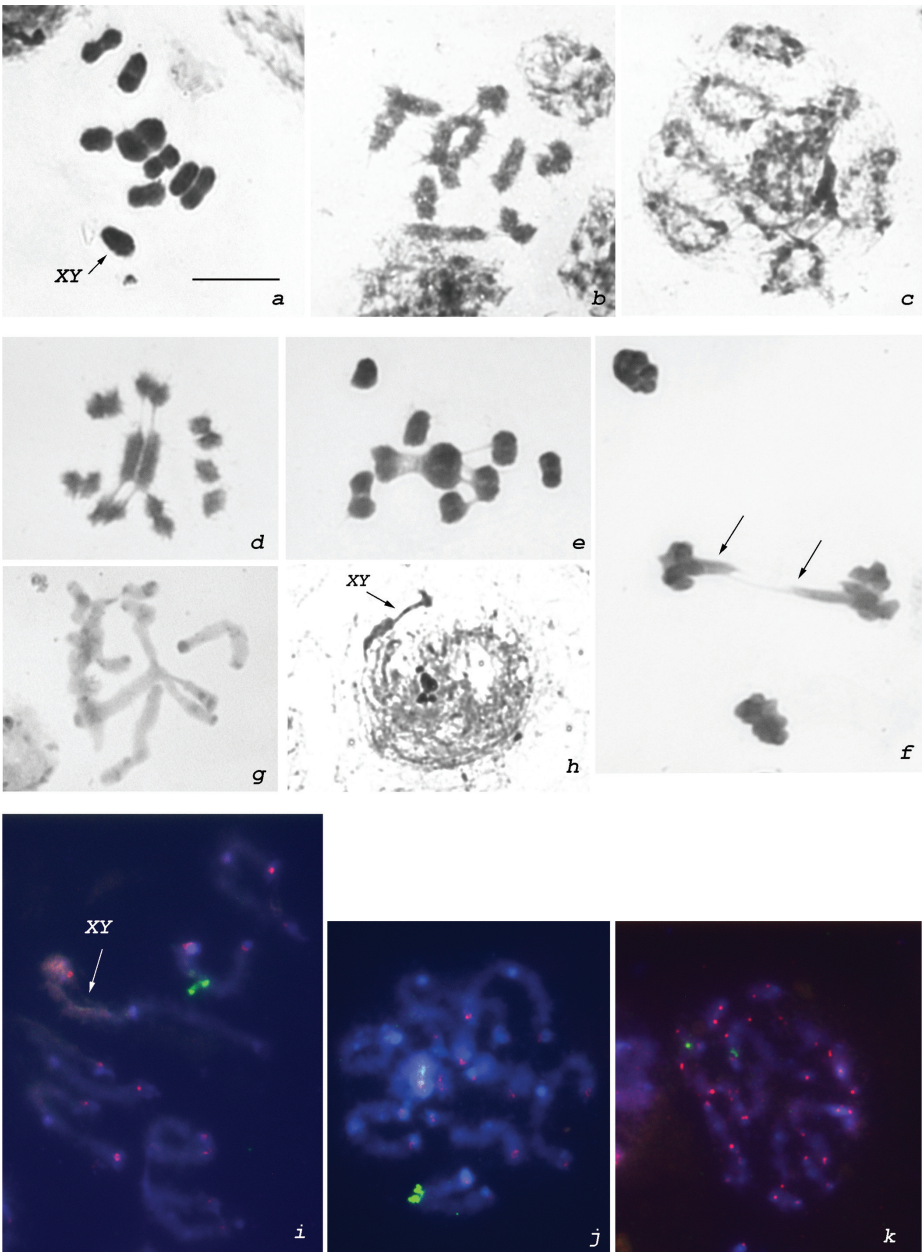

Figure 2. Male karyotype of *Mapuchea chilensis*. **a** metaphase I, n = 8AA + XY **b** diakinesis, largest bivalent with two chiasmata **c** diplotene, 5 bivalents with two chiasmata each **d** metaphase II with the largest chromosome in the center of a ring formed by autosomes. Note chromatin associations between non-homological chromosomes **e** metaphase I showing associations between bivalents **f** anaphase II with lagging chromosomes (arrows) **g** diplotene (C-banding) showing terminal C-bands in chromosomes **h** early prophase (NOR-banding) showing argyrophilic granules associated with autosomes **i–k** diplotenes (**i, j**) and mitotic metaphase (**k**) after FISH with rDNA-probe (green signals) and $(TTAGG)_n$ telomeric probe (red signals). rDNA sites are located on a medium-sized pair of autosomes. Bar = 10μm.

(Maryańska-Nadachowska et al. 2006, Kuznetsova et al. 2010). The phylogenetic importance of this character in Auchenorrhyncha has been discussed (Emelyanov and Kuznetsova 1983, D'Urso et al. 2005, Kuznetsova et al. 2009, 2010). Despite some intraindividual variation observed in the four examined males, 6 follicles per testis predominated and can thus be considered characteristic of *M. chilensis*. In Cicadellidae, this number varies from 3 to 14, with low numbers (6 and 4) predominating (Bednarczyk 1993). In other families of Membracoidea, testes with 9 follicles have been recorded in Aetalionidae (Kuznetsova and Kirillova 1993) and testes with 4, 6 and 8 follicles in Membracidae (Emelyanov and Kuznetsova 1983). The number of follicles is higher in other superfamilies of cicadomorphan Auchenorrhyncha: 12-35 in Cercopoidea (Emelyanov and Kuznetsova 1983) and very high (over 100) in Cicadoidea (Glasgow 1908, Moulds 2005).

Among Cicadellidae, chromosome numbers in males vary from 2n = 7(6 + X) to 2n = 27(26 + X) and both X(0) and XY sex chromosome systems occur, the latter being found only occasionally (Kirillova 1988, Wei 2010, Juan 2011). The complement of 2n = 18 (16 + XX/XY), determined for *M. chilensis*, has been previously described only in two cicadellids, *Taslopa montana* Evans, 1941 from the subfamily Ulopinae (Whitten 1965) and *Hecalus porrectus* (Walker, 1858) from Deltocephalinae (as *Thomsoniella (Parabolocratus) albomaculata* Distant, 1908 and *Th. (Parabolocratus) porrecta* Distant, 1908, see Kirillova 1988). This karyotype has not been recorded so far among Aetalionidae, Membracidae, Cercopoidea, or Cicadoidea (Kirillova 1988, Kuznetsova and Kirillova 1993, Tian and Yuan 1997, Perepelov and Bugrov 2002, Maryańska-Nadachowska et al. 2013).

Therefore, in both the karyotype and the number of follicles, *M. chilensis* falls within the spectrum of variation observed in Cicadellidae.

Other cytogenetic characters have so far been examined in only a few representatives of cicadomorphan Auchenorrhyncha and thus do not inform on the relationships of Myerslopiidae. *M. chilensis* was found to have small subterminal C-blocks, the pattern described, with the exception of large blocks in *Philaenus italosignus* Drosopoulos & Remane, 2000 (Cercopoidea: Aphrophoridae) (Maryańska-Nadachowska et al. 2013), in all previously examined species of Cercopoidea (Maryańska-Nadachowska et al. 2013) and Cicadoidea (Perepelov and Bugrov 2002), which are the only other cicadomorphans in which the amount and distribution of C-heterochromatin have been studied. The amount and distribution of C-heterochromatin were found to vary among species of *Philaenus* Stål, 1864 (Maryańska-Nadachowska et al. 2013).

In *M. chilensis*, rDNA loci were detected by FISH on one of the medium-sized pairs of autosomes, this location being confirmed by AgNOR-staining, which suggested presence of a single autosomal NOR (per haploid set). The latter technique has previously been used to demonstrate variation in the number and position of NORs in four genera of Cercopoidea (Castanhole et al. 2010, Maryańska-Nadachowska et al. 2013); for one of these genera, *Philaenus*, the results have been confirmed using FISH (Maryańska-Nadachowska et al. 2013).

The telomeric sequence $(TTAGG)_n$, identified in *M. chilensis*, is known to be characteristic of the majority of insect groups and is considered to be ancestral for Insecta

(Frydrychová et al. 2004, Vitková et al. 2005) and Arthropoda as a whole (Lukhtanov and Kuznetsova 2010). Among Hemiptera, this canonical motif is not present (lost) in the advanced heteropteran infraorders Cimicomorpha and Pentatomomorpha (Grozeva et al. 2011), but has been reported in *Lethocerus patruelis* (Stal, 1854) from the more basal heteropteran infraorder Nepomorpha (Kuznetsova et al. 2012), in coccids (Mohan et al. 2011), aphids (Monti et al. 2011) and the auchenorrhynchan genus *Philaenus* (Maryańska-Nadachowska et al. 2013).

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RESEARCH ARTICLE

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Karyotype diversity and patterns of chromosomal evolution in *Eigenmannia* (Teleostei, Gymnotiformes, Sternopygidae)

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Abstract

Conventional (Giemsa, C-banding, Ag – NORs) and molecular [5S rDNA, 18S rDNA, $(TTAGGG)_n$] cytogenetic techniques were employed to study six species of the genus *Eigenmannia* Jordan & Evermann, 1896. They exhibited diploid chromosome numbers ranging from 2n=28 (*Eigenmannia* sp.1) to 2n=38 (*E. virescens* (Valenciennes, 1836)). The C-banding results revealed that species with the lowest 2n have less heterochromatin content and that morphologically differentiated sex chromosomes observed in two species showed distinct patterns of heterochromatin. While the X_1, X_2 and Y-chromosomes of *Eigenmannia* sp.2 showed only centromeric heterochromatin, the XY sex chromosomes of *E. virescens* possessed large heterochromatic blocks in the terminal position, particularly on the X chromosome. The nucleolus organizer regions (NORs) were located in different positions when compared to the 5S rDNA sites. Additionally, the presence of minor ribosomal gene sites on the sex chromosome pair of *E. virescens* represented a new type of the sex chromosomes in this group. The telomeric probe $(TTAGGG)_n$ hybridized to the terminal portion of all chromosomes in all species examined however, interstitial telomeric sites were found in the metacentric pair No. 2 in *Eigenmannia* sp.1. The analyzes confirmed some hypotheses about karyotype evolution in the genus *Eigenmannia* , and brought new information about the distribution of the genetic material in the chromosomes of the samples analyzed providing new insights for understanding the process differentiation in the genomes of species under study.

Keywords
Fish comparative cytogenetics, electric fishes, chromosome banding, rDNA variability, sex chromosomes

Introduction

Fishes of the Gymnotiformes order, known as "electric knifefishes", constitute an endemic group in Neotropical freshwaters (Albert and Crampton 2003). This group comprises more than 100 species classified into five families, namely Gymnotidae, Rhamphichthyidae, Hypopomidae, Sternopygidae, and Apteronotidae (Reis et al. 2003). The genus *Eigenmannia* Jordan & Evermann, 1896, family Sternopygidae, is represented by eight widely distributed species (Albert 2001). However, the actual taxonomic diversity of this genus is still unclear, mainly because presently recognized species very likely include other undescribed species, i.e. they represent catch-all taxa.

Available cytogenetic data for *Eigenmannia* species show a remarkable karyotype diversification, including the occurrence of distinct diploid chromosome numbers, ranging from 2n = 28 to 38 chromosomes, and several sex chromosome systems (Almeida Toledo and Foresti 2001; Henning et al. 2008; Silva et al. 2009). However, studies on the distribution of repetitive sequences are scarce and still restricted to chromosomes of a single species – *Eigenmannia virescens* (Valenciennes, 1836) (Silva et al. 2009).

Remarkably, the distribution of repetitive DNAs in the genomes of *Gymnotus* Linnaeus, 1758, another genus within the order Gymnotiformes, is well known and showed that individual multigene families may be extremely variable (e.g. 5S rDNA) or conserved (U2 snDNA and 18S rDNA) at the species level (Scacchetti et al. 2011, 2012; Milhomem et al. 2013; Utsunomia et al. 2014). Therefore, the cytogenetic mapping may be a valuable tool to provide insights into the evolutionary relationships among close species and allow a better comprehension of the distribution and organization of repetitive sequences in the genomes of several species.

The main aim of the present study was to increase the knowledge about karyotype structure of six different *Eigenmannia* species. Additionally, the chromosomal location of telomeric repeats and ribosomal genes was revealed by fluorescence *in situ* hybridization (FISH).

Materials and Methods

Fishes were collected in distinct river basins (Table 1, Fig. 1). The fishes were collected in accordance with Brazilian environmental protection legislation (Collection Permission MMA / IBAMA / SISBIO – number 3245) and the procedures for collection, maintenance and analysis of fish samples were performed with the international protocols on animal experimentation followed by the Universidade Estadual Paulista. The sampled individuals analyzed were fixed in 10% formaldehyde, preserved in 70% ethanol and deposited in the collection of the Laboratory of Fish Biology and Genetics

Table 1. Individuals of *Eigenmannia* species analyzed, diploid chromosome number 2n, collecting localities. LBP – deposit voucher number at the fish collection of the Laboratório de Biologia e Genética de Peixes, Instituto de Biociências de Botucatu, UNESP.

Species	2n	Materials	Sample localities	Coordinates	LBP
E. virescens	38	11♀ 09♂	Mogi-Guaçu river, Araras, São Paulo	S21°56'35", W47°23'04"	12307
E. virescens -XY	38-XY	01♀ 01♂	Ribeirão Claro stream, Rio Claro, São Paulo	S22° 21'28.3", W47°30'51.4"	12308
E. cf. *trilineata*	34	08♀ 06♂	Acre river, Rio Branco, Acre	S09°56'16.6", W67°52'43.6"	12303
Eigenmannia sp.	36	01♀ 01♂	Hortelã river, Botucatu, São Paulo	S22°55'23.22", W48°32'40.46"	12304
Eigenmannia sp.1	28	05♀ 06♂	Mogi-Guaçu river, Araras, São Paulo	S21°56'35", W47°23'04"	12305
Eigenmannia sp.2	31/32 -$X_1X_1X_2X_2$-X_1X_2Y	10♀ 08♂	Araquá river, Botucatu, São Paulo	S22°47'13", W48°28'89"	12306

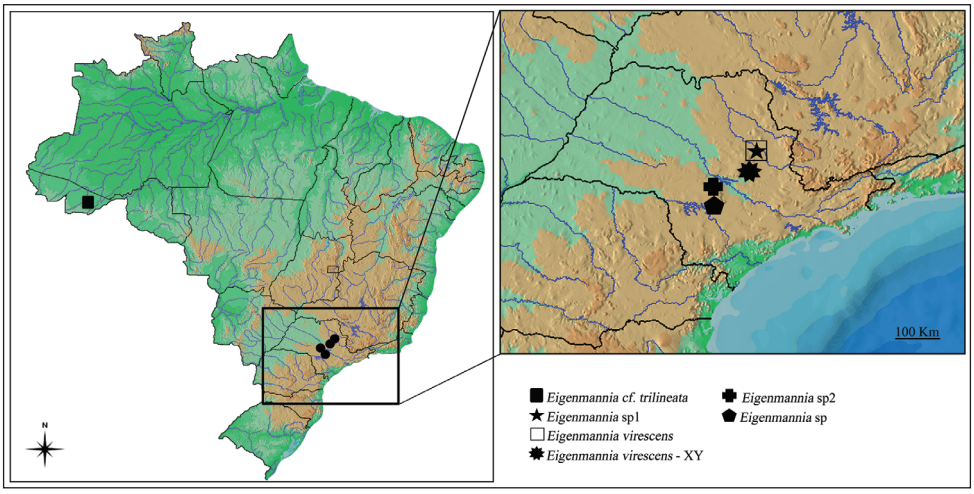

Figure 1. Map of Brazil showing the collection sites of the *Eigenmannia* populations analyzed.

(LBP), UNESP, Botucatu, São Paulo, Brazil under the identification number 521 LBP (Table 1).

Mitotic chromosomes were obtained from cell suspensions of the anterior kidney (Foresti et al. 1981). Nucleolus organizer regions (NORs) were identified by silver (Ag) nitrate staining (Howell and Black 1980), and C-banding patterns were obtained following the protocol by Sumner (1972). Genomic DNA was obtained from muscle using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Fluorescence *in situ* hybridization (FISH) was accomplished according to Pinkel et al. (1986).

(TTAGGG)n, major (18S rDNA) and minor (5S rDNA) ribosomal probes were isolated from the genome of *Eigenmannia* sp. 2 by PCR using previously described

primers (White et al. 1990; Ijdo et al. 1991; Pendás et al. 1994). The 18S rDNA sequences were labeled with Digoxigenin-11-dUTP (Roche Applied Science), and the 5S rDNA and (TTAGGG)n probes probe were labeled with biotin-16-dUTP (Roche Applied Science). Detection of hybridization signals was performed using anti-digoxigenin-rhodamine (Roche Applied Science) and avidin-FITC.

The chromosomes were cut using Adobe Photoshop version 11.0 software - Adobe Systems and organized were arranged in putative homologous pairs in the karyotypes, and classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) (Levan et al. 1964) and disposed in order of decreasing size in two groups consisting of metacentric-submetacentric and subtelocentric-acrocentric chromosomes.

Results

Diploid chromosome numbers ranged from 2n=28 chromosomes in *Eigenmannia* sp.1 to 2n=38 in *Eigenmannia virescens* (Table 1). Moreover, the individuals of *Eigenmannia* sp. 2 from the Araquá River had a multiple sex chromosome system of $X_1X_1X_2X_2/X_1X_2Y$ type.(Fig. 2C–D), while *E. virescens* had an XY sex chromosome system (Fig. 3C–D). The constitutive heterochromatin was preferentially located in the pericentromeric regions of all chromosomes of the analyzed species. Additionally, a conspicuous accumulation

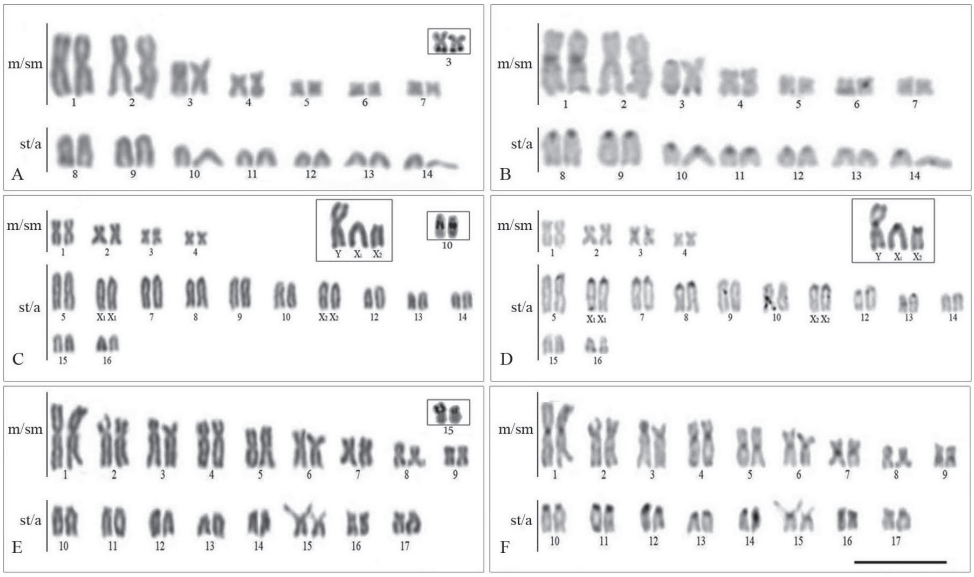

Figure 2. Karyotypes of *Eigenmannia* sp.1 (**a, b**), *Eigenmannia* sp.2 (**c, d**), *E.* cf. *trilineata* (**e, f**), arranged from Giemsa stained (**a, c, e**) and C-banded chromosomes (**b, d, f**). Inset shows the Ag-NOR-bearing chromosomes (**a, c, e**). Inset shows the male sex chromosomes of *Eigenmannia* sp.2 (**c, d**). Bar =10 μm.

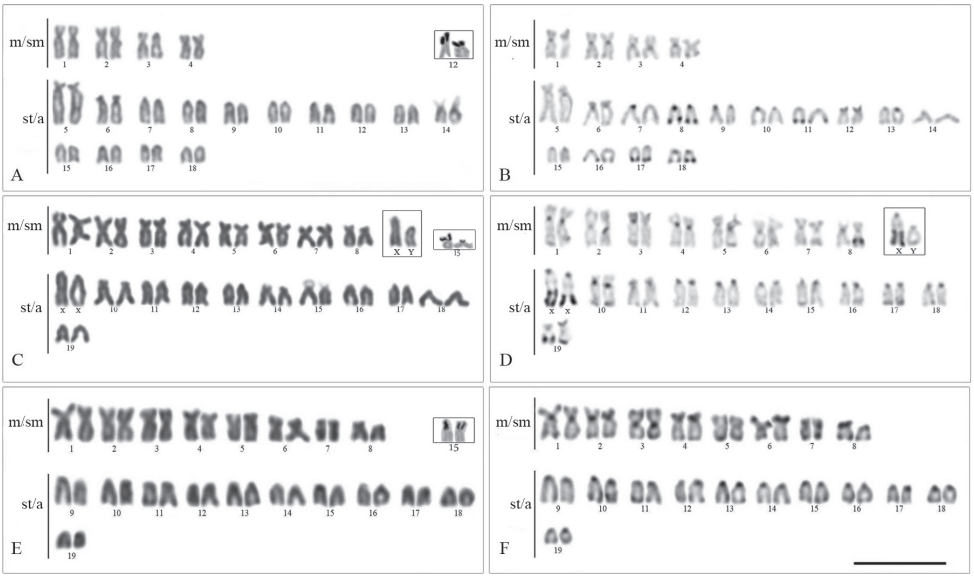

Figure 3. Karyotypes of *Eigenmannia* sp. (**a, b**), *E. virescens* - XY (**c, d**), *E. virescens* (**e, f**), arranged from Giemsa stained (**a, c, e**) and C-banded chromosomes (**b, d, f**). Inset shows the Ag-NOR-bearing chromosomes (**a, c, e**). Inset shows the male sex chromosomes of *E. virescens* - XY (**c, d**). Bar =10 μm.

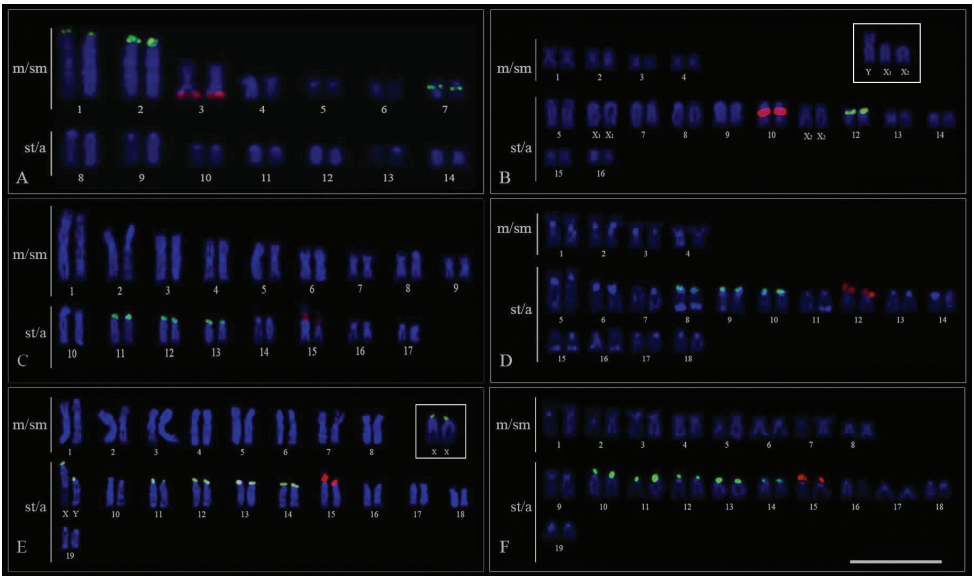

Figure 4. Karyotypes of the analyzed *Eigenmannia* species after FISH with 5S (green) and 18S (red) ribosomal probes and counterstained with DAPI. **a** *Eigenmannia* sp.1 **b** *Eigenmannia* sp.2 **c** *E.* cf. *trilineata* **d** *Eigenmannia* sp. **e** *E. virescens* –XY **f** *E. virescens.* Inset shows the male sex chromosomes of *Eigenmannia* sp.2 (**b**) and *E. virescens* – XY (**e**). Bar =10 μ.

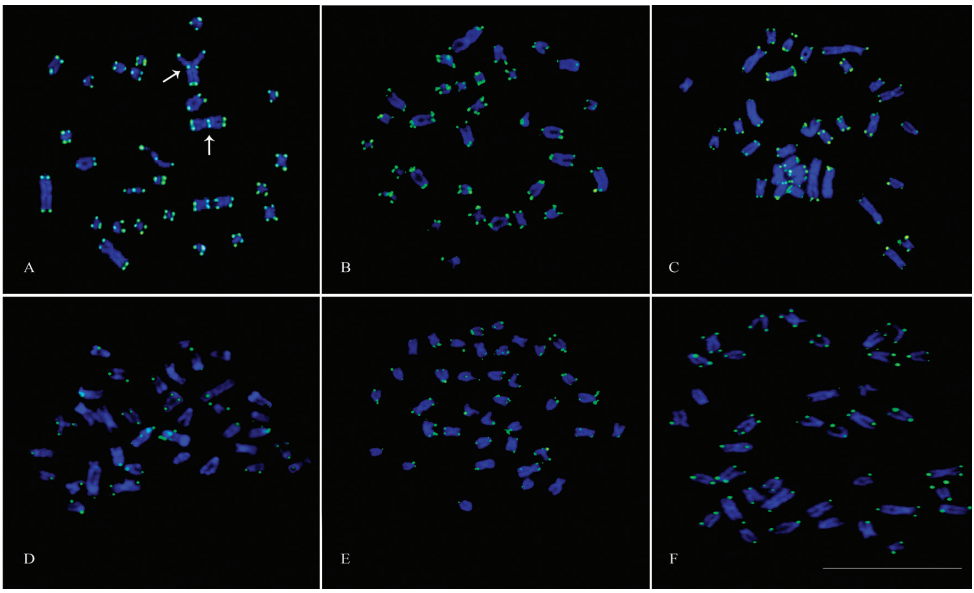

Figure 5. Mitotic metaphase chromosomes of *Eigenmannia* species hybridized with telomeric probes. **a** *Eigenmannia* sp.1 note interstitial telomeric sites (ITS) in chromosome pair 2 **b** *Eigenmannia* sp.2 **c** *E.* prope *trilineata* **d** *Eigenmannia* sp. **e** *E. virescens* **f** *E. virescens*–XY. Bar =10 μm.

of heterochromatin in the X chromosome of *E. virescens* was also observed (Fig. 3D). Ag-NORs were located in a single chromosome pair in all species.

FISH analyses using 18S rDNA probes confirmed the Ag-NOR sites (Fig. 4). Conversely, the minor ribosomal sites presented a distinct number of sites per genome, from 2 to 10, in different species. However, the position of these sites, mostly located in the centromeric region of st/a chromosomes, was conserved, except for *Eigenmannia* sp.1 (Fig. 4a–f).

Telomeric probes revealed hybridization signals in the terminal position of almost all chromosomes in all species examined (Fig. 5). Additionally, interstitial sites were observed in the m pair 2 of *Eigenmannia* sp.1 (Fig. 5a).

Discussion

The genus *Eigenmannia* is a fish group that shows complex morphological patterns. Cytogenetic studies performed in this group revealed great karyotype diversity among species and populations, including the occurrence of karyomorphs with different heteromorphic sex chromosomes (Silva et al. 2009; Henning et al. 2010). Considering their territorial behavior the fixation of different karyotypes via chromosomal rearrangements could be promoted by reproductive isolation and low levels of interchange among individuals from different small rivers (Moysés et al. 2010).

The significant chromosomal variability observed in the present study is consistent with previous studies of this genus (Almeida-Toledo et al. 1985; Almeida-Toledo et al.1996) and highlights the importance of cytogenetics as a tool in the study of relationships among knifefish representatives. Since the 2n is remarkably diversified in *Eigenmannia*, it has been suggested that chromosomal fusions and fissions are mechanisms that played an important role in the karyotype diversification within this group (Almeida-Toledo et al. 2000, 2001, 2002; Almeida-Toledo and Foresti 2001; Henning et al. 2008). FISH analyses corroborated this hypothesis and indicated that pair No. 2 of *Eigenmannia* sp.1 probably originated via a centric fusion, due to its decreased number of st/a chromosomes when compared to other species.

In a pioneer study, Milhomem et al. (2013) showed that despite the occurrence of a high karyotype variability in *Gymnotus* species, the NOR-bearing chromosomes are homologous in distinct species. Our analyses documented that a similar situation may occur in *Eigenmannia*, since the NOR-bearing chromosomes of *Eigenmannia* sp, *E. virescens* and *E.* cf. *trilineata* López & Castello, 1966 are very similar and possibly homologous among them, bearing the major ribosomal sites in the terminal position on the p arms. However, in the species with lower 2n, the location of these sites is species-specific, indicating that the NOR-bearing chromosomes might have been involved in chromosomal rearrangements during their differentiation process. *Eigenmannia* sp.2 is the only species showing NORs located at the interstitial position, conceivably indicating that pair No. 10 of this species may have arisen through fusion events involving ancestral chromosomes carrying ribosomal sequences.

The chromosomal location of 5S rRNA sites was described for the first time in *Eigenmannia* and showed that unlike 18S rDNA, the minor ribosomal sites present an extensive evolutionary variation in this group. A similar scenario was also observed in *Gymnotus*, in which chromosomal location of 5S rDNA is diversified among different species, probably because of its association with transposable elements (da Silva et al. 2011; Scacchetti et al. 2011, 2012). However, the chromosomal location of these sites does not seem to have changed in a short span of time in *E. virescens* because various cytotypes of 5S rDNA sites diverged recently (<0.6mya) (Henning et al. 2010). Actually, the ribosomal sites 5S are probably conserved in the same five pairs (Fig. 4E–F), including the XX/XY sex chromosomes. Such organization implies that these sex chromosomes are not yet well differentiated, with the accumulation of heterochromatin in the X being the primary cause of diversification of the sex chromosomes, as suggested by previous studies (Henning et al. 2010).

The present study confirmed the high diversity in the chromosome structure among the representatives of *Eigenmannia.* It also corroborates the occurrence of sex-linked chromosome polymorphisms, indicating the presence of extensive chromosomal rearrangements with *Eigenmannia* species at the genome macro and microstructure levels, of the genetic material, providing new insight for understanding the contributing evidently to speciation processes. (Fig. 6).

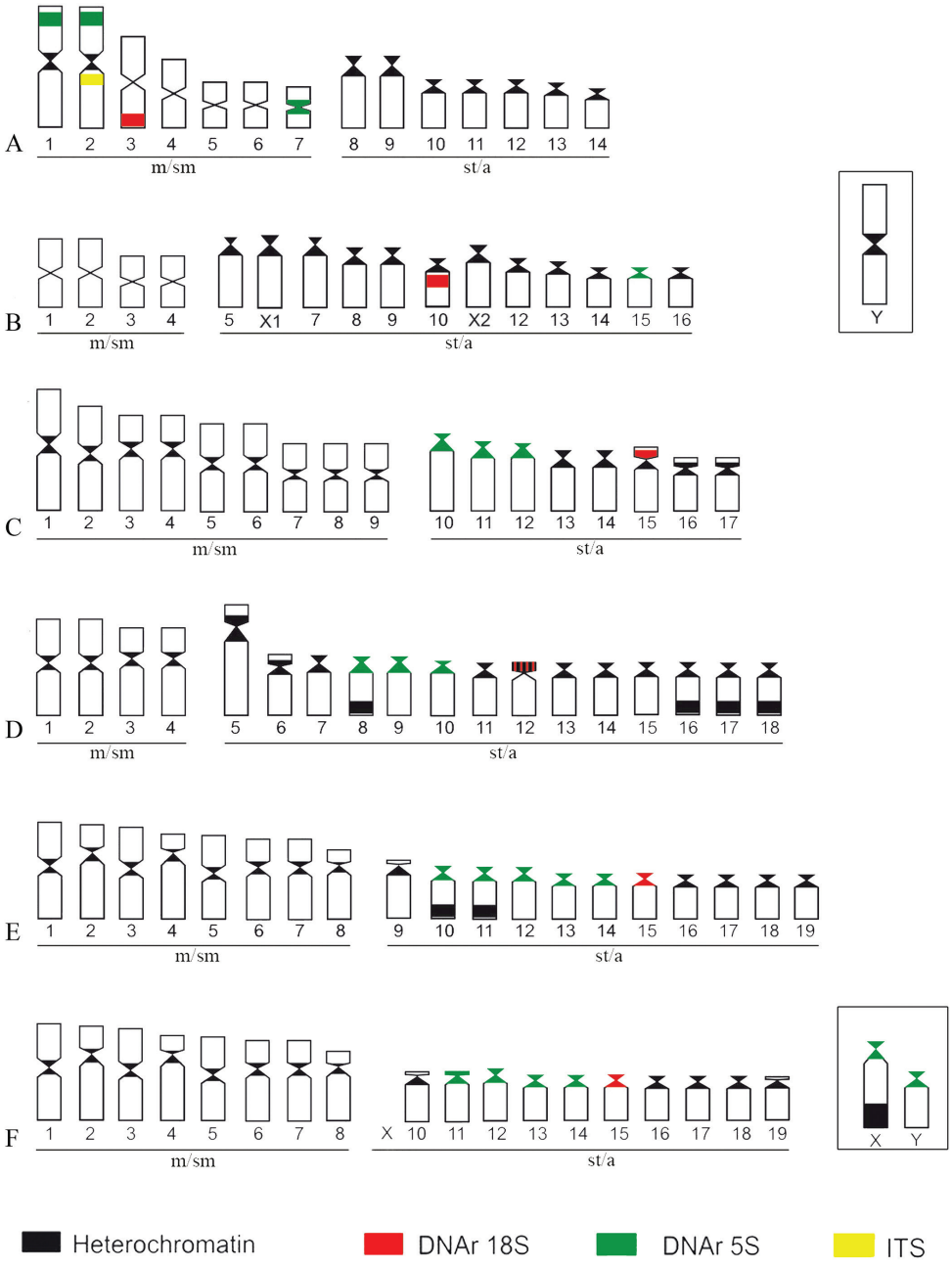

Figure 6. Ideograms showing C-heterochromatin and hybridization patterns described in this study for: **a** *Eigenmannia* sp.1 **b** *Eigenmannia* sp.2 **c** *E.* cf. *trilineata* **d** *Eigenmannia* sp. **e** *E. virescens* **f** *E. virescens*–XY.

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RESEARCH ARTICLE

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Taxonomic position of several enigmatic *Polyommatus* (*Agrodiaetus*) species (Lepidoptera, Lycaenidae) from Central and Eastern Iran: insights from molecular and chromosomal data

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Abstract

The species-rich subgenus *Polyommatus* (*Agrodiaetus*) has become one of the best studied groups of Palearctic blue butterflies (Lepidoptera, Lycaenidae). However, the identity and phylogenetic position of some rare taxa from Iran have remained unclear. An enigmatic, recently described Central Iranian species *P.* (*A.*) *shirkuhensis* ten Hagen et Eckweiler, 2001 has been considered as a taxon closely related either to *P.* (*A.*) *eckweileri* ten Hagen, 1998 or to *P.* (*A.*) *baltazardi* (de Lesse, 1962). *P.* (*A.*) *baltazardi*, in its turn, was treated as a taxon close to Iranian-Pakistani *P.* (*A.*) *bogra* Evans, 1932. Here we used a combination of molecular and chromosomal markers to show that none of these hypotheses was true. Instead, *P.* (*A.*) *shirkuhensis* was recovered as a member of a species group close to *P.* (*A.*) *cyaneus* (Staudinger, 1899). From genetically closest species, *P.* (*A.*) *kermansis* (de Lesse, 1962), *P.* (*A.*) *cyaneus* and *P.* (*A.*) *sennanensis* (de Lesse, 1959), it differs by the wing coloration. From morphologically similar *P.* (*A.*) *mofidii* (de Lesse, 1963) and *P.* (*A.*) *sorkhensis* Eckweiler, 2003, it differs by its chromosome number, n=21. *P.* (*A.*) *bogra* and *P.* (*A.*) *baltazardi* were found to be members of two different species groups and, thus, are not closely related.

Keywords

Agrodiaetus, chromosome number, *COI*, karyotype, Lycaenidae, *Polyommatus*

Introduction

Agrodiaetus Hübner, 1822, a subgenus of the species-rich Palearctic genus *Polyommatus* Latreille, 1804 (Talavera et al. 2013) is a model system in studies of speciation (Lukhtanov et al. 2005, Wiemers et al. 2009), intraspecific differentiation (Dinca et al. 2013, Przybyłowicz et al. 2014), and rapid karyotype evolution (Lukhtanov and Dantchenko 2002, Kandul et al. 2007, Vershinina and Lukhtanov 2010, 2013). Despite this fact the taxonomy of the subgenus is poorly understood, and using of molecular markers in combination with cytogenetic studies resulted in recent years in discovery of new species (Lukhtanov et al. 2003, 2008) and numerous taxonomic and nomenclatural changes (Lukhtanov1989, Lukhtanov et al. 2006, Vila et al. 2010).

Here we use a combination of molecular mitochondrial (*COI*), molecular nuclear (*ITS2*) and nuclear chromosomal (karyotype) markers to analyze two recently described and little known taxa *P.* (*A.*) *shirkuhensis* ten Hagen et Eckweiler, 2001 (ten Hagen and Eckweiler 2001) and *P.* (*A.*) *bogra birjandensis* Eckweiler, 2003 (Eckweiler 2003) which status and taxonomic position is disputed in literature (ten Hagen and Eckweiler 2001, Skala 2002).

Material and methods

The taxa *P.* (*A.*) *shirkuhensis* (Iran, Yazd Province, Shirkuh Mts., Deh-Bala village, 2900-3150 m, 12 July 2005, samples J299-1, J299-2 and J299-3, J302 and J304) and *P.* (*A.*)*bogra birjandensis* (Iran, South Khorasan Province, 26 km N of Birjand, 1900-2000 m, 14 July 2005, samples J305, J306, J307, J307-1, J307-2, J307-3, J307-4, J315, J318 and J319) were collected exactly in their type localities.

Fresh (not worn) adult males were used to investigate the karyotypes. After capturing a butterfly in the field, it was placed in a glassine envelope for 1-2 hours to keep it alive until we processed it. Testes were removed from the abdomen and placed into a small 0.5 ml vial with a freshly prepared fixative (ethanol and glacial acetic acid, 3:1). Then each wing was carefully removed from the body using forceps. The wingless body was placed into a plastic, 2 ml vial with pure 96% ethanol. The samples are kept in the Zoological Institute of the Russian Academy of Sciences.

Testes were stored in the fixative for 1-12 months at +4°C. Then the gonads were stained in 2% acetic orcein for 30-60 days at +18-20°C. Different stages of male meiosis were examined by using a light microscope Amplival, Carl Zeiss. We have used an original two-phase method of chromosome analysis (Lukhtanov and Dantchenko 2002, Lukhtanov et al. 2006).

A 643 bp fragment of mitochondrial gene *cytochrome oxidase subunit I* (*COI*) and 592 bp fragment of *nuclear internal transcribed spacer 2* (*ITS2*) were used to analyze clustering of the specimens. Primers and the protocol of DNA amplification were given in our previous publication (Lukhtanov et al. 2008). The sequences were edited

and aligned using BioEdit 7.0.3 (Hall 1999). Since *P. icarus* (Rottemburg, 1775) and *P. stempfferi* (Brandt, 1938) were earlier inferred as outgroups to the subgenus *Agrodiaetus* (Talavera et al. 2013), we used them to root the phylograms.

Sequences of the following additional representatives of the subgenus *Agrodiaetus* were found in GenBank (Wiemers 2003, Wiemers and Fiedler 2007, Wiemers et al. 2009, Kandul et al. 2004, 2007, Lukhtanov et al. 2005) and used for phylogenetic inference: *P.* (*A.*) *ainsae* (Forster, 1961), *P.* (*A.*) *achaemenes* Skala, 2002, *P.* (*A.*) *actinides* (Staudinger, 1886), *P.* (*A.*) *admetus malievi* (Dantchenko et Lukhtanov, 2005), *P.* (*A.*) *aereus* Eckweiler, 1998, *P.* (*A.*) *alcestis karacetinae* (Lukhtanov et Dantchenko, 2002), *P.* (*A.*) *altivagans* (Forster, 1956), *P.* (*A.*) *antidolus* (Rebel, 1901), *P.* (*A.*) *ardschira* (Brandt, 1938), *P.* (*A.*) *baltazardi* (de Lesse, 1963), *P.* (*A.*) *baytopi* (de Lesse, 1959), *P.* (*A.*) *bilgini* (Dantchenko et Lukhtanov, 2002), *P.* (*A.*) *birunii* Eckweiler et ten Hagen, 1998, *P.* (*A.*) *caeruleus* (Staudinger, 1871), *P.* (*A.*) *carmon carmon* (Herrich-Schäffer, 1851), *P.* (*A.*) *carmon munzuricus* (Rose, 1978), *P.* (*A.*) *ciscaucasicus* (Forster, 1956), *P.* (*A.*) *cyaneus* (Staudinger, 1899), *P.* (*A.*) *dagestanicus* (Forster, 1960), *P.* (*A.*) *dagmara* (Grum-Grshimaïlo, 1888), *P.* (*A.*) *damocles* (Herrich-Schäffer, 1844), *P.* (*A.*) *damon* (Dennis et Schiffermüller, 1775), *P.* (*A.*) *damone altaicus* (Elwes, 1899), *P.* (*A.*) *damone damone* (Eversmann, 1841), *P.* (*A.*) *damone irinae* (Dantchenko, 1997), *P.* (*A.*) *dantchenkoi* Lukhtanov et Wiemers, 2003, *P.* (*A.*) *demavendi* (Pfeiffer, 1938), *P.* (*A.*) *dizinensis* (Schurian, 1982), *P.* (*A.*) *dolus vittata* (Oberthür, 1892), *P.* (*A.*) *ectabanensis* (de Lesse, 1964), *P.* (*A.*) *elbursicus* (Forster, 1956), *P.* (*A.*) *eriwanensis* (Forster, 1960), *P.* (*A.*) *erschoffii* (Lederer, 1869), *P.* (*A.*) *faramarzii* Skala, 2001, *P.* (*A.*) *femininoides* (Eckweiler, 1987), *P.* (*A.*) *firdussii* (Forster, 1956), *P.* (*A.*) *fulgens* (Sagarra, 1925), *P.* (*A.*) *glaucias* (Lederer, 1870), *P.* (*A.*) *gorbunovi* (Dantchenko et Lukhtanov, 1994), *P.* (*A.*) *haigi* (Dantchenko et Lukhtanov, 2002), *P.* (*A.*) *hamadanensis* (Lesse, 1959), *P.* (*A.*) *hopfferi* (Gerhard, 1851), *P.* (*A.*) *huberti* (Carbonell, 1993), *P.* (*A.*) *iphidamon* (Staudinger, 1899), *P.* (*A.*) *iphigenia* (Herrich-Schäffer, 1847), *P.* (*A.*) *iphigenides* (Staudinger, 1886), *P.* (*A.*) *karatavicus* Lukhtanov, 1990, *P.* (*A.*) *karindus* (Riley, 1921), *P.* (*A.*) *kendevani* (Forster, 1956), *P.* (*A.*) *kermansis* (de Lesse, 1963), *P.* (*A.*) *khorasanensis* (Carbonell, 2001), *P.* (*A.*) *klausschuriani* ten Hagen, 1999, *P.* (*A.*) *kurdistanicus* (Forster, 1961), *P.* (*A.*) *lorestanus* Eckweiler, 1997, *P.* (*A.*) *lukhtanovi* (Dantchenko, 2005), *P.* (*A.*) *luna* Eckweiler, 2002, *P.* (*A.*) *magnificus* (Grum-Grshimaïlo, 1885), *P.* (*A.*) *masulensis* ten Hagen et Schurian, 2000, *P.* (*A.*) *mediator* (Dantchenko et Churkin, 2003), *P.* (*A.*) *menalcas* (Freyer, 1837), *P.* (*A.*) *merhaba* De Prins, van der Poorten, Borie, van Oorschot, Riemis et Coenen, 1991, *P.* (*A.*) *mithridates* (Staudinger, 1878), *P.* (*A.*) *mofidii* (de Lesse, 1963), *P.* (*A.*) *ninae* (Forster, 1956), *P.* (*A.*) *peilei* (Bethune-Baker, 1921), *P.* (*A.*) *pfeifferi* (Brandt, 1938), *P.* (*A.*) *phyllides* (Staudinger, 1886), *P.* (*A.*) *phyllis* (Christoph, 1877), *P.* (*A.*) *pierceae* (Lukhtanov et Dantchenko, 2002), *P.* (*A.*) *poseidon* (Herrich-Schäffer, 1851), *P.* (*A.*) *poseidonides* (Staudinger, 1886), *P.* (*A.*) *pulcher* (Sheljuzhko, 1935), *P.* (*A.*) *putnami* (Dantchenko et Lukhtanov, 2002), *P.* (*A.*) *ripartii* (Freyer, 1830), *P.* (*A.*) *ripartii paralcestis* (Forster, 1960), *P.* (*A.*) *rjabovi* (Forster, 1960), *P.* (*A.*) *rovshani* (Dantchenko et Lukhtanov, 1994), *P.* (*A.*) *senna-*

nensis (de Lesse, 1959), *P.* (*A.*) *shahkuhensis* (Lukhtanov, Shapoval et Dantchenko, 2008), *P.* (*A.*) *shahrami* Skala, 2001, *P.* (*A.*) *shamil* (Dantchenko, 2000), *P.* (*A.*) *sorkhensis* Eckweiler, 2003, *P.* (*A.*) *surakovi* (Dantchenko et Lukhtanov, 1994), *P.* (*A.*) *tankeri* (de Lesse, 1960), *P.* (*A.*) *tenhageni* Schurian et Eckweiler, 1999, *P.* (*A.*) *transcaspica* (Heyne, 1895), *P.* (*A.*) *turcicolus* (Koçak, 1977), *P.* (*A.*) *turcicus* (Koçak, 1977), *P.* (*A.*) *urmiaensis* Schurian et ten Hagen, 2003, *P.* (*A.*) *vanensis sheljuzhkoi* (Forster, 1960), *P.* (*A.*) *vaspurakani* (Lukhtanov et Dantchenko, 2003) and *P.* (*A.*) *zarathustra* Eckweiler, 1997.

Bayesian analysis was performed using the program MrBayes 3.2.2 (Ronquist et al. 2012). A GTR substitution model with gamma distributed rate variation across sites and a proportion of invariable sites was specified before running the program for 5,000,000 generations with default settings. The first 1250 trees (out of 5000) were discarded as a burn-in prior to computing a consensus phylogeny and posterior probabilities.

Results

Molecular markers

Bayesian analysis of the gene *COI* resulted in a consensus phylogram which displayed a high level of posterior probability for the majority of the clades revealed. A fragment of this tree demonstrating the position of the target species *P.* (*A.*) *shirkuhensis*, *P.* (*A.*) *eckweileri* ten Hagen, 1998, *P.* (*A.*) *baltazardi* (de Lesse, 1962) and *P.* (*A.*) *bogra birjandensis* is shown on Fig. 1.

Bayesian analysis of the sequence *ITS2* resulted in a mostly unresolved consensus phylogram (Fig. 2), however some clades, including the clade demonstrating the position of *P.* (*A.*) *shirkuhensis*, were revealed with moderate level of posterior probability.

Karyotypes

P. (*A.*) *shirkuhensis* (Table 1, Fig. 3). The haploid chromosome number n=21 was found in MI and MII cells of three studied individuals (J299-1, J299-2 and J299-3). In the specimen J299-2, the number 2n=42 was found in diploid chromosome set observed in male asynaptic meiosis. In MI cells, all bivalents formed a gradient size row. The karyotype contained no exceptionally large or small bivalents.

P. (*A.*) *bogra birjandensis* (Table 1). Only one (J305) of nine studied specimens displayed metaphase plates acceptable for chromosome analysis. In this specimen we were able to count approximately 2n=ca105-106 in male asynaptic meiosis. The count was done with approximation due to the overlapping of some chromosomes. The diploid set included one pair of exceptionally large chromosomes. Other chromosomes formed a gradient size row.

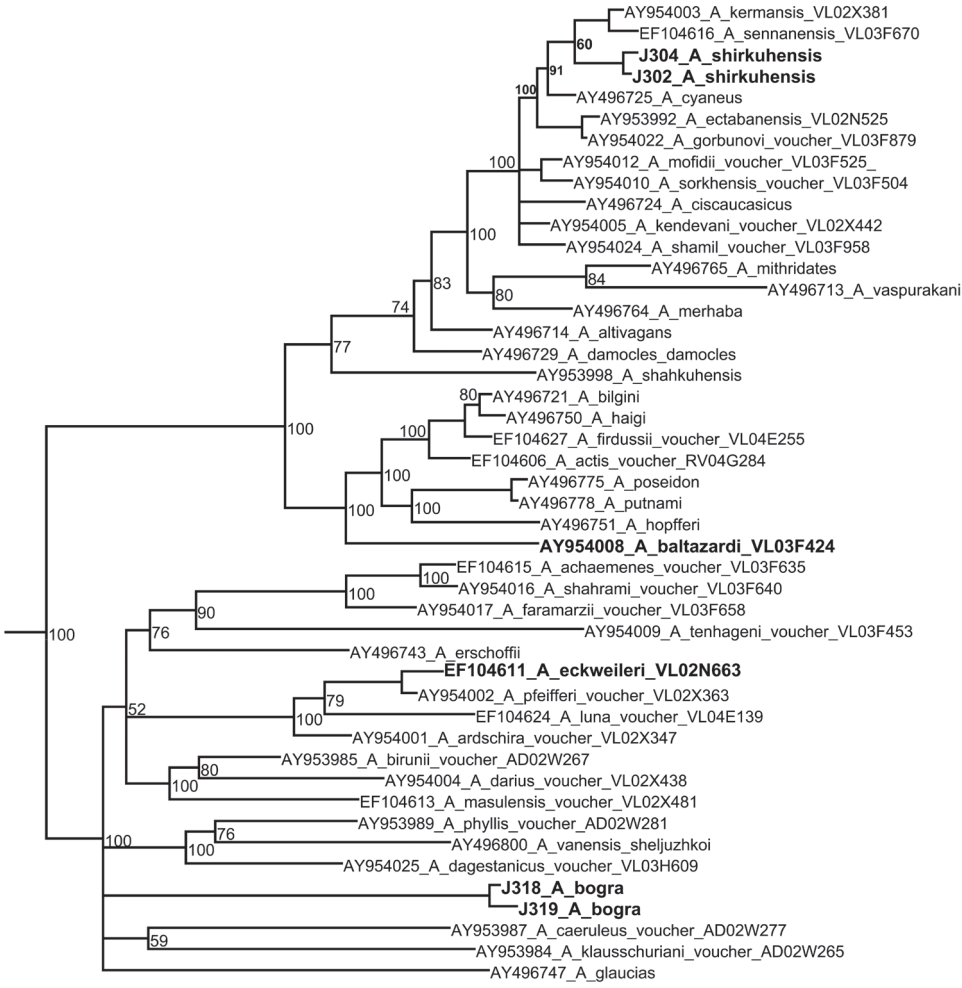

Figure 1. Fragment of consensus Bayesian tree of the subgenus *Agrodiaetus* inferred from *COI* sequences. Posterior probability values >50% are shown. Names of the target species are in bold. The complete tree is given online in the Suppl. material 1.

Discussion

P. (*A.*) *shirkuhensis* is the only species of the subgenus *Agrodiaetus* known from Shirkuh Mts massif in Central Iran (province Yazd) (ten Hagen and Eckweiler 2001). Immediately after its description, it attracted attention of lepidopterists (Skala 2002) because of its unusual combination of morphological characters such as loss of the white streak on the underside of the hind wings (most important apomorphy of the subgenus *Agrodiaetus* as a whole) and exaggerated elements of the wing underside pattern. A similar wing pattern is known in three other *Agrodiaetus* species from Central and

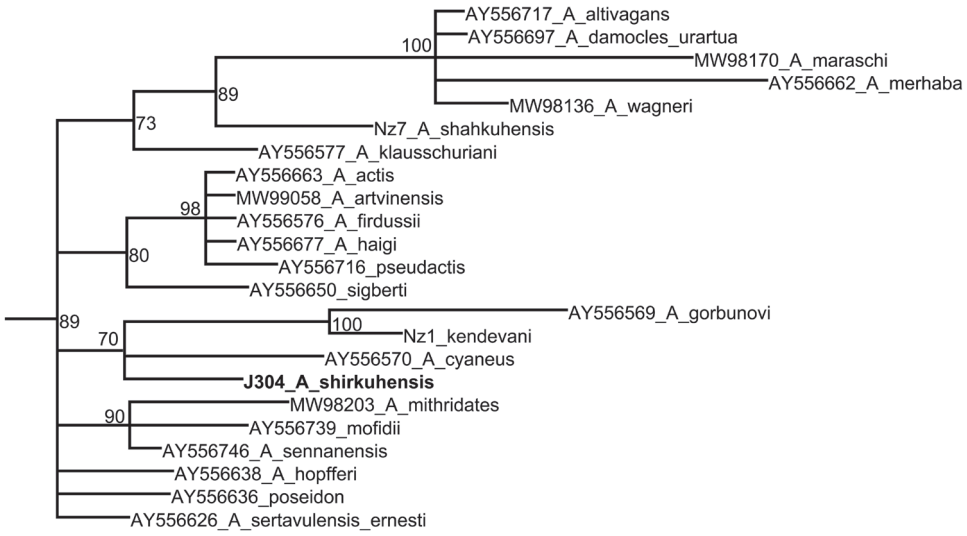

Figure 2. Fragment of consensus Bayesian tree of the subgenus *Agrodiaetus* inferred from *ITS2* sequences. Posterior probability values >50% are shown. Names of the target species are in bold. The complete tree is given online in the Suppl. material 2.

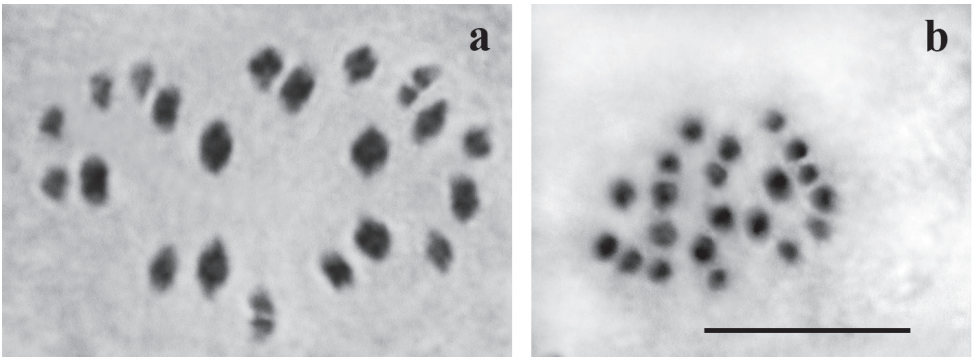

Figure 3. Male karyotype of *Polyommatus* (*Agrodiaetus*) *shirkuhensis.* **a** metaphase I, n = 21 **b** metaphase II, n = 21. Bar = 10μm.

Eastern Iran: *P.* (*A.*) *eckweileri, P.* (*A.*) *baltazardi* and *P.* (*A.*) *bogra* Evans, 1932. From these three species, *P.* (*A.*) *bogra* has the white streak on the hind wing underside, whereas *P.* (*A.*) *eckweileri* and *P.* (*A.*) *baltazardi* do not (Eckweiler and Häuser 1997, ten Hagen and Eckweiler 2001, Skala 2002). All four species are allopatric in their distribution ranges (ten Hagen and Eckweiler 2001).

Ten Hagen and Eckweiler (2001) hypothesized that *P.* (*A.*) *shirkuhensis* was a taxon closely related either to *P.* (*A.*) *eckweileri* (distributed in province Esfahan) or to *P.* (*A.*) *baltazardi* (distributed in province Kerman). *P.* (*A.*) *baltazardi*, in its turn, was treated by them as a taxon close to East Iranian – Pakistani species *P.* (*A.*) *bogra*.

Table 1. Haploid chromosome number (n) of the taxa discussed and the species groups to which these taxa belong in classifications by Eckweiler and Häuser (1997) and Kandul et al. (2004).

Taxon	n	Species group (classification after Eckweiler and Häuser)	Species group (classification after Kandul et al.)	Reference
P. (*A.*) *baltazardi*	45	*P.* (*A.*) *erschoffii*	*P.* (*A.*) *poseidon*	Lukhtanov et al. 2005
P. (*A.*) *bogra birjandensis*	ca52–53	*P.* (*A.*) *erschoffii*	*P.* (*A.*) *erschoffii*	This paper
P. (*A.*) *cyaneus*	from 18 to 20	*P.* (*A.*) *damon*	*P.* (*A.*) *cyaneus*	de Lesse 1963, Kandul et al. 2007
P. (*A.*) *eckweileri*	ca106	unclear	*P.* (*A.*) *erschoffii*	Kandul et al. 2007
P. (*A.*) *kermansis*	22	*P.* (*A.*) *damon*	*P.* (*A.*) *cyaneus*	Lukhtanov et al. 2005
P. (*A.*) *mofidii*	35	*P.* (*A.*) *damon*	*P.* (*A.*) *cyaneus*	Lukhtanov et al. 2005
P. (*A.*) *sennanensis*	28–31	*P.* (*A.*) *dolus* (Hübner, 1823)	*P.* (*A.*) *cyaneus*	Kandul et al. 2007
P. (*A.*) *shirkuhensis*	21	unclear	*P.* (*A.*) *cyaneus*	This paper
P. (*A.*) *sorkhensis*	43	*P.* (*A.*) *damon*	*P.* (*A.*) *cyaneus*	Lukhtanov et al. 2005

However, analysis of *COI* clusters in the Bayesian tree (Fig. 1) showed that none of these hypotheses was true. Among the major species groups recognized within the subgenus *Agrodiaetus* by Kandul et al. (2004, 2007) (Table 1), *P.* (*A.*) *eckweileri* is recovered by us as a member of *P. pfeifferi* (Brandt, 1938) – *P. ardschira* (Brandt, 1938) – *P. luna* Eckweiler, 2002 species complex belonging to *P. erschoffii* (Lederer, 1869) group.

P. (*A.*) *baltazardi* is found to be a member of *P.* (*A.*) *poseidon* (Herrich-Schäffer, [1851]) group and, thus, is not related to *P.* (*A.*) *bogra.* The latter species has very isolated position within the *P. erschoffii* group. The karyotypes of *P.* (*A.*) *baltazardi* and *P.* (*A.*) *bogra* are also different (Table 1).

Finally, our target species, *P.* (*A.*) *shirkuhensis*, is found to be a member of *P.* (*A.*) *cyaneus* (Staudinger, 1899) group and is especially close to *P.* (*A.*) *kermansis* (de Lesse, 1962), *P.* (*A.*) *sennanensis* (de Lesse, 1959) and *P.* (*A.*) *cyaneus* (Fig. 1). The position of *P.* (*A.*) *shirkuhensis* on the *ITS2* tree (Fig. 2) also does not contradict the conclusion that *P.* (*A.*) *shirkuhensis* belongs to *P.* (*A.*) *cyaneus* species group.

From *P.* (*A.*) *kermansis, P.* (*A.*) *cyaneus* and *P.* (*A.*) *sennanensis*, which possess closest *COI* haplotypes, *P.* (*A.*) *shirkuhensis* differs by blue upper side of wings in males (it is deep violet in *P.* (*A.*) *kermansis*, violet in *P.* (*A.*) *cyaneus* and whitish in *P.* (*A.*) *sennanensis*) (see figures in Eckweiler and Häuser 1997). The wing color in *P.* (*A.*) *shirkuhensis* is similar to those found in *P.* (*A.*) *mofidii* (de Lesse, 1963) and *P.* (*A.*) *sorkhensis* Eckweiler, 2003 (see figs 18-25 in Eckweiler 2003), two other members of the *P.* (*A.*) *cyaneus* group. *P.* (*A.*) *mofidii*, *P.* (*A.*) *sorkhensis* and *P.* (*A.*) *shirkuhensis* are allopatric in their distribution ranges (ten Hagen and Eckweiler 2001, Eckweiler 2003) and significantly different in their karyotypes (Table 1).

To conclude, our study demonstrates that four allopatric taxa known from Central and East Iran, *P.* (*A.*) *shirkuhensis*, *P.* (*A.*) *eckweileri, P.* (*A.*) *baltazardi* and *P.* (*A.*) *bogra birjandensis*, which possess significant elements of morphological similarity, are not only specifically distinct from each other, but even belong to different distantly related groups of species within the subgenus *Agrodiaetus*.

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Supplementary material 1

Consensus Bayesian tree of the subgenus *Polyommatus* (*Agrodiaetus*) inferred from COI sequences

Authors: Vladimir A. Lukhtanov, Nazar A. Shapoval, Alexander V. Dantchenko

Data type: image

Explanation note: Consensus Bayesian tree of the subgenus *Polyommatus* (Agrodiaetus) inferred from *COI* sequences. Posterior probability values >50% are shown. Names of the target species are in bold.

Supplementary material 2

Consensus Bayesian tree of the subgenus *Polyommatus* (*Agrodiaetus*) inferred from ITS2 sequences

Authors: Vladimir A. Lukhtanov, Nazar A. Shapoval, Alexander V. Dantchenko

Data type: image

Explanation note: Consensus Bayesian tree of the subgenus *Polyommatus* (Agrodiaetus) inferred from *ITS2* sequences. Posterior probability values >50% are shown. Names of the target species are in bold.

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RESEARCH ARTICLE

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Use of laser microdissection for the construction of *Humulus japonicus* Siebold et Zuccarini, 1846 (Cannabaceae) sex chromosome-specific DNA library and cytogenetics analysis

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Abstract

Dioecy is relatively rare among plant species, and distinguishable sex chromosomes have been reported in few dioecious species. The multiple sex chromosome system (XX/XY1Y2) of *Humulus japonicus* Siebold et Zuccarini, 1846 differs from that of other members of the family Cannabaceae, in which the XX/XY chromosome system is present. Sex chromosomes of *H. japonicus* were isolated from meiotic chromosome spreads of males by laser microdissection with the P.A.L.M. MicroLaser system. The chromosomal DNA was directly amplified by degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR). Fast fluorescence *in situ* hybridization (FAST-FISH) using a labeled, chromosome-specific DOP-PCR product as a probe showed preferential hybridization to sex chromosomes. In addition, the DOP-PCR product was used to construct a short-insert, *H. japonicus* sex chromosomes-specific DNA library. The randomly sequenced clones showed that about 12% of them have significant homology to *H. lupulus* and 88% to *Cannabis sativa* Linnaeus, 1753 sequences from GenBank database. Forty-four percent of the sequences show homology to plant retroelements. It was concluded that laser microdissection is a useful tool for isolating the DNA of sex chromosomes of *H. japonicus* and for the construction of chromosome-specific DNA libraries for the study of the structure and evolution of sex chromosomes. The results provide the potential for identifying unique or sex chromosome-specific sequence elements in *H. japonicus* and could aid in the identification of sex chromosome-specific repeat and coding regions through chromosome isolation and genome complexity reduction.

Keywords
Laser microdissection, plant sex chromosomes, fluorescence in situ hybridization, chromosome-specific DNA

Introduction

Dioecy is relatively rare in the plant kingdom, in which only approximately 4% of angiosperm species are dioecious (Yampolsky and Yampolsky 1922). Most of these species lack morphologically distinguishable sex chromosomes and posses sex-determining loci on homologous chromosomes or utilize environmental cues to determine sex ratios (Ainsworth 2000, Charlesworth and Guttman 1999, Tanurdzic and Banks 2004). Distinguishable sex chromosomes have been reported in several dioecious species belonging to five angiosperm families. One of these, *Humulus japonicus* Siebold et Zuccarini, 1846 (Japanese hop), is a dioecious species of the family Cannabaceae. The chromosome number is 2n=16=14+XX for females and 2n=17=14+XY1Y2 for males (Winge 1929). The multiple sex chromosome system (XX/XY1Y2) of *H. japonicus* differs from other members of the family Cannabaceae, such as the common hop (*Humulus lupulus* Linnaeus, 1753, 2n=20) and hemp (*Cannabis sativa* Linnaeus, 1753, 2n=20), in which the XX/XY chromosome system is present. Additionally, the genome sizes of these three related species vary widely: *H. lupulus* – 2.90 pg (Zonneveld et al. 2005), *H. japonicus* – 1.7 pg (Grabowska-Joachimiak et al. 2006) and *C. sativa* - 0.9 pg (Bennett and Leitch 2010; Sakamoto et al. 1998). Therefore, the family Cannabaceae can be used as a model to study the evolution of plant sex chromosomes in addition to plants from the genera *Silene* Linnaeus, 1753 and *Rumex* Linnaeus, 1753, which are classically used in this regard. In spite of recent progress in the *H. lupulus, H. japonicus* and *C. sativa* molecular cytogenetics (Alexandrov et al. 2012; Divashuk et al. 2011, 2014; Grabowska-Joachimiak et al. 2011; Karlov et al. 2003; Kim et al. 2008;) and *C. sativa* genomics (van Bakel et al. 2012), we know little about the genetics of sex determination in these species (Ming et al. 2011).

The most widespread method for the detection of new sex-specific DNA sites is to search for molecular markers that are linked to sex (Alexandrov et al. 2011; Danilova and Karlov 2006; Gao et al. 2010; Polley et al. 1997), but this method does not allow for the study of multiple chromosome-specific sequences. In complex plant genomes containing widespread repetitive sequences, it is important to establish genomic resources that enable us to focus on a particular part of the genome. There are several methods available that can be used to dissect a particular chromosome or subchromosomal region. The direct strategy for isolating sequences from chromosomes of interest is to separate them by a flow-sorting procedure or by microdissection. The main disadvantage of the flow-sorting approach is contamination of dissected material by chromosomes of similar size and the presence of particles with the same DNA content as sorted chromosomes (Dolezel et al. 2001). Currently, microdissection constitutes one of the most direct approaches to ascertain the molecular composition of certain chromosomes or chromosome regions (Houben

2012). Fine glass needles are commonly used for the mechanical dissection of chromosomes. Alternatively, laser microdissection results in the isolation of extremely pure pools of chromosomes, from which DNA can be amplified by DOP-PCR (degenerate oligonucleotide primed PCR) both to generate chromosome-specific DNA libraries and to be applied as complex probes for FISH (Fukui et al. 1992; Hobza et al. 2004; Houben 2012).

In plants, Sandery et al. (1991) first applied the microdissection technique toward isolating B-chromosomes from rye (*Secale cereale* Linnaeus, 1753) and were able to identify a DNA sequence on these rye B-chromosomes. With the development of PCR, microdissection techniques have widely been used with genetic studies of *Secale cereale* (Houben et al. 1996; Zhou et al. 1999), *Triticum aestivum* Linnaeus, 1753 (Hu et al. 2004), *Zea mays* Linnaeus, 1753 (Stein et al. 1998), *Avena sativa* Linnaeus, 1753 (Chen and Armstrong 1995; Sanz et al. 2012), *Gossypium arboreum* Linnaeus, 1753 (Renhai et al. 2012), *Citrus grandis* Osbeck, 1757 (Huang et al. 2004a,b), *Silene latifolia* Poiret, 1789 (Hobza et al. 2004, 2007), *Populus tremula* Linnaeus, 1753 (Zhang et al. 2005), an addition line of wheat-*Thinopyrum intermedium* Barkworth & Dewey, 1985 (Deng et al. 2013a) and *Spinacia oleracea* Linnaeus, 1753 (Deng et al. 2013b). Chromosome microdissection and cloning are powerful tools that combine cytogenetics with molecular genetics and have played an important role in research on genome structure (Fominaya et al. 2005; Hobza and Vyskot 2007). By generating a DNA probe for fluorescent *in situ* hybridization (FISH) with the DNA microdissected from a certain chromosome, it is possible to obtain an idea of the DNA sequences shared among different chromosomes within the same genome. The microdissection technique was used to study the structure and evolution of sex chromosomes from two model species, *Rumex acetosa* and *Silene latifolia* (Mariotti et al. 2006, Matsunaga et al. 1996, 1999; Shibata et al. 1999). These species possess heteromorphic sex chromosomes that can be microscopically distinguished from the remaining complement chromosomes (Vyskot and Hobza 2004). Painting of sex chromosomes has been performed in *Rumex acetosa* Linnaeus, 1753 by Shibata et al. (1999) and in *Silene latifolia* by Hobza et al. (2004). Hobza et al. (2004) used a modified FAST-FISH protocol, based on a short hybridization time combined with a low concentration of probe, and successfully distinguished the sex chromosomes by differential labeling patterns.

Identification of specific chromosomes for microdissection is difficult in many plant species. It can be achieved by choosing a plant with chromosomes bearing a prominent morphological feature, for example, a large somatic chromosome such as the Y chromosome in *Silene.* In *H. japonicus*, sex chromosomes are difficult to distinguish from autosomes at the mitotic metaphase plate (Grabowska-Joachimiak et al. 2011; Kim et al. 2008). During meiosis in the male plants of *H. japonicus*, a trivalent chromosome configuration is observed (Jacobsen 1957). This can be most clearly observed at diakinesis and metaphase I, which allows for reliable identification of sex chromosomes from autosomes in pollen mother cells (PMC). PMC at these stages of meiosis can easily be isolated in large quantities from immature male flowers.

To investigate the structure of the sex chromosomes in *H. japonicus,* the XY1Y2 chromosomes were isolated by laser microdissection of the meiotic trivalent at the diakinesis and metaphase I stages and the DOP-PCR products were used for FISH and the creation of the DNA library.

Materials and methods

Plant material and chromosome preparation

The male *H. japonicus* plants (2n=17=14+XY1Y2) were grown in a greenhouse from seeds of cultivar "Samuray" ("Gavrish seeds", Moscow, Russia) and were used to prepare the meiotic chromosomes. The one month old plants were exposed to a short day photoperiod (8 h day and 16 h night) to induce flowering.

For the preparation of *H. japonicus* meiotic diakinesis and metaphase I chromosomes, the significantly modified method of Zhong et al. (1996) was used. Young floral buds from male plants, approximately 3–5 mm long, were selected for meiotic chromosome preparation and the appropriate meiotic stage of development was determined. One anther from a bud was squashed in 1% Carmine in 45% acetic acid on a slide and observed under a phase microscope. The remaining anthers with pollen mother cells (PMCs) in metaphase I were fixed in a mixture of glacial acetic acid and absolute ethanol (1:3) for 1 h, washed twice on the surface of distilled water in a Petri dish (5 cm in diameter) and placed on 50 μmol L^{-1} citrate buffer (pH 4.5) for 10 minutes. Digestion was carried out on the surface of an enzyme mixture containing 3 % (w/v) cellulase R-10 (Sigma), 0.3% (w/v) pectinase (Sigma) and 0.3 % (w/v) cytohelicase (Sigma). A cell spreading technique was used for meiotic chromosome preparation on microscope slides covered with a polyethylene naphthalate membrane (P.A.L.M. GmbH, Bernried, Germany), and the slides were used for microdissection.

For FISH experiments, the chromosome preparations were made as described above, except that conventional slides were used instead of the polyethylene naphthalate membrane-coated slides.

Microdissection

The P.A.L.M. MicroLaser system (P.A.L.M. GmbH) was used to dissect Y1-X-Y2 trivalent figures at diakinesis. The microscopic stage, micro-manipulator and laser micromanipulation procedures were computer controlled. All procedures for the dissection of chromosomes are adapted from experiments performed by Kubickova et al. (2002). The membrane around the chromosome of interest is cut, and the chromosome is then catapulted by a single laser pulse directly into the cap of an Eppendorf tube. Energy of

1.5–11.7 mJ per pulse is used for microdissection and 2 mJ per pulse is used for catapulting. Fifty trivalents were collected in each experiment. The isolated chromosomes were collected in 20 µl of distilled water in a tube.

DOP-PCR

Chromosomes were used directly (without any enzymatic treatment) for amplification by DOP-PCR with regular primers designed by Telenius et al. (1992). Amplification reactions containing 50 isolated sex chromosomes were brought to volumes of 25 µL containing final concentrations of 1 x Taq DNA polymerase buffer, 0.2 mM each of four deoxynucleotides, 1.5 pM DOP primer and 0.02 U/µL Taq DNA polymerase. Amplifications were performed in a Tetrad PCR machine. An initial incubation of 94°C for 4 min was followed by eight thermal cycles of 94°C for 1 min, 28°C for 1 min, and 72°C for 2 min, in which the duration of the heating step between 28 and 72°C was set to 2 min. This was followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, with a single final incubation at 72°C for 7 min.

A male-specific DNA marker (Gao et al. 2010) was used to check the quality of DOP-PCR product from sex chromosomes. The PCR was performed using primers Sex164F 5'- AGAGAGAGAGAGAGAGCGAGAAAG-3' and Sex164R 5'-AGAGAGAGAGAGAGAGCGGAAATG-3'. Amplification reactions were performed using a Tetrad PCR machine after initial incubation at 94 C for 4 min, which was followed by 30 cycles at 94°C for 30 s, 60°C for 45 s, and 72 C for 45 s, with a single final incubation at 72°C for 7 min.

DOP-PCR product labeling and FISH

For FISH experiments, the DOP-PCR products were labeled with dioxigenin-11-dUTP (Roche Diagnostics GmbH). One-half of a microliter of the primary PCR reaction was added as a template to 20 µl of DOP-labeling PCR mix. Cycling parameters were: 3 min at 95°C for initial denaturation; 30 cycles of 15 s at 94°C, 30 s at 56°C; and 2 min at 72°C, followed by a 5 min final extension at 72°C.

FISH was performed using a modified version of the method of Fransz et al. (1996). The slides were preheated at 60°C for 30 min, pretreated with 100 µg mL^{-1} DNase-free RNase in 2 x SSC at 37 °C for 1 h and then washed three times in 1xPBS for five minutes each. 30 µL of hybridization mixture containing 50% formamide, 2x SSC, 10% sodium dextran sulphate, 50 mmol L^{-1} phosphate buffer (pH 7.0) and 10-20 ng μL^{-1} of DNA probe was used for each slide. *In situ* hybridization was performed at 37°C overnight, followed by post-hybridization wash for 15 minutes in 0.1x SSC at 42°C.

The FAST-FISH was performed as described by Hobza et al. (2004). The pretreatment and hybridization mixture preparation for the slides was as described above. The time of *in situ* hybridization was shortened to 1 h.

The slides were counterstained with 4,6-diamino-2-phenylindole (DAPI, 0,5 μg/ml) in Vectashild (Vector). The hybridization signals were observed under a fluorescence microscope (Zeiss AxioImager.M1, Germany). Images were captured by a charge-coupled device (CCD) system (AxioCam MRm) and AXIOVISION software.

Library preparation and sequencing

The DOP-PCR products were cloned into the pGEM®-T Easy Vector System (Promega, USA) as described by manufacturer. Clones were picked into 96 well plates, grown for 18 h, replicated and frozen at -80° C. One hundred randomly selected clones were tested by PCR with M13 primers on the insert present, and 24 randomly selected clones were sequenced using ABI Big Dye Mix v3.1 (Applied Biosystems Inc) with M13 primers, according to the manufacturer's instructions. Products were resolved on an ABI 3130xl sequencer. BLAST analysis was performed according to the standard procedure. BLAT analysis was used to find homology of sequences against the *C. sativa* genome (http://genome.ccbr.utoronto.ca/index.html). BLAT on DNA is designed to quickly find sequences of 95% and greater similarity of length 25 bases or more.

Results

The sex chromosomes from PMCs at meiotic diakinesis and metaphase I stages of *H. japonicus* can easily be distinguished from autosomes under a light microscope without any staining procedures, which allows for reliable identification and rapid isolation of pure chromosomes of interest (Fig. 1a). The sex chromosomes were bordered and cut using a laser beam of low energy, transferred by a single laser pulse directly into the cap of an Eppendorf tube (Fig. 1b) and then directly (without any enzymatic treatment) used as template for DNA amplification. This procedure minimizes the level of contamination. On one slide, we were able to collect up to approximately 50 sex trivalents (Y1-X-Y2). After amplification by DOP-PCR, agarose gel electrophoresis showed that DNA fragments varied in size from approximately 200 bp to 3000 bp. The conditions of DOP-PCR were optimized to minimize any preferential amplification (Fig. 1c). The absence of banding on the gel indicates preferential amplification.

To ensure that DOP-PCR product was obtained from sex chromosomes the male specific SCAR marker was used. The PCR product of expected size was obtained from DOP-PCR DNA and DNA from male plants only. No amplification was detected from female DNA and DOP-PCR product obtained after microdissection of autosomes (Fig. 1d), indicating no cross contamination.

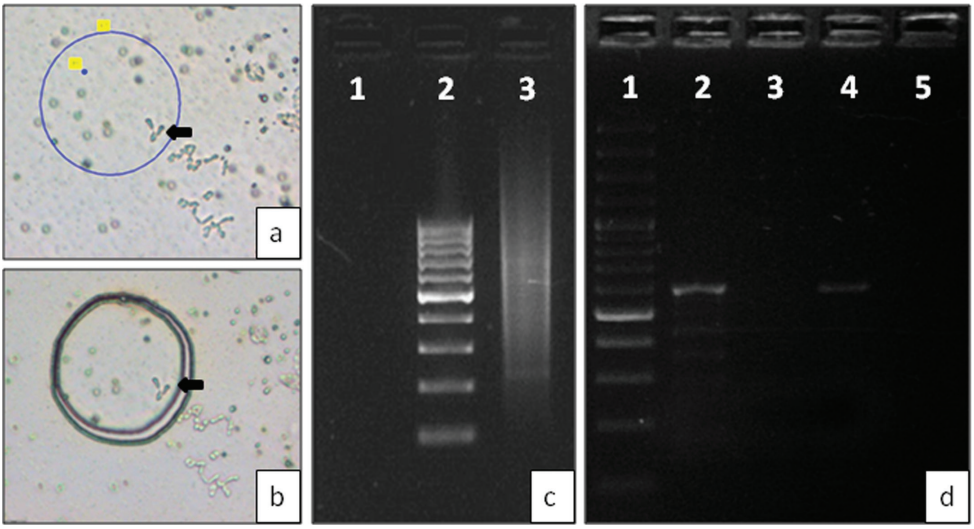

Figure 1. Microdissection of *H. japonicus* sex chromosomes at meiotic diakinesis-metaphase I stage. **a** Selection of sex chromosomes (Y1-X-Y2 trivalent formation indicated by arrow) **b** Cutting out of the sex chromosomes **c** The gel electrophoresis of the microdissected sex chromosomes DOP-PCR product: 1 – negative control, 2 – 100 bp DNA ladder, 3 - DOP-PCR **d** The gel electrophoresis after PCR with the male sex specific marker on different DNA templates: 1 – 100 bp DNA ladder, 2 – DOP-PCR product from sex chromosomes, 3 – DOP-PCR product from autosomes, 4 – DNA of male plant, 5 – DNA of female plant.

To examine the quality of the DOP-PCR product, the standard FISH procedure was performed. DIG-labeled DOP-PCR products hybridized to the chromosomes of male plants in the absence of a competitor. Signals were observed uniformly on all chromosomes (data not shown).

The application of FAST-FISH, using lower concentrations of DIG-labeled DOP-PCR probe per slide and reducing the hybridization time from 16 h to 1 h, allowed for the differentiation of chromosomes by FISH signal (Fig. 2). Analysis of the 25 meiotic metaphase I chromosome plates shows that the intensity of FISH signal on the Y1 and Y2 chromosomes was higher compared to chromosome X and autosomes.

The DOP-PCR product was used to construct a short-insert *H. japonicus* sex chromosomes-specific DNA library. Cloning of the DOP-PCR products resulted in 5 x 10^3 recombinant colonies per 100 µl PCR reaction mixture. The length of the cloned DNA fragments ranged from 450 to 3000 bp, with an average fragment length of 1000 bp. Twenty-four clones were randomly selected for sequencing. When we compared sequences with the NCBI database, using BLAST, 11 of them showed homology to sequences of plant retrotransposons (Table 1).

Three sequences show homology to some sequences of *H. lupulus* and 13 sequences show homology to *C. sativa*. Two sequences show homology to hypothetical proteins or mRNA. Additionally, a database search of the recently sequenced *C. sativa* [14] using BLAT (http://genome.ccbr.utoronto.ca) showed homology in 21 of 24 sequences with the *Cannabis* genome (Table 1).

Table 1. Similarity of the sequenced *Humulus japonicus* sex chromosome specific clones to GenBank accessions, *Cannabis sativa* draft genome and RepBase database.

№	Similarity to GenBank accessions	Tool	Similarity to *Cannabis sativa* ***
1	*Humulus lupulus* clone HlAT9 microsatellite sequence (AY588370.1)	blastn *	+
	gag-pol polyprotein [*Phaseolus vulgaris*] (AAR13317.1)	blastx *	
2	*Medicago truncatula* DNA sequence from clone MTH2-46C14 on chromosome 3, complete sequence (CT962505.9)	blastn	+
	pol protein [*Cucumis melo* subsp. *melo*] (AAO45752.1)	blastx	
3	No homology in GenBank and RepBase		+
4	*Medicago truncatula* chromosome 5 clone mte1-70c24, COMPLETE SEQUENCE (CR932962.2)	blastn	+
5	retrotransposon gag protein [*Cucumis melo* subsp. *melo*] (ADN33993.1)	blastn	+
	integrase [*Populus trichocarpa*] (ABG37658.1)	blastn	
6	*Populus trichocarpa* clone POP065-M23, complete sequence (AC209187.1)	blastn	+
	pol protein [*Cucumis melo* subsp. *melo*] (AAO45752.1)	blastx	
	rve superfamily: Integrase core domain (pfam00665)	blastx	
7	No homology in GenBank and RepBase		+
8	*Serratia proteamaculans* 568, complete genome (CP000826.1)	blastn	-
9	No homology in GenBank and RepBase		-
10	*Nicotiana benthamiana* mRNA for PME inhibitor (FN432042.1)	blastn	+
11	A family of autonomous Polinton DNA transposons (CR1-6_BF)	CENSOR **	+
12	*Gossypium raimondii* clone GR__Ba0005I14-jfn, complete sequence (AC243106.1)	blastn	+
	Amphioxus CR1-6_BF autonomous Non-LTR Retrotransposon - consensus.	CENSOR	
13	*Lotus japonicus* cDNA, clone: LjFL1-045-CB01, HTC (AK337120.1)	blastn	+
	integrase [Populus trichocarpa] (ABG37658.1)	blastx	
	LTR retrotransposon from the western balsam poplar: internal portion. (Gypsy-39_PT-I)	CENSOR	
14	No homology in GenBank and RepBase		-
15	*Humulus lupulus* vps gene for valerophenone synthase, complete cds (AB047593.2)	tblastx *	+
	gag-pol polymerase [Arabidopsis lyrata subsp. lyrata] (ABW81018.1)	blastx	
16	gag-protease polyprotein [*Cucumis melo* subsp. *melo*] (AAO45751.1)	blastx	+
17	hypothetical protein VITISV_026408 [*Vitis vinifera*] (CAN60970.1)	blastx	+
18	*Humulus lupulus* clone GT2-P16-8 microsatellite sequence (EU094990.1)	blastn	+
	HLUTR3CH_T3_051_H10_24JUL2006_066 HLUTR3CH *Humulus lupulus* cDNA, mRNA sequence (GD252950.1)	blastn	
19	*Cannabis sativa* strain Purple Kush scaffold130939_1, whole genome shotgun sequence (AGQN01284755.1)	blastn (wgs)	+
20	No homology in GenBank and RepBase		+
21	gag-protease polyprotein [*Cucumis melo* subsp. *melo*] (AAO45751.1)	blastx	+
	Vitis vinifera contig VV78X146750.38, whole genome shotgun sequence (AM458430.2)	tblastx	
22	No homology in GenBank and RepBase		+
23	No homology in GenBank and RepBase		+
24	*Daucus carota subsp. sativus* clone BAC C235O6O genomic sequence (FJ148580.1)	blastn	+
	Retrotransposon gag protein [*Asparagus officinalis*] (ABD63156.1)	blastx	

* GenBank database

** RepBase database (http://www.girinst.org/censor/index.php)

*** Seach with BLAT tool in *Cannabis sativa* genome (http://genome.ccbr.utoronto.ca)

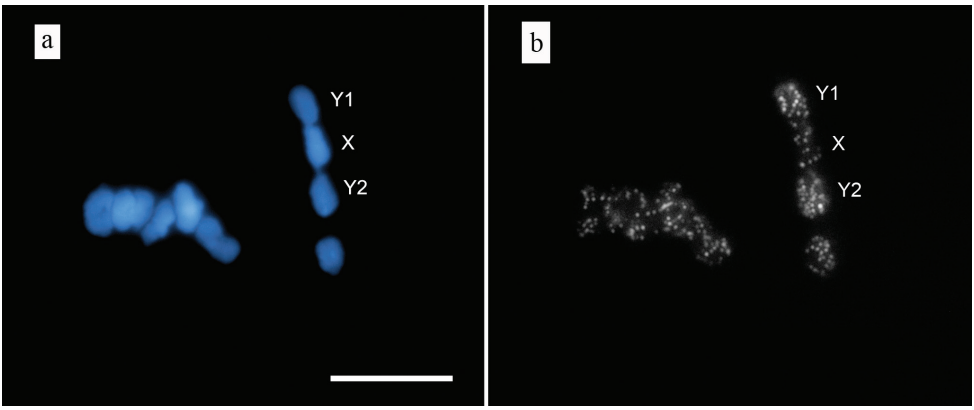

Figure 2. FISH with DOP-PCR probe on meiotic chromosomes of *H. japonicus*. **a** DAPI-stained chromosomes at meiotic metaphase I stage **b** The result of FAST-FISH with DOP-PCR probe. The Y1-X-Y2 trivalent formation is indicated. Bar = 10 μm.

Discussion

To isolate sex chromosomes, we used a technique based on laser beam microdissection with the P.A.L.M. MicroLaser system. An accurate identification of the target chromosomes is the first step in microdissection and microcloning. Additionally, the quality of microdissected chromosomal DNA depends critically on the pretreatment, chromosome fixation and staining of the samples (Houben 2012). On mitotic metaphase plates, the sex chromosomes of *H. japonicus* are difficult to distinguish from autosomes without special staining procedures. C-banding/DAPI or FISH with subtelomeric repeat were proposed to identify the X-, Y1-and Y2-chromosomes (Alexandrov et al. 2012; Grabowska-Joachimiak et al. 2011). Pretreatment and UV-light can damage chromosomal DNA when using these methods (Houben 2012). In our study, the chromosomes from PMCs at meiotic diakinesis -and metaphase I stages were used. At these stages, the sex chromosomes of *H. japonicus* (trivalent chromosome configuration) can easily be distinguished from autosomes under a light microscope without any staining procedures, which allows for reliable identification and rapid isolation of pure chromosomes of interest. Sufficient dispersion of chromosomes suitable for laser microdissection was achieved by spreading procedure of PMCs on microscopic slides covered with a polyethylene naphthalate membrane. Another advantage of the use of PMCs is the high level of synchronization of the cells.

The results of standard FISH procedure with DIG-labeled DOP-PCR products is in agreement with previous observations showing that the DNA of microdissected plant chromosomes hybridized to all chromosomes as a result of widespread repetitive sequences contained in plant genomes (Hobza et al. 2004). The use of complex subgenomic probes often leads to a nonspecific FISH signal on all chromosomes due to the difference in complexity of genomes and organization of repetitive sequences in plants compared to animals (Heslop-Harrison and Schwarzacher 2011; Schmidt and Heslop-Harrison 1998; Schubert et al. 2001).

The preferential, uneven distribution of DOP-PCR probes on the Y1 and Y2 sex chromosomes in FAST-FISH experiments is indicative of an abundance of dispersed repeats, such as retrotransposons, on Y chromosomes. These results agree with Grabowska-Joachimiak et al. (2011) where DAPI/C-banding shows brighter staining of the Y1 and Y2 chromosomes. Additionally, it may indicate accumulation on Y chromosomes-specific repetitive DNA. The accumulation of different repetitive DNA sequences was detected on Y chromosomes of *Rumex* and *Silene* species (Hobza et al. 2006; Kejnovsky et al. 2009; Shibata et al. 1999; Steflova et al. 2013).

The observation that about 12% of the sequences show significant homology to *H. lupulus* and 88% to *C. sativa*, whose genome is closely related to *H. japonicus*, indicates efficient amplification of DNA from *H. japonicus* chromosomes by DOP-PCR. Less apparent homology between *H. japonicus* and *H. lupulus,* compared to *C. sativa,* can be explained by the lack of sequence representation in the GenBank database. FISH with DOP-PCR probes led to a hybridization signal on all chromosomes, which suggests that a large amount of dispersed repeated DNA sequences are present in the genome of this species and in the DOP-PCR product. This was confirmed by sequencing, which showed that 44% of sequences were homologous to plant retroelements. The presence of multiple sequences with homology to plant retrotransposons is in agreement with FISH experiments in which a dispersed signal was seen on all chromosomes, given that retroelements are usually distributed throughout the genomes of plants (Heslop-Harrison and Schwarzacher 2011). The preferential hybridization to Y chromosomes of sex chromosome-specific DOP-PCR probes in FAST-FISH experiments indicates the presence of chromosome-specific repeated sequences.

It was concluded that laser microdissection is a useful tool for isolating the DNA of individual chromosomes, including the relatively small chromosomes of *H. japonicus,* and for the construction of chromosome-specific libraries for the study of the structure and evolution of the sex chromosomes. This is the first time a DNA library of the sex chromosomes Japanese hop has been constructed.

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RESEARCH ARTICLE

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A proposal for a multivariate quantitative approach to infer karyological relationships among taxa

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Abstract

Until now, basic karyological parameters have been used in different ways by researchers to infer karyological relationships among organisms. In the present study, we propose a standardized approach to this aim, integrating six different, not redundant, parameters in a multivariate PCoA analysis. These parameters are chromosome number, basic chromosome number, total haploid chromosome length, M_{CA} (Mean Centromeric Asymmetry), CV_{CL} (Coefficient of Variation of Chromosome Length) and CV_{CI} (Coefficient of Variation of Centromeric Index). The method is exemplified with the application to several plant taxa, and its significance and limits are discussed in the light of current phylogenetic knowledge of these groups.

Keywords

Comparative cytogenetics, cytotaxonomy, karyotype asymmetry, karyotype variation, PCoA

Introduction

Chromosomes, especially those of plants, have been efficient material for almost every kind of cytogenetic research (Guerra 2005, 2012). The genetic information of an organism is transferred via chromosomes, and changes in their number (e.g. polyploidy, dysploidy) and structure (rearrangements such as inversions, deletions, or translocations) are important contributors to plant evolution and speciation (Levin 2002, Doyle et al. 2004, Schubert 2007, Leitch and Leitch 2008, Weiss-Schneeweiss et al. 2009). Since the putative discovery of a constant species-specific chromosome number by Strasburger (1910), several times researchers posed the question, whether basic karyotype structure might provide information about the systematic position of a species (Venora et al. 2008). As a result, vast amounts of data on chromosome number have been collected until now (Stace 2000, Garbari et al. 2012) and chromosome data are constantly used for karyosystematic purposes. More recently, efforts to process this huge quantity of chromosome numbers accumulated in literature have been made, producing interesting results (Peruzzi et al. 2011, 2012, 2014, Bedini et al. 2012, Góralski et al. 2013, 2014). However, it is well known that chromosome numbers alone are not sufficient to exactly trace the evolutionary history of a group (Weiss-Schneeweiss and Schneeweiss 2003). Also, when considering some genera with many species, the ecological and the morphological data may not be an efficient tool to provide a clear representation of the systematic relationships between species. In these cases cytotaxonomy (or comparative cytogenetics), together with molecular data, can be an effective tool and it can allow a more accurate knowledge of the relationships (Coutinho 1952, Dewey 1984, Venora et al. 2008). In such cases, more detailed information about the karyotype is essential besides the chromosome number.

The karyotype of a species is generally subject to little variation and it is generally assumed that two similar species can be different for a number of chromosome rearrangements correlated with phylogenetic distance among them (Stebbins 1966, Venora et al. 2008). Karyomorphological traits are evaluated by many authors as important taxonomic characters which not only provide additional characters but also allow conclusions about evolutionary events in the group of interest (Greilhuber and Speta 1978, Greilhuber 1982, Cerbah et al. 1998, Weiss-Schneeweiss and Schneeweiss 2003). A karyotype clarifies the phenotypic aspects of the chromosome complement of a species in terms of number, size, arm ratio, centromere position, and other basic landmark features of its chromosomes (Levin 2002). In recent years, in the light of the great positive impact of the molecular phylogeny, the knowledge on the chromosome complement is still a fundamental aid to evaluate the phylogenetic relationships among taxa (Garbari et al. 2012 and literature cited therein). The karyotype asymmetry is a good expression of the general morphology of plant chromosomes. It is therefore very important to have a uniform system to compare karyotypes on correct statistical grounds (Paszko 2006). The position of centromere and the relative chromosome size are the two most important

karyotype features which allowed reasonable assessment of chromosomal affinities based on the concept of symmetry (Lavania and Srivastava 1999). Hence the use of statistically correct parameters as characters for the reconstruction of karyological relationships is fundamental. Some authors also tried to reconstruct phylogenetic relationships using only the highest possible number of karyological parameters (Caputo et al. 2013 and literature cited therein). However, until now two main problems were, more or less consciously, encountered by researchers: a) a lack of agreement in which karyotype asymmetry parameters have to be used, often leading to their misuse (e.g. redundancy etc.); b) the use of taxon-specific parameters, not of general applicability (for instance the comparison of each chromosome pair in a karyotype, which can be carried out only among closely related taxa with equal chromosome number). Concerning karyotype asymmetry, we think that the revisions of Paszko (2006), Zuo and Yuan (2011) and Peruzzi and Eroğlu (2013) were decisive, in definitely showing how and what to measure (see beyond, in Materials and methods, for more details). Despite this, many researchers – even in the very last year – continued to use outdated and often not statistically correct parameters to quantify karyotype asymmetry (Gao et al. 2012, Eroğlu et al. 2013, Wang et al. 2013, Altınordu et al. 2014, Morales et al. 2014, De Oliveira et al. 2014, Jafari et al. 2014, Chen et al. 2014). In addition, a number of basic karyological parameters (besides karyotype asymmetry) are of general applicability and can be compared among taxa: chromosome number, basic chromosome number (*x*, as defined by Peruzzi 2013), and total length of chromosomes (which is a rough proxy of genome size; Peruzzi et al. 2009).

Hence, the aims of our study were (1) to propose a standardized use of basic karyological characters as a valid, of general use, complement to other source of systematic data to understand the relationships among taxonomic groups as families, tribes, genera, sections and species, and (2) to demonstrate the using of this new quantitative method in cytotaxonomy in selected groups, for which data were available in literature.

Materials and methods

Data source

The data about Smilacaceae, Liliaceae and its tribes and genera were derived by Kong et al. (2007) and by the supplementary material published along with Peruzzi et al. (2009), Gao et al. (2012), and by Peruzzi (2012), concerning specifically the genus *Gagea* Salisbury, 1806. For *Cyananthus* Wallich ex Bentham, 1836 (Campanulaceae) and for *Crocus* Linnaeus, 1753 ser. *Verni* Mathew, 1982 (Iridaceae), the data were derived by the recent papers by Chen et al. (2014) and Harpke et al. (2014), respectively. Most of these papers report also information on the phylogenetic relationships among groups (for *Cyananthus* available in Zhou et al. 2013), as inferred from molecular systematic studies. All the datasets are available as Supplementary material 1.

Karyological parameters

To determine the karyological relationships among taxa, we used chromosome number ($2n$), basic chromosome number (x), and other basic karyomorphological characters such as genome size, grossly estimated as total haploid length of the chromosome set, THL (Peruzzi et al. 2009). Also karyotype symmetry indices were used, such as M_{CA} (Mean Centromeric Asymmetry) which gives a measure of intrachromosomal asymmetry, and CV_{CL} (Coefficient of Variation of Chromosome Length) which gives a measure of interchromosomal asymmetry, together with CV_{CI} (Coefficient of Variation of Centromeric Index), which gives a measure of centromere position heterogeneity (Paszko 2006; Zuo and Yuan 2011, Peruzzi and Eroğlu 2013). For a karyotype, M_{CA} is calculated as the mean (L-S)/(L+S) ×100 where, for each chromosome, L is the length of long arm and S is the length of short arm; CV_{CL} as the standard deviation of (L+S) divided by the mean (L+S) ×100; CV_{CI} as the standard deviation of S/(L+S) divided by the mean S/(L+S) ×100. These three parameters estimate quantitatively three different features of a karyotype, so that any redundancy of data is avoided. Moreover, they were shown to be the only quantitative parameters correct on statistical grounds (Peruzzi and Eroğlu 2013). For these reasons, other parameters proposed earlier to estimate the intrachromosomal (TF%, AsK%, AsI%, Syi, A_1, CG; for details and references see Peruzzi and Eroğlu 2013) or the interchromosomal asymmetry (Rec, R; for details and references see Peruzzi and Eroğlu 2013) were discarded. The same applied also to semi-quantitative methods such as that of Stebbins (1971) or to indices trying to summarize both kind of asymmetries (intra- and inter-chromosomal) in a single value (i.e. DI, AI; for details and references see Paszko 2006, and Peruzzi et al. 2009 for criticisms). Also karyomorphometric measurements of single chromosome pairs (as for instance those used by Caputo et al. 2013 and in previous works of the same research team) were not considered, to guarantee a general applicability of the method independent from chromosome number.

Other karyological characters might have been used, such as number of 45S and 5S sites or "best practice" genome size estimations, but this kind of data is not yet widespread (Roa and Guerra 2012; Garcia et al. 2012, 2014a, 2014b) and would also limit the applicability of the method.

Data analysis

Since our main objective was to highlight correctly karyological relationships among objects (e.g. single accessions) and not to form groups, we avoided multivariate classification techniques such as cluster analysis etc. and focused on a general ordination method as PCoA (Principal Coordinate Analysis). In cases where specific *a priori* grouping hypotheses (based on independent sources of systematic data) needed to be tested, this approach was complemented by subjecting the same data matrix to DA (Discriminant Analysis). To perform PCoA, a similarity matrix was created using Gower's (1971)

general coefficient similarity to summarize relationship among accessions (Sneath and Sokal 1973), which can be used directly with a mixture of character types (binary, qualitative, and quantitative characters) as well as taking into account missing values (St-Laurent et al. 2000). To perform these kind of analyses, the software Past 3.03 (Hammer et al. 2001, Hammer 2013), freely available online, was used.

Results

Testing the new approach at family level

We analyzed 434 accessions for Liliaceae and 35 accessions for Smilacaceae by PCoA (cumulative variance explained by the first two axes: 54.21%). Only a modest overlap among the two families was evident (Fig. 1). Indeed, DA correctly attributed objects (accessions) to the two families in 95.24% of cases (jackknifed). The most important characters in recognizing the two families as distinct resulted THL, CV_{CI}, and M_{CA}.

Testing the new approach at tribe level

Within Liliaceae, 103 accessions for Tulipeae tribe, 252 accessions for Lilieae tribe, 14 accessions for Medeoelae tribe, 13 accessions for Streptopeae tribe, 27 accessions for Tricyrtideae tribe and 25 accessions for Calochorteae tribe were analyzed by PCoA (cumulative variance explained by the first two axes: 53.96%). Also in this case, the accessions belonging to the same tribe clearly tend to cluster together (Fig. 2). Indeed,

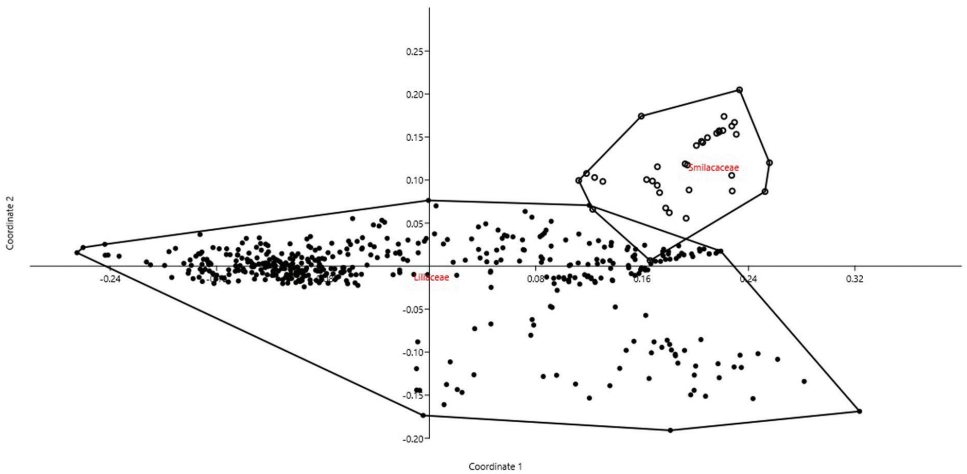

Figure 1. PCoA for Liliaceae and Smilacaceae based on 6 quantitative karyological parameters (Axis 1 vs. Axis 2).

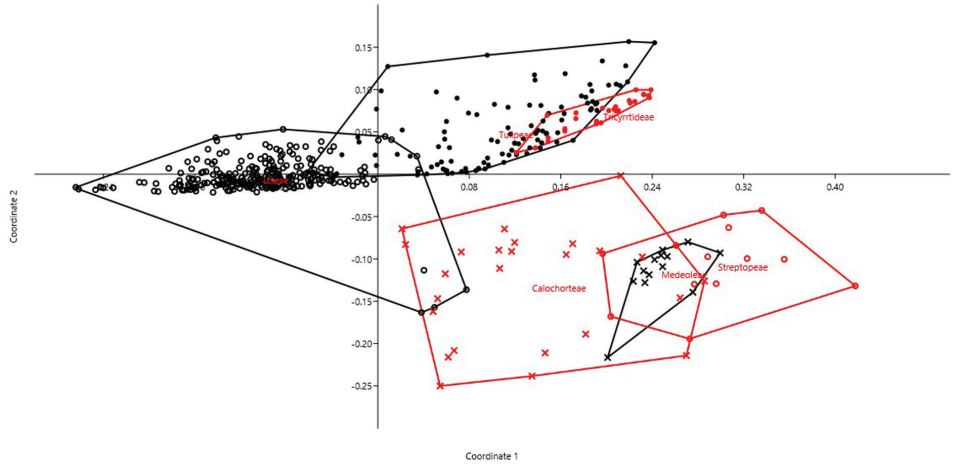

Figure 2. PCoA for Liliaceae tribes based on on 6 quantitative karyological parameters (Axis 1 vs. Axis 2).

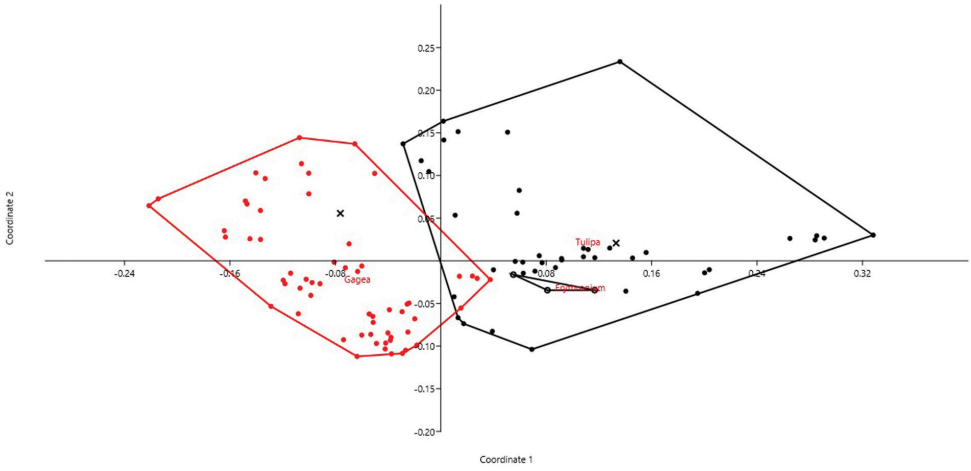

Figure 3. PCoA for Tulipeae genera based on 6 quantitative karyological parameters (Axis 1 vs. Axis 2). The two *Amana* accessions are represented by the "×" symbol.

DA correctly attributed objects (accessions) to the two families in 93.97% of cases (jackknifed). The most important characters in recognizing the two families as distinct resulted THL, CV_{CL}, and M_{CA}.

Testing the new approach at genus level

Within Liliaceae tribe Tulipeae, *Erythronium* Linnaeus, 1753 (3), *Tulipa* Linnaeus, 1753 (42), *Amana* Honda, 1935 (2), *Gagea* (56) accessions were analyzed by PCoA (cumulative variance explained by the first two axes: 48.3%). The isolated position of *Gagea* respect with other genera was particularly evident (Fig. 3). The DA, restricted to

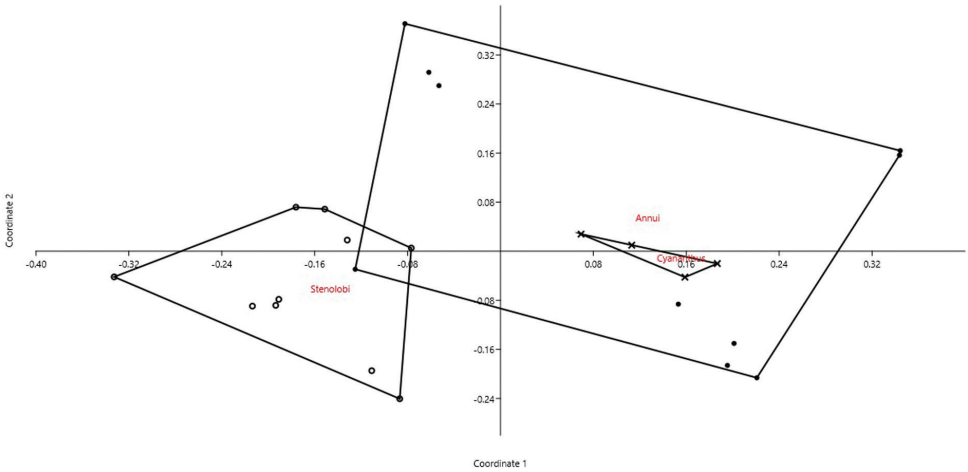

Figure 4. PCoA for *Cyananthus* accessions based on 6 quantitative karyological parameters (Axis 1 vs. Axis 2).

Gagea and *Tulipa*, correctly attributed objects (accessions) to the two genera in 94.12% of cases (jackknifed). The most important characters in recognizing the two families as distinct resulted THL, M_{CA}, and CV_{CL}.

Testing the new approach at section level

We analyzed 24 accessions belonging to three sections (*Annui*, *Cyananthus*, and *Stenolobi*) representing 15 species of the genus *Cyananthus* (Campanulaceae) by PCoA (cumulative variance explained by the first two axes: 65.52%). We can see a certain overlap among all sections, with *Stenolobi* seemingly more isolated and *Cyananthus* forming a homogeneous group within of *Annui* (Fig. 4). However, when the first axis is plotted against the third one, also these two sections appear well separated (Fig. 5). Indeed, DA correctly attributed objects (accessions) to the three sections in 87.5% of cases (jackknifed). In this case, the most important characters in recognizing the three sections resulted 2*n*, CV_{CI}, and THL.

Testing the new approach for relationships among closely related species

We analyzed 36 accessions belonging to nine species of *Crocus* ser. *Verni* (Iridaceae): *C. etruscus* Parlatore, 1858 (1), *C. heuffelianus* Herbert, 1847 (9), *C. ilvensis* Peruzzi et Carta, 2011 (4), *C. kosaninii* Pulević, 1976 (1), *C. neapolitanus* (Ker Gawler) Loiseleur-Deslongchamps, 1817 (6), *C. neglectus* Peruzzi et Carta, 2014 (5), *C. siculus* Tineo, 1832 (3), *C. tommasinianus* Herbert, 1847) (3) and *C. vernus* (Linnaeus) Hill, 1765 (4) (cumulative variance explained by the first two axes: 58%). We can see the

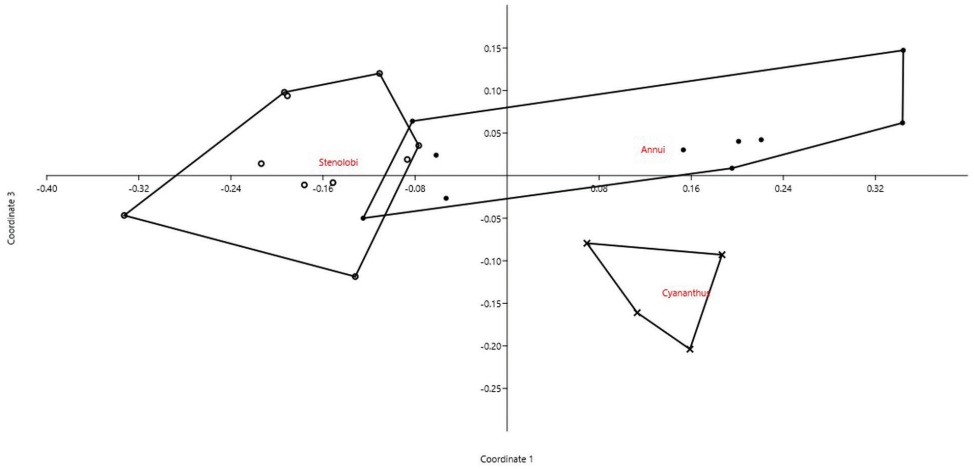

Figure 5. PCoA for *Cyananthus* accessions based on 6 quantitative karyological parameters (Axis 1 vs. Axis 3).

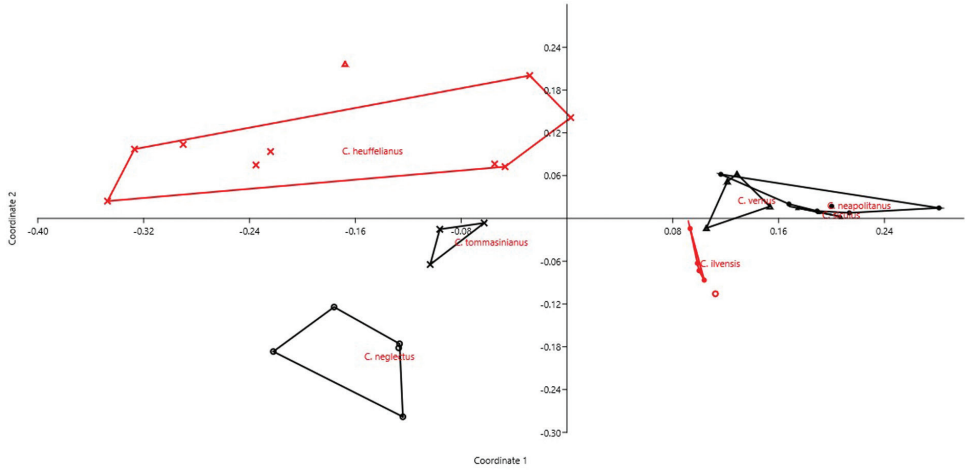

Figure 6. PCoA for *Crocus* accessions based on 6 quantitative karyological parameters (Axis 1 vs. Axis 2). The red circle and the red triangle depict the single accessions of *C. etruscus* and *C. kosaninii*, respectively.

accessions belonging to same species close each other (Fig. 6). DA correctly attributed objects (accessions) to each species in 69.44% of cases (jackknifed). The most important characters in recognizing the three sections resulted THL, CV_{CL}, and M_{CA}.

Discussion

Our method allows to describe basic karyological relationships among taxa in a correct way, avoiding redundant data or the use of statistically not well founded parameters. Concerning

the examples presented, there is always a certain degree of agreement among the information resulting from karyological multivariate analysis and the available phylogenetic information (used to form the groups highlighted in the PCoA and tested by means of DA). Liliaceae and Smilacaceae are sister families (Peruzzi et al. 2009 and literature cited therein), and despite their closeness show very modest overlap on karyological grounds. This is true also at tribe level within Liliaceae, albeit for instance Tricyrtideae are karyologically closer to Tulipeae, while on phylogenetic grounds they result an independent lineage (Peruzzi et al. 2009). This can be easily explained by the striking overall similarity in karyotype structure among *Gagea* (within Tulipeae) and Tricyrtideae, albeit chromosome numbers are different ($x = 12$ the former, $x = 13$ the latter; Peruzzi et al. 2009). As far infrageneric taxa are concerned, *Cyananthus* sections show a certain degree of karyological separation. Zhou et al. (2013) showed that sect. *Cyananthus* is sister to *Annui* + *Stenolobi*. Our data point towards a higher karyological affinity between *Annui* and *Stenolobi* (Figs 4 and 5), as already evidenced by Chen et al. (2014). PCoA, however, highlights a certain karyological heterogeneity within sect. *Annui*, which is partly close to *Cyananthus* and in part overlapping to *Stenolobi*. The accessions falling close to *Cyananthus* in the PCoA share the same basic chromosome number with the latter. Also the karyological relationships among the species of *Crocus* ser. *Verni*, as evidenced here, are fully congruent with the current systematic knowledge of the group (Harpke et al. 2014). In particular, *C. neapolitanus*, *C. siculus* and *C. vernus* resulted karyologically very closely related species and this is supported by available phylogeny. The resolution of karyological relationships is much better than that obtained by simply plotting karyotype asymmetry parameters against each other, as done by Harpke et al. (2014).

Conclusions

For various reasons, researchers used until very recently outdated, wrong or redundant parameters in order to establish relationships among taxa. We propose here a standardized method, taking into account six quantitative parameters: $2n$ (somatic chromosome number), x (basic chromosome number), THL (total length of haploid chromosome set), CV_{CI} (Coefficient of Variation of Centromeric Index, measuring the heterogeneity in the centromere position), M_{CA} and CV_{CL} (Mean Centromeric Asymmetry and Coefficient of Variation of Chromosome Length, both measuring the karyotype asymmetry). We used a multivariate ordination approach (PCoA), eventually complemented by DA, if specific grouping hypotheses need to be tested. We think this method is best suited to establish karyological relationships, relationships, compared with classification approaches (i.e. clustering, used for instance by Caputo et al. 2013, Chen et al. 2014 and many others), which may be misinterpreted concerning their real significance (i.e. a dendrogram can resemble a phylogenetic tree). We applied our method to several taxa at various ranks from family to species, showing that the discriminatory power of karyological parameters is very variable among groups. As already highlighted by Siljak-Yakovlev and Peruzzi (2012) and Peruzzi and Eroğlu (2013), basic karyological data alone are not sufficient to definitely establish systematic

and phylogenetic relationships among taxa, and should always be complemented by independent sources of systematic data. However, karyological data significantly contribute to understanding evolutionary relationships, jointly with morphological and molecular approaches. To this aim, our method is better than others because it is easy to use, based on correct, not redundant parameters of general use, and also because the data are treated with ordination and not classification techniques.

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Supplementary material 1

Karyological parameters available for the studied taxa

Authors: Lorenzo Peruzzi, Fahim Altınordu

Data type: measurement

Explanation note: Excel file with three different worksheets (Liliaceae+Smilacaceae; *Cyananthus*; *Crocus* ser. *Verni*).

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Karyotype diversity among predatory Reduviidae (Heteroptera)

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Abstract

Species of infraorder Cimicomorpha of Heteroptera exhibit holokinetic chromosomes with inverted meiosis for sex chromosomes and high variation in chromosome number. The family Reduviidae, which belongs to this infraorder, is also recognized by high variability of heterochromatic bands and chromosome location of 18S rDNA loci. We studied here five species of Reduviidae (Harpactorinae) with predator habit, which are especially interesting because individuals are found solitary and dispersed in nature. These species showed striking variation in chromosome number (including sex chromosome systems), inter-chromosomal asymmetry, different number and chromosome location of 18S rDNA loci, dissimilar location and quantity of autosomal C-heterochromatin, and different types of repetitive DNA by fluorochrome banding, probably associated with occurrence of different chromosome rearrangements. Terminal chromosome location of C-heterochromatin seems to reinforce the model of equilocal dispersion of repetitive DNA families based in the "bouquet configuration".

Keywords

Cimicomorpha, DAPI/CMA_3 banding, Heteroptera, holokinetic chromosomes, 18S rDNA

Introduction

Species of the suborder Heteroptera share several cytogenetic features such as the occurrence of holokinetic chromosomes, inverted meiosis for sex chromosomes and variation in chromosome number (Ueshima 1979, Manna 1984, Pérez et al. 2000, Papeschi and Bressa 2006, Bardella et al. 2014a). Chromosome numbers vary from 2n = 4 in Nepomorpha to 2n = 80 in Cimicomorpha (Ueshima 1979, Manna 1984, Papeschi and Bressa 2006), and the latter infraorder displays the greatest karyotype diversity among the Heteroptera (Kuznetsova et al. 2011). These insects also exhibit diversity in heterochromatin distribution (Grozeva and Nokkala 2003, Grozeva et al. 2004, Ituarte and Papeschi 2004, Bressa et al. 2005, Franco et al. 2006, Panzera et al. 2010, Bressa et al. 2008, Chirino et al. 2013, Bardella et al. 2014a). Previous reports on C-heterochromatin in heteropterans showed that C-bands are terminally located. However, intercalary patterns are described in several species (Camacho et al. 1985, Dey and Wangdi 1990, Pérez et al. 1997, Papeschi et al. 2003, Ituarte and Papeschi 2004, Grozeva and Nokkala 2003, Angus et al. 2004, Grozeva et al. 2004, Waller and Angus 2005, Angus 2006, Bressa et al. 2008).

The 18S rDNA locus is the principal marker on chromosomes of Nepomorpha, Pentatomomorpha and Cimicomorpha (González-García et al. 1996, Papeschi et al. 2003, Cattani et al. 2004, Cattani and Papeschi 2004, Dias de Campos Severi-Aguiar and Azeredo-Oliveira 2005, Severi-Aguiar et al. 2006, Morielle-Souza and Azeredo-Oliveira 2007, Bressa et al. 2008, 2009, Grozeva et al. 2010, 2011, Poggio et al. 2011, 2013a, 2014, Panzera et al. 2012, Chirino et al. 2013a, Bardella et al. 2013). Of the 36 species of Pentatomomorpha studied until now, the rDNA loci are preferably located in autosomes with only four species with rDNA loci on the sex chromosomes (González-García et al. 1996, Bressa et al. 2009, Grozeva et al. 2011, Bardella et al. 2013). On the contrary, in Cimicomorpha, the location of rDNA loci are more heterogeneous: the hybridization sites are observed on autosomes, sex chromosomes or both simultaneously (Dias de Campos Severi-Aguiar and Azeredo-Oliveira 2005, Severi-Aguiar et al. 2006, Morielle-Souza and Azeredo-Oliveira 2007, Grozeva et al. 2010, 2011, 2013, 2014, Panzera et al. 2012, 2014, Poggio et al. 2011, 2013a, 2013b).

According to Schuh and Slater (1995), Cimicomorpha includes species with different habits, such as predatory and hematophagous (Reduviidae), phytophagous (Miridae) and ectoparasitic (Cimicidae and Polyctenidae). Predators are interesting because they act in the biological control of other insects, either in natural or agricultural environments (Schaefer and Panizzi 2000). The study of these insects is difficult because they are always found scattered in nature, without the formation of colonies. The small number of individuals obtained is a limiting factor for comparative analyses of relatedness and karyotype evolution, as well as for population approaches. We made great efforts to obtain a large number of predators of Cimicomorpha to increase our knowledge of the karyotypical structure of these insects. Our goal was to generate a good volume of data and to compare them with the results previously reported for other heteropteran groups. The results presented here for the family Reduviidae provide information on karyotype organization, including the distribution of heterochromatin

and location of 18S rDNA sites. These analyses reinforce the model of equilocal dispersion of repetitive DNA families based in the "bouquet organization".

Materials and methods

Five species of Heteroptera belonging to the family Reduviidae (subfamily Harpactorinae) were collected in the South and Southeast regions of Brazil, and information about the collection localities is given in Fig. 1 and Table 1. Conventional karyotypes of *Apiomerus lanipes* (Fabricius, 1803) and *Cosmoclopius nigroannulatus* (Stål, 1860) were previously described (Poggio et al. 2007), while all cytogenetic information on *Zelus laticornis* (Herrich-Schäffer, 1853), *Montina confusa* (Stål, 1859) and *Repipta flavicans* (Amyot & Serville, 1843) is new. Gonads were dissected out and the seminiferous tubules were fixed in a solution of methanol-acetic acid (3:1, v:v) and stored at 20°C below zero. For the preparation of slides, tubules were incubated in 45% acetic acid for 10 min at room temperature, and squashed in a drop of 45% acetic acid. Coverslips were removed after freezing in liquid nitrogen, and the slides air-dried. For conventional staining the slides were treated with 1N HCl for 6 min at room temperature and stained with 2% Giemsa for 1 min at room temperature. The samples were air-dried and mounted with Entellan. Chromosome measurements were made in five metaphases I, with similar chromosome condensation, for each species. The measurement was performed manually, using a needle point compass. Chromosome pairs were arranged in decreasing size, according to the average size and standard deviation. The sex chromosomes were distinguished by the characteristic arrangement in metaphase I and were measured separately since they exhibit univalent behavior.

For chromosome C-banding (Sumner 1982, with modifications), slides were aged for three days after removal of coverslips. Afterwards, the slides were incubated in 0.2 N HCl for 10 min at room temperature, 5% barium hydroxide at 60°C for 2 min, and 2× SSC, pH 7.0, at 60°C for 60 min. Samples were treated with 30 µl of each fluorochrome: 0.5 mg/ml chromomycin A_3 (CMA_3/Sigma) for 1.5 h at room temperature and 2 µg/ml 4'6-diamidino-2-phenylindole (DAPI/Invitrogen) for 30 min at room temperature. Preparations were mounted with a medium composed of glycerol/McIlvaine buffer, pH 7.0 (1:1, v:v), plus 2.5 mM $MgCl_2$.

Fluorescent *in situ* hybridization (FISH) was done as described in Bardella et al. (2010) and performed on samples of at least two individuals per species. The p*At*05 clone, containing a partial sequence of the 18S rDNA of *Antiteuchus tripterus* (Fabricius, 1787) (Pentatomidae, Pentatomomorpha), was labeled with digoxigenin-11-dUTP by nick translation (DIG-nick translation mix Roche prepared according to the procedures recommended by the manufacturer). Preparations were treated with 30 µl of hybridization mixture containing 4 µl of labeled probe (100 ng), 15 µl of 100% formamide, 6 µl of 50% polyethylene glycol, 3 µl of 20×SSC, 1 µl of 10% SDS and 1 µl of water. Chromosome denaturation/renaturation was done at 90°C for 10 min using a thermal cycler, and hybridization was performed for 12 h at 37°C in a humidified chamber. Post-hybridization washes were carried out at different concentrations of SSC buffer (3.17M

Table 1. Information about predatory Reduviidae predators. The numbers before the city names indicate the position on the map (Fig. 1) and capital letters refer to the Brazilian states: SP: São Paulo, MS: Mato Grosso do Sul and PR: Paraná. The average sizes of chromosomes of all species are presented in µm. with standard deviation. Asterisk indicates the size of sex chromosomes, CN = chromosome number, CP = chromosome pairs (univalent for sex chromosomes), SC = sex chromosome, LSC = large sex chromosome, SSC = small sex chromosome and FSC = fragmented sex chromosome.

Species	***A. lanipes***		***C. nigroannulatus***		***Z. laticornis***		***M. confusa***		***R. flavicans***	
Number of Males	6		4		5		3		5	
Localities	(1) Nova Alvorada do Sul-MS		(2) Londrina-PR		(3) Assis-SP		(3) Assis-SP		(4) Borrazópolis-PR	
Coordinates	21°23.058'S, 54°23.012'W		23°18.394'S, 51°12.139'W		22°28.645'S, 50°20.983'W		22°28.645'S, 50°20.983'W		23°56.225'S, 51°35.280'W	
CN	2n = 22+XY		2n = 24+$X_1X_2X_3Y$		2n = 24+XY		2n = 12+XY		2n = 18+XY	
CP	2*n*	*n*	2*n*	*n*	2*n*	*n*	2*n*	*n*	2*n*	*n*
1	4.64 ± 0.33	2.32	2.89 ± 0.27	1.45	3.79 ± 0.62	1.90	4.95 ± 0.64	2.48	3.83 ± 0.45	1.92
2	3.76 ± 0.29	1.88	2.74 ± 0.33	1.37	3.13 ± 0.40	1.57	4.83 ± 0.53	2.42	3.09 ± 0.28	1.55
3	3.50 ± 0.27	1.75	2.58 ± 0.11	1.29	2.99 ± 0.48	1.50	4.10 ± 0.35	2.05	2.96 ± 0.17	1.48
4	3.50 ± 0.27	1.75	2.50 ± 0.00	1.25	2.88 ± 0.52	1.44	2.63 ± 0.47	1.32	2.79 ± 0.17	1.40
5	3.40 ± 0.22	1.70	2.26 ± 0.13	1.13	2.70 ± 0.35	1.35	2.63 ± 0.47	1.32	2.65 ± 0.10	1.33
6	3.30 ± 0.00	1.67	2.20 ± 0.00	1.10	2.54 ± 0.09	1.27	2.56 ± 0.35	1.28	2.60 ± 0.12	1.30
7	3.30 ± 0.00	1.65	2.14 ± 0.13	1.07	2.54 ± 0.09	1.27	2.05 ± 0.31*	1.02 LSC	2.48 ± 0.21	1.24
8	3.25 ± 0.11	1.65	2.14 ± 0.13	1.07	2.42 ± 0.22	1.21	1.95 ± 0.31*	0.97 SSC	2.40 ± 0.24	1.20
9	3.08 ± 0.25	1.63	1.96 ± 0.25	0.98	2.36 ± 0.23	1.18	-		2.33 ± 0.35	1.17
10	2.96 ± 0.26	1.54	1.96 ± 0.25	0.98	2.26 ± 0.25	1.13	-		1.80 ± 0.33*	0.9 LSC
11	2.64 ± 0.43	1.48	1.78 ±0.16	0.89	2.20 ± 0.21	1.10	-		1.75 ± 0.33*	0.87 SSC
12	3.35 ± 0.17*	1.68 SC	1.60 ± 0.00	0.80	2.08 ± 0.34	1.04	-		-	
13	3.35 ± 0.17*	1.68 SC	1.48 ± 0.18*	0.74 LSC	1.56 ± 0.58*	0.78 LSC	-		-	
14			0.60 ± 0.18*	0.30 FSC	1.32 ± 0.58*	0.66 SSC				
15			0.40 ± 0.18*	0.20 FSC						
16			0.38 ± 0.18*	0.19 FSC						

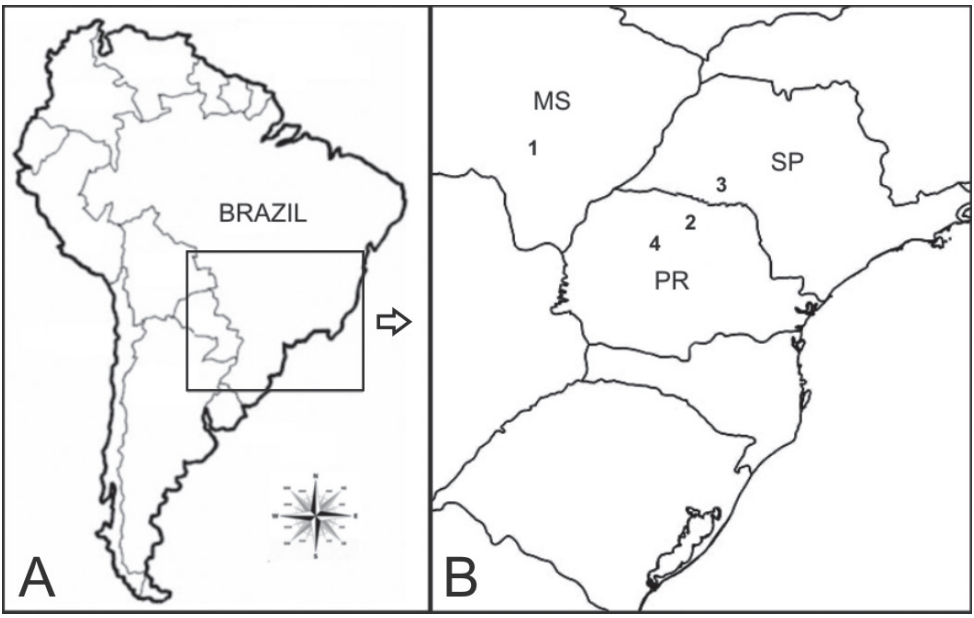

Figure 1. Maps of South America and Brazil (**A**). The section in **B** indicates the position of the states with collection points (SP: São Paulo, MS: Mato Grosso do Sul and PR: Paraná). The locations 1, 2, 3 and 4, which indicate the cities where heteropterans were collected, are specified in Table 1.

NaCl and 0.34M $Na_3C_6H_5O_7$), with 60% stringency due to heterologous hybridization. For detection, anti-digoxigenin-rhodamine in 5% BSA/4× SSC/0.2% Tween 20 (1:100, v:v) was used. The post-detection washes were performed in 4× SSC/0.2% Tween 20 at room temperature. Slides were mounted with 26 µl of DABCO solution (1,4-diaza-bicyclo (2.2.2)-octane (2.3%), 20 mM Tris-HCl, pH 8.0, (2%) and glycerol (90%) in distilled water), 2 µl of 2 µg/ml DAPI and 1 µl of 50 mM $MgCl_2$.

All chromosome images were acquired separately in grayscale mode using a Leica DM 4500 B epifluorescence microscope equipped with a very high sensitivity, 1.4 MPixel resolution, firewire interface Leica DFC300 FX camera. Pseudo coloration of blue/red colors for DAPI, greenish for CMA_3 and greenish-yellow for rhodamine were done using Leica IM50 4.0 software, as well as the overlapping of images.

Results

The chromosome numbers found for the five species of Reduviidae were 2n = 22 + XY in *A. lanipes*, 2n = 24 + $X_1X_2X_3Y$ in *C. nigroannulatus*, 2n = 24 + XY in *Z. laticornis*, 2n = 12 + XY in *M. confusa* and 2*n* = 18 + XY in *R. flavicans* (Fig. 2A–E, respectively). In *Z. laticornis* and *R. flavicans* the size of chromosomes decreased gradually (Fig. 2 and Fig. 3). In *A. lanipes,* chromosome asymmetry was due to the existence of a larger autosomal pair. *Cosmoclopius nigroannulatus* exhibited three sex chromosomes (X) with

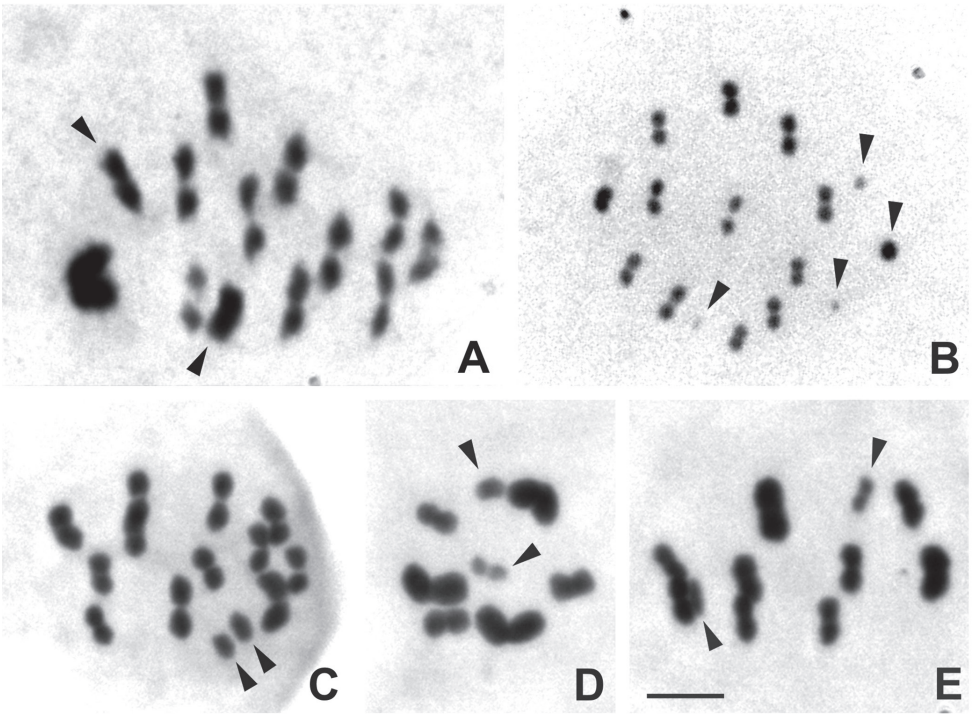

Figure 2. Conventional staining with 2% Giemsa of meiotic chromosomes of five species of Reduviidae. **A** *Apiomerus lanipes*: Metaphase I. 2*n* = 22 + XY **B** *Cosmoclopius nigroannulatus*. Metaphase I. 2n = 24 + $X_1X_2X_3Y$ **C** *Zelus laticornis* Metaphase I. 2*n* = 24 + XY **D** *Montina confusa*. Metaphase II. 2*n* = 12 + XY **E** *Repipta flavicans*. Metaphase I. 2n = 18 + XY. The arrowheads indicate the sex chromosomes. Bar = 5μm.

reduced size, and *M. confusa* showed three larger autosomal pairs (Fig. 3). In all species, the sex chromosomes were smaller of the chromosome complement; except in *A. lanipes*, where the sex chromosomes exhibited intermediate relative sizes (Fig. 3).

The predominant sex determination system was the simple XY in the species studied, except *C. nigroannulatus,* which displayed $X_1X_2X_3Y$ (Fig. 2B). The difficulty of keeping these species in captivity made it impossible to obtain eggs, and this prevented the differentiation of the sex chromosomes X and Y for the species with a simple sex chromosome system. Therefore, these chromosomes are named here generically as only "sex chromosomes". The comparison of measurements of sex chromosomes showed that $X_1X_2X_3$ of *C. nigroannulatus* were five times smaller than the sex chromosomes of *M. confusa, R. flavicans* and *Z. laticornis*, and up to ten times smaller than the sex chromosomes of *A. lanipes* (Table 1 and Figs 2–4).

Fluorescent C-chromosome banding exhibited a large variability in the occurrence of C-DAPI$^+$/CMA_3^+ bands among the five species:

A. lanipes: Only the largest autosomal pair showed terminal C-DAPI$^+$/CMA_3^+ bands (Fig. 4A–B). The heterochromatic sex chromosomes of this species exhibit different

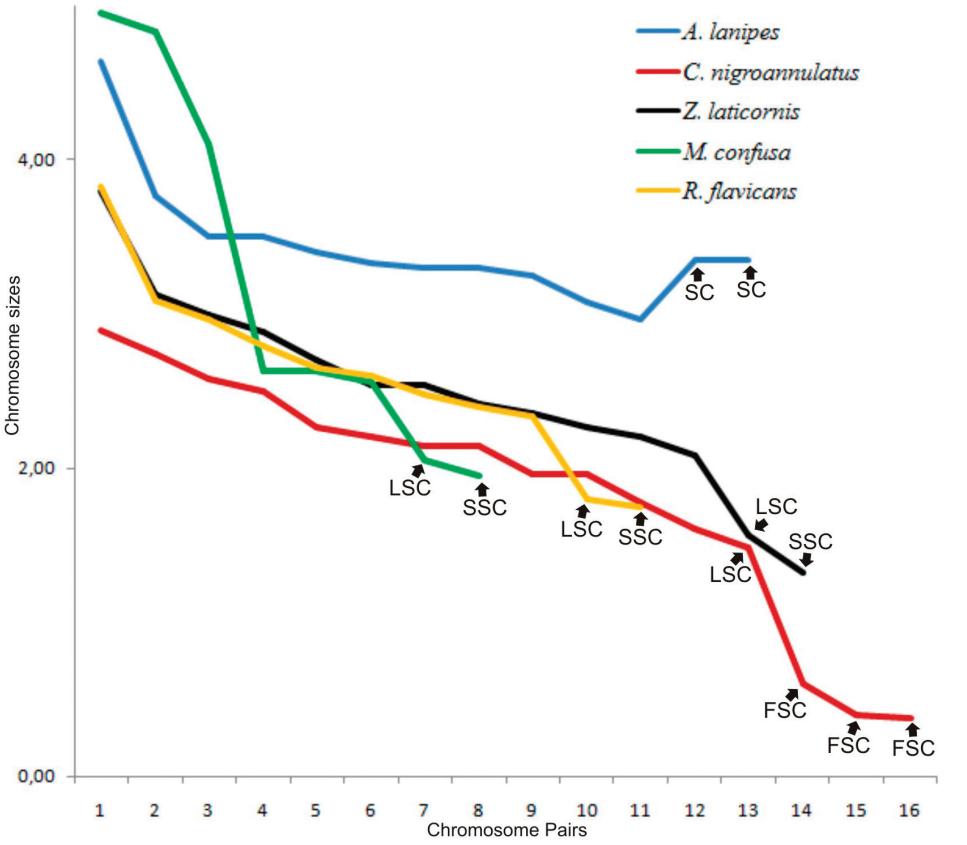

Figure 3. Graph showing the difference in karyotype in five species of Reduviidae, based on the decrease in chromosome size. SC indicates the position of the sex chromosomes of similar size, LSC points to the large sex chromosome, SSC shows the small sex chromosome, and FSC indicates the fragmented sex chromosomes. Note that *Montina confusa* displays the karyotype with a great sized variation among the five species analyzed, and *Apiomerus lanipes* is the only species with sex chromosomes of intermediate size relative to the autosomes.

fluorescent patterns (Fig. 4A–B). One sex chromosome appeared totally C-DAPI$^+$/CMA$_3^+$, and the other was totally C-DAPI$^+$ with C-CMA$_3^+$ band observed as subterminal dots (arrowheads in the Fig. 4A–B).

M. confusa: A large number of heterochromatic bands is observed: the two largest autosomes and both sex chromosomes exhibited C-DAPI$^+$/CMA$_3^+$ bands in both terminal regions. The third autosomal pair showed a C-DAPI$^+$/CMA$_3^+$ band in only one terminal region, whereas the three smaller pairs were totally C-DAPI$^+$/CMA$_3^+$ (Fig. 4D–E).

C. nigroannulatus: Autosomal complement not exhibit fluorescence banding. The Y chromosome is totally C-DAPI$^+$/CMA$_3^+$ (Fig. 4G-H),

*Z. laticornis:*only one sex chromosome was totally C-DAPI$^+$/CMA$_3^+$ (Fig. 4J–K).

R. flavicans exhibited no fluorescent bands in autosomes and sex chromosomes (Fig. 4M–N).

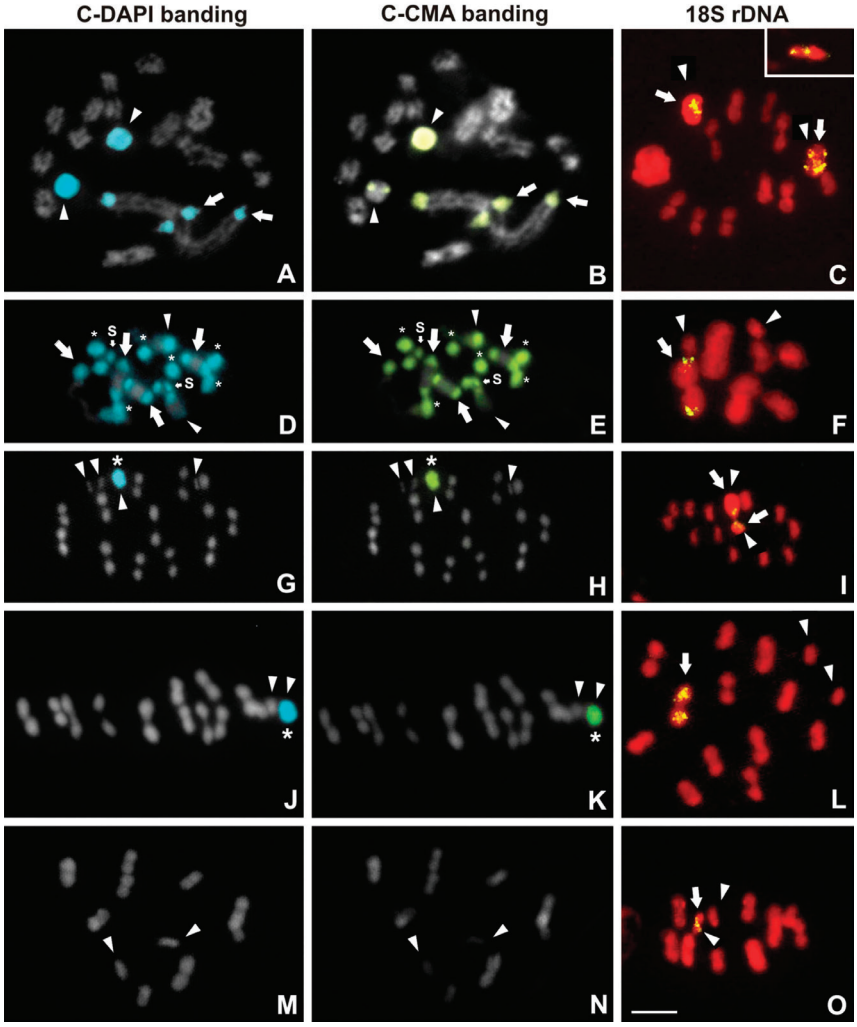

Figure 4. C-DAPI/CMA banding and FISH with 18S rDNA probe in five predatory species of Reduviidae. **A–C** *Apiomerus lanipes*. Diplotene: terminal DAPI$^+$/CMA$^+$ bands in the largest bivalent (arrows), one sex chromosomes totally DAPI$^+$/CMA$^+$ (arrowhead) and the other sex chromosome totally DAPI$^+$ (arrowhead) with a terminal CMA$^+$ dot. In metaphase I, the hybridization rDNA signals are located at terminal positions of both sex chromosomes (arrow and box - metaphase II) **D–F** *Montina confusa.* Gonial mitosis with two autosomal pairs and both sex chromosomes exhibiting DAPI$^+$/CMA$^+$ bands at both terminal regions (arrow and the word S, respectively), one autosomal pair with DAPI$^+$/CMA$^+$ band at one terminal region (arrowhead) and three autosomal pairs totally DAPI$^+$/CMA$^+$(asterisk). In metaphase **I**, ribosomal loci are located on the largest bivalent (arrow) **G–I** *Cosmoclopius nigroannulatus*. In metaphase I, the Y chromosome appear entirely DAPI$^+$/CMA$^+$ (asterisk) and hybridization signals of rDNA on two sex chromosomes in metaphase II (arrows). Note the aggregation of the three X chromosomes **J–L** *Zelus laticornis*. Metaphase I has one sex chromosome totally DAPI$^+$/CMA$^+$ (asterisk), and the rDNA sites are situated on one bivalent (arrow) **M–O** *Repipta flavicans*. Diakinesis without heterochromatic regions. The hybridization signals are located on one sex chromosome in metaphase I (arrow). Arrowheads indicate the sex chromosomes. Bar = 5μm.

FISH experiments with the 18S rDNA probe showed variation in number, location, and signal intensity. In all species the hybridization signals always appeared at terminal chromosome positions. In *A. lanipes*, both sex chromosomes showed hybridization signals (Fig. 4C). In *C. nigroannulatus,* one of the signals of 18S rDNA was located on the largest sex chromosome (Y), whereas the other ribosomal signal was observed on one of the fragmented X chromosomes (Fig. 4I), which had a CMA_3-negative signal after C-CMA banding (Fig. 4H). In *R. flavicans,* a hybridization signal was observed on one sex chromosome (Fig. 4O). In *M. confusa* (Fig. 4F) and *Z. laticornis* (Fig. 4L), hybridization signals were observed on a large autosomal bivalent.

Discussion

Species of Reduviidae show low variation in chromosome number, from 2n = 12 in the genus *Polididus* Stål, 1858 (Manna and Deb-Mallich 1981) up to 2n = 34 in the genus *Bagauda* Bergroth, 1903 (Ueshima 1979), when compared with other families of Cimicomorpha, such as Miridae (2n = 14 to 80) and Cimicidae (2n = 14 to 50) (Kuznetsova et al. 2011). Many of these chromosome variations have been associated with chromosomal rearrangements such as fusion and fragmentation (Ueshima 1979, Papeschi and Bidau 1985, Papeschi 1988, 1994, Rebagliati et al. 2001, Bressa et al. 2002, Papeschi and Bressa 2006, Poggio et al. 2007, 2009, 2014, Grozeva et al. 2010, Chirino et al. 2013, Chirino and Bressa 2014). Although these arguments have been proposed considering the occurrence of rearrangements, there is not much evidence of these changes in Heteroptera. Rearrangements are more precisely evidenced when trivalents, multivalents or robust cytogenetic markers (heterochromatin, rDNA sites or others) are noted. Samples of these events were reported for species of *Belostoma* Latreille, 1807 (Papeschi 1994, 1996) and *Triatoma infestans* (Klug, 1834) (Poggio et al. 2013b). Other examples of chromosome changes were reported in insects of the family Aradidae, where fusions were important for karyotype evolution (Jacobs 2003), as well as the dysploidy that originated the neoXneoY sex system in *Dysdercus albofasciatus* Guérin Meneville, 1831 (Bressa et al. 2009). Dysploidy is recognized as an important evolutionary mechanism for karyotype differentiation in organisms with holokinetic chromosomes, for both plants (Guerra 2008) and animals (Bardella et al. 2014a). Due to the lack of phylogenetic analyses as well as the absence of chromosome markers for most heteropterans, the evolutionary direction for certain rearrangements is very speculative, especially in heteropteran predators. However, there are sporadic examples where chromosome rearrangements can be supposed, as observed in *C. nigroannulatus*, where numerical diversity is clearly linked to the fragmentation of sex chromosomes (Papeschi 1994, 1996, Poggio et al. 2007, 2013a, 2014).

Of the five karyotypes of Reduviidae studied here, two (*R. flavicans* and *Z. laticornis*) showed a gradual decrease in size. This feature is common in Heteroptera, and it has been observed in species of different families, such as *Holhymenia rubiginosa* Breddin, 1904, Coreidae (Bressa et al. 2008) and *Edessa rufomarginata* (De Geer, 1773),

Pentatomidae (Rebagliati et al. 2003). On the other hand, the substantial dissimilarities in the autosomal size or between sex chromosomes and autosomes were marked in three of the species here analyzed. In *A. lanipes,* the presence of a greater bivalent could be associated with a reduction in their chromosome number (2n = 24), when compared with the modal number of the subfamily Harpactorinae, 2n = 26 (Poggio et al. 2007). A similar situation was observed in *Dichelops furcatus* (Fabricius, 1775), (Rebagliati et al. 2001), and in *Lygaeus alboornatus* Blanchard, 1852 (Bressa et al. 2002), in which a very large bivalent probably originated from a chromosome fusion. In *C. nigroannulatus*, as discussed above, the reduced size of three X chromosomes is due to fragmentation events, as reported by Poggio et al. (2007). The most striking case found here was the karyotype of *M. confusa*. Grozeva et al. (2006) reported more than one large chromosome in *Macrolophus costalis* Fieber, 1858 (Miridae). In heteropterans, significant variation in karyotype size may be associated not only with chromosomal rearrangements, but also with differential accumulation of heterochromatin, able to change the set size (Panzera et al. 1995, 2004, Bressa et al. 2008, Chirino et al. 2013, Bardella et al. 2014b). However, this does not seem be the case for *A. lanipes* and *M. confusa*, because if we disregard the heterochromatin, these chromosomes are still very large.

The variation in the content and distribution of heterochromatin in autosomes and sex chromosomes is well documented in heteropteran species, and occurs mainly in the terminal chromosomal regions (Grozeva and Nokkala 2002, Bressa et al. 2005, Panzera et al. 2010, Grozeva et al. 2010, Chirino et al. 2013, Suman and Kaur 2013, Poggio et al. 2014, Bardella et al. 2014a). This common feature was observed only in *M. confusa* among the predator species studied here. On the other hand, *C. cosmoclopius* and *Z. laticornis* showed heterochromatin located only in one of the sex chromosomes. The heterochromatic profile reported in *A. lanipes* is similar to that observed for *T. infestans*, but the latter displays a greater number of bivalents with terminal heterochromatic regions (Panzera et al. 1995, 2010, Bardella et al. 2014b). *T. infestans* was the best studied species of Reduviidae in relation to the distribution of heterochromatin. This species exhibits bands in terminal chromosome regions, but there is a variation in the chromosome pairs carrying bands, which is associated with the geographic distribution of each population in South America (Panzera et al. 1992, 1995, 2004, 2014). High interspecific variation in distribution of heterochromatin has also been reported for other species of Cimicomorpha (Grozeva and Nokkala 2001, Panzera et al. 2010) and Pentatomomorpha (Bardella et al. 2014a). Despite the high variability found in the content and distribution of heterochromatin, the constancy in the positioning of bands in terminal chromosome regions suggests that mechanisms of dispersion of heterochromatin could be associated with positioning of satDNA in interphase. The model of "bouquet polarization," which postulates that chromosomes can be closely associated with the nuclear envelope through their ends, could support the idea of the sharing of repetitive DNA families at terminal chromosomal regions. The "bouquet polarization" model was proposed by Rodriguez Iñigo et al. (1996) when cells in the transition interphase-prophase I of *Dociostaurus genei* (Ocskay, 1832) (Orthoptera) were studied. Among Heteroptera, the "bouquet" has been mentioned

for *Pyrrhocoris apterus* Linnaeus, 1758. (Suja et al. 2000) and *Graphosoma italicum* (O.F. Muller, 1766) (Vieira et al. 2009). Except for *Holhymenia histrio, H. rubiginosa, Macrolophus costalis* and *Spartoceras batatas* (Fabricius, 1758), which show interstitial bands on some chromosomes (Franco et al. 2006, Grozeva et al. 2006, Bressa et al. 2008, Bardella et al. 2014a), the terminal pattern of heterochromatin distribution, such as that found here in *M. confusa*, was also found in almost all species of Heteroptera. The total absence of bands, as found here in *R. flavicans,* has been seen in different families of Heteroptera: Reduviidae (Poggio et al. 2011); Belostomatidae (Papeschi and Bidau 1985, Papeschi 1994, Papeschi and Bressa 2006), Coreidae (Bressa et al. 2005, Bardella et al. 2014a), Pentatomidae and Pyrrhocoridae (Bressa et al. 2009, Bardella et al. 2014a). This suggests that the presence or not of heterochromatin may be intrinsic in each genome, regardless of the phylogenetic relationships of the species studied to date.

In heteropteran species, many C-heterochromatic bands can be AT or GC-rich, such as in *M. confusa* (Rebagliati et al. 2003, Bressa et al. 2005, Franco et al. 2006, Bardella et al. 2010, 2012, 2014a, Chirino et al. 2013). In this way, the example of *T. infestans* can be highlighted because the distinct repetitive DNA families (AT- and GC-rich) appear adjacently arranged at terminal chromosome regions (Bardella et al. 2014b). On the other hand, species with small amounts of constitutive heterochromatin generally exhibit only CG-rich bands or dots associated with the nucleolar organizer regions (NORs), as observed in *Graphosoma italicum* (González-García et al. 1996), among others (Cattani et al. 2004, Papeschi and Bressa 2006, Bardella et al. 2010, Grozeva et al. 2013, Chirino et al. 2013). Only in few species, NORs associated with AT-rich regions have been observed (Fossey and Liebenberg 1995, Bardella et al. 2010). Differently, *Z. laticornis* showed CG and AT-rich heterochromatin completely restricted to only one of the sex chromosomes without association with the NORs. Similar cases have been reported in *T. brasiliensis* Neiva, 1911 and *T. rubrovaria* Blanchard, 1834 (Bardella et al. 2010).

The FISH studies in five species of predators studied here showed a variation in number (1-3) and distribution (autosomes and/or sex chromosomes) of 18S rDNA sites. These variations are included within the range previously reported for Reduviidae (Bardella et al. 2010, Panzera et al. 2012). For this group, Poggio et al. (2011) suggested that the 18S rDNA sites are generally located at the terminal position on the X chromosome, or on both sex chromosomes in species with simple sex chromosome system (XY). However, in most cases the ribosomal loci are located at terminal position on an autosomal pair in species with multiple sex chromosomes (X_nY). However, our data on *C. nigroannulatus*, which shows fragmentation of the X chromosome, suggests an additional situation for the distribution of 18S rDNA sites, since the rDNA signals appeared on both one of the fragmented X chromosomes and Y chromosome. The presence of 18S or 45S rDNA loci in one or more sex chromosomes has also been observed in several reduviid species from the subfamilies Triatominae (Severi-Aguiar et al. 2006, Panzera et al. 2012) and Reduviinae (Poggio et al. 2013a) with multiple sex chromosome system. There is at least one example, *Dysdercus albofasciatus,* where

the original X chromosome was inserted into the NOR-autosome next to the rDNA cluster in an ancestor carrying the X0 system, resulting in a neo-sex-chromosome system (Bressa et al. 2009). We did not observe chromosomal rearrangements associated directly with the mobility of 18S rDNA sites in the reduviids. However, the variation in the chromosomal location of rDNA loci seems to be more common in reduviids from the Cimicomorpha infraorder than in the Pentatomomorpha infraorder (Panzera et al. 2012, Bardella et al. 2013, Poggio et al. 2013a). This variability indicates different evolutionary pressures for the 18S rDNA distribution in the suborder Heteroptera, as in other insect groups (Nguyen et al. 2010).

Despite the five analyzed species belong to the same subfamily (Harpactorinae) and share the predatory habit (Zhang and Weirauch 2013), we observe different evolutionary pathways in their chromosomes based on the extensive cytogenetic differences: i) great variation in chromosome number, ii) inter-chromosomal asymmetry, iii) simple and multiple sex systems, iv) different number and chromosome location of 18S rDNA loci, v) dissimilar location and quantity of autosomal C-heterochromatin, and vi) different types of repetitive DNA by fluorochrome banding. The chromosome diversity found in this study clearly shows the need for analysis of a large number of species to establish evolutionary patterns in predator reduviids.

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RESEARCH ARTICLE

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Karyotypic variation in the Andean rodent *Phyllotis xanthopygus* (Waterhouse, 1837) (Rodentia, Cricetidae, Sigmodontinae)

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Abstract

Phyllotis xanthopygus (Waterhouse, 1837) is an Andean rodent endemic to South America. Despite its wide geographical distribution in Argentina, few individuals have been studied on the cytogenetic level and only through conventional staining. In this work, chromosome characterization of Argentine samples of this species was performed using solid staining, C-banding and base-specific fluorochromes. Twenty two specimens were analyzed, collected in the provinces of Jujuy, Catamarca, and the north and south of Mendoza. All studied specimens showed 2n=38, having mostly the bi-armed autosomes, metacentric or submetacentric. Fundamental Number varied between 70 and 72. These changes were due to the presence of chromosome heteromorphisms in individuals from southern Mendoza and Jujuy. C-banding revealed pericentromeric blocks of constitutive heterochromatin in most chromosomes. Acrocentric chromosomes involved in heteromorphisms showed high variation in the amount of heterochromatin within and among populations. Additionally, banding with fluorochromes (DAPI and chromomycin A_3) revealed homologous localization of AT and GC rich regions among chromosomes of the different populations analyzed. Comparisons among heteromorphic pairs suggested, however, that the variation might be the result of complex chromosome rearrangements, involving possibly amplifications and/or deletions of heterochromatic segments. These results are in accordance with molecular studies that indicate genetic variability within and among the populations of this taxon.

Keywords
Chromosomes, constitutive heterochromatin, fluorochromes, genetic variability, mammals

Introduction

The sigmodontine rodents constitute one of the most diverse and broadly distributed Neotropical mammalian groups. Within the subfamily Sigmodontinae, the genus *Phyllotis* Waterhouse, 1837 (leaf-eared mice, or pericores) includes about 13 species and its geographic range extends from Ecuador to southern Argentina (Musser and Carleton 2005). *Phyllotis xanthopygus* has a broad distribution in Peru, Bolivia, Chile, and Argentina. Characterized as a montane species, it occupies a variety of habitats among which are grassland and desert regions (Kramer et al. 1999). It is distributed over an extensive elevation gradient ranging from high elevations in the central Andes (5000 m.a.s.l) to sea level. This distribution pattern provides an excellent natural experiment for exploring the effects of mountain topography on phylogeography and speciation (Albright 2004).

The taxonomic history of *P. xanthopygus* has been intertwined with that of *P. darwini* (Waterhouse, 1837), principally in the area of Central Chile where populations of *P. xanthopygus* were assigned to *P. darwini* at the species level (Spotorno and Walker 1983, Walker et al. 1984, Kramer et al. 1999). However, the specific recognition of *P. xanthopygus* is supported by studies based on morphometric, chromosomal and molecular differences (Spotorno and Walker 1983, Walker et al. 1984, Steppan et al. 2007). Six subspecies of *P. xanthopygus* have historically been recognized: *P. x. chilensis* Mann, 1945, *P. x. posticalis* Thomas, 1912, *P. x. ricardulus* Thomas, 1919, *P. x. rupestris* (Gervais, 1841), *P. x. vaccarum* Thomas, 1912 and *P. x. xanthopygus* (Waterhouse, 1837), which have typically been described by morphological traits (Steppan 1993). The species *P. limatus* Thomas, 1912 and *P. bonariensis* Crespo, 1964 are embedded in a *P. xanthopygus* complex within the *P. darwini* species group (Steppan et al. 2007). Data from phylogenetic analysis of both mitochondrial and nuclear DNA support that the *P. xanthopygus* complex is characterized by deep divergences and high genetic diversity (Kim et al. 1998, Steppan et al. 2007).

The genus *Phyllotis* has a high degree of karyotypic diversification. The diploid number shows variations from maximal 2n=68 in *Phyllotis osilae* Allen, 1901 to minimal 2n=38 shared by several species of the genus. The chromosome complement of *P. xanthopygus* is 2n=38 with all chromosomes biarmed (Pearson and Patton 1976, Spotorno et al. 2001). However, some departures from such a "common" karyotype have been reported. Pearson and Patton (1976) using routine solid staining described a karyotype with a single acrocentric element in two specimens of *P. xanthopygus* from the Central Andes. This was interpreted as a possible pericentric inversion. These changes represent one of the most frequent chromosome rearrangements and, consequently, a very common source of karyotypic variation in rodents (Patton and Sherwood 1983).

Constitutive heterochromatin (CH) is a feature that is often variable among *Phyllotis* and other rodents (Walker et al. 1991). Studies of related *Phyllotis* species have shown substantial differences in amount of CH. Comparative C-banding studies performed in

three subspecies of *P. xanthopygus* from Chile showed intra and interspecific CH variation. An important difference was found between *P. xanthopygus xanthopygus*, which exhibits most autosomes with very tiny pericentromeric C-bands, and *P. x. rupestris* and *P. x. vaccarum*, with large pericentromeric C-bands on all their autosomes. On the other hand, the G-banding patterns of these subspecies were similar, with the exception of the sex chromosomes from *P. x. xanthopygus* (Walker et al. 1984, 1991). Additionally, patterns of fluorescent bands, which identify sequences rich in AT and GC base pairs, are very informative and have been useful for determining chromosome homologies comparable to G- and R-banding respectively. Moreover, they provide information about the distribution of these sequences within the genome (Veyrunes et al. 2007).

Studies of banding patterns are important to establish karyotype homology and specify the chromosome rearrangements accompanying processes of taxonomic diversity and karyotype evolution in a taxon. Differential chromosome banding in *Phyllotis* species has been published only for Chilean specimens (Walker et al. 1991). Previous cytogenetic studies on Argentine populations of *P. xanthopygus* are very scarce and used routine techniques only (Pearson and Patton 1976, Albright 2004). We are presenting here a wider intraspecies spectrum of data on the karyotype of this species which will allow us to assess the distribution of chromosomal variation in *P. xanthopygus* along provinces of Argentina.

Material and methods

For chromosome study, 22 individuals of *Phyllotis xanthopygus* were collected across the Puna and Monte desert biomes. Fig. 1 shows north-south distribution of 7 of 9 listed below collection sites, with couples of neighboring localities being united under the numbers 4 and 6, from 3 provinces of western Argentina. Geographic data and number of cytogenetically studied individuals (N) are as follows: Jujuy province (sites 1–3): Loma Blanca (N=2), 22°26'30.25"S; 66°26'28.57"W, Abra Pampa (N= 1), 22°46'4.80"S; 65°42'10.08"W, Susques (N=1), 23°23'8.88"S; 66°32'15.72"W. Catamarca province (site 4): Cortaderas (N=3), 27°35'3.84"S; 68°8'57.12"W and Pastos Largos (N=1), 27°40'8.40"S; 68°9'36.00"W. Mendoza province (sites 5–7): Uspallata (N=1), 32°46'30.01"S; 69°36'14.39"W, Las Heras (N=2), 32°49'12.00"S; 69°65'52.79"W and Quebrada del Toro (N=1), 32°31'12.00"S; 69°0'36.01"W, Malargüe (N=10), 36°4'26.40”S; 69°32'2.40"W.

Animals were captured using Sherman traps and the voucher specimens are housed in the mammal collection of the Instituto Argentino de Zonas Áridas (CMI – IADIZA), CCT-Mendoza, CONICET. Catalog numbers of studied specimens correspond to the Colección Mastozoológica del IADIZA: CMI. Provincia de Jujuy: Localidad Loma Blanca (07508, 07509); Abra Pampa (006998); Susques (006999). Provincia de Catamarca: Localidad Cortaderas (007132, 007134, 007177); Pastos Largos (007186). Provincia de Mendoza: Localidad Uspallata (007395), Las Heras (007391, 007398); Quebrada del Toro (006797); Malargüe (006794, 006792, 006791, 006790, 007400, 07505, 07506, 07422, 07421, 07507).

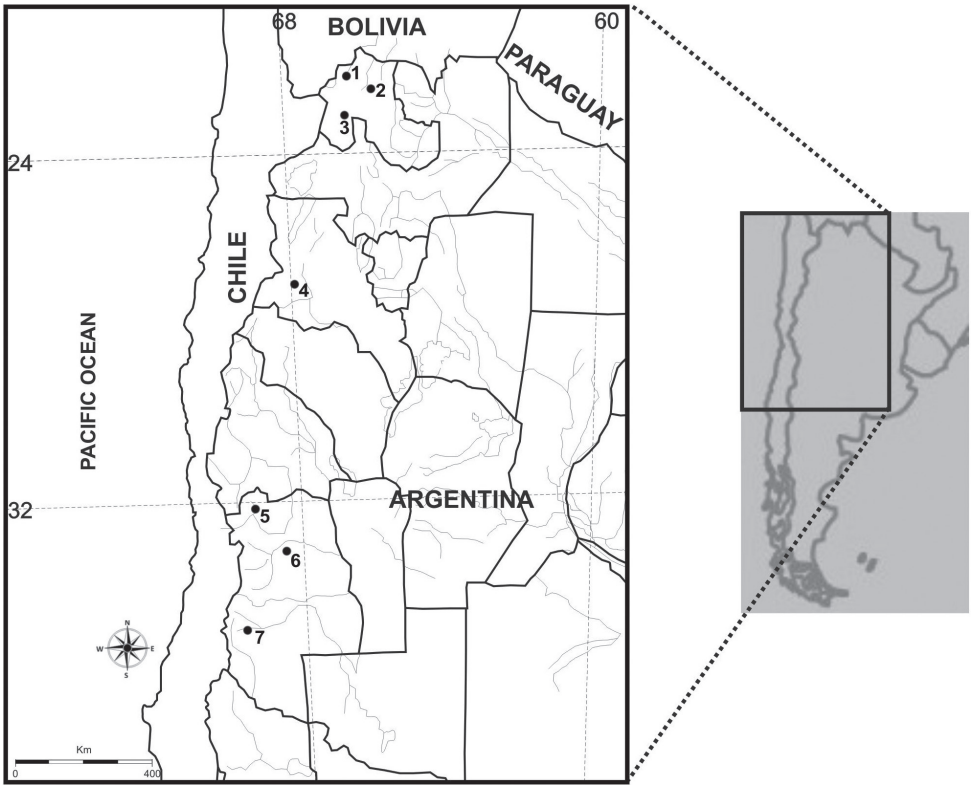

Figure 1. Map showing collection sites of *Phyllotis xanthopygus* in 3 provinces of Argentina: Jujuy, **1**) Loma Blanca, **2**) Abra Pampa, **3**) Susques; Catamarca, **4**) Cortaderas and Pastos Largos; Mendoza, **5**) Uspallata, **6**) Las Heras and Quebrada del Toro, **7**) Malargue. Localities **5** and **6** correspond to the north and **7** to the south of Mendoza province, respectively.

Mitotic chromosome preparations were obtained from bone marrow using the traditional cell suspension technique (Ford and Hamerton 1956). Chromosomes were stained with Giemsa (pH=6.8). Ten metaphase spreads were counted for each specimen. The distribution of constitutive heterochromatin (C-bands) was determined according to the Sumner's (1972) method. The technique of Schweizer (1978, 1980) was used for CMA_3/DAPI staining. Photomicrographs were obtained using an Olympus BX 50 photomicroscope, with a Sony Exwave Had digital camera.

Results

Solid staining

Karyotypes of all individuals of *Phyllotis xanthopygus* analyzed had 2n=38, with 18 autosomal pairs which can be arranged by decreasing size, and a pair of XY sex chromosomes.

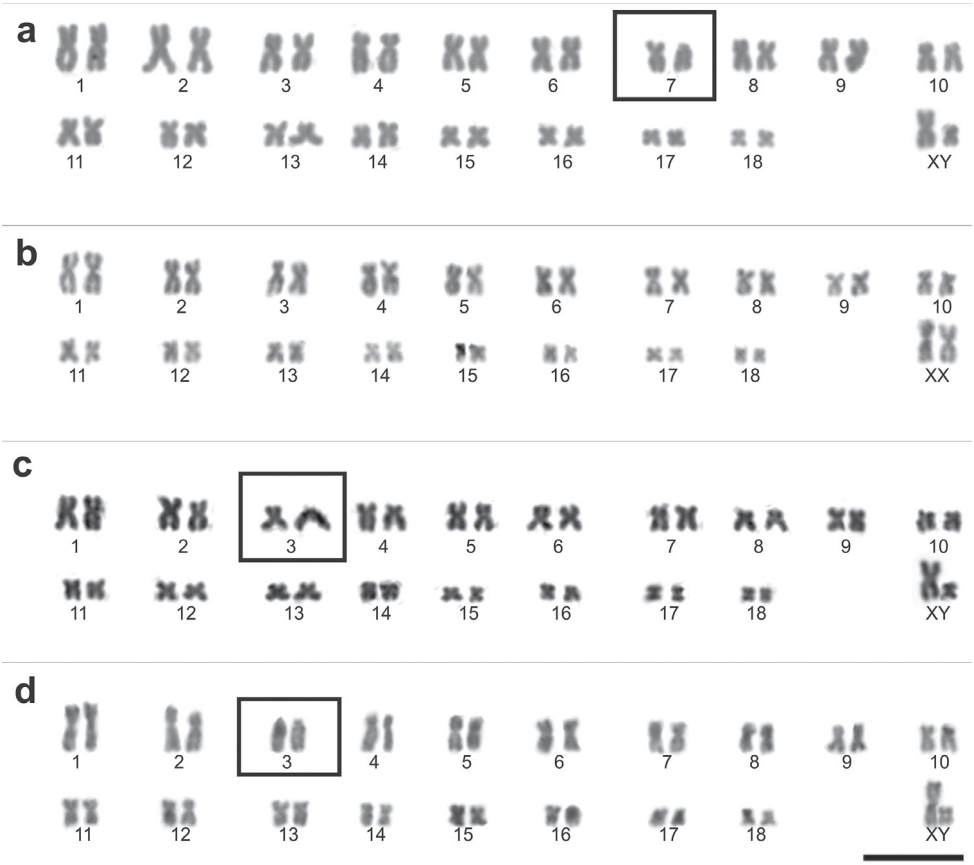

Figure 2. Karyotype variation in *Phyllotis xanthopygus*, 2n=38, from provinces: Mendoza, (**a** site 7), FNa=71; Catamarca (**b** site 4), FNa=72; Jujuy (**c** sites 1 and 3) FNa=71 and (**d** sites 1 and 2) FNa=70. Chromosome heteromorphisms are in boxes. Routine Giemsa staining. XX, XY – sex chromosomes. Bar = 10 μm.

Most of the autosomal complement was characterized by meta-submetacentric chromosomes. But, due to the presence of chromosome heteromorphisms, the fundamental number of autosomal arms (FNa) varied between 70 and 72. The X chromosome is one of the largest elements and the Y chromosome one of the smallest, both metacentric.

In Malargüe, southern Mendoza province, two different karyotypes were observed. Four individuals (two females and two males) had FNa=72 with all chromosomes biarmed, and another six specimens (two females and four males) had FNa=71 with one heteromorphic pair (number 7 when arranged by size) composed of one acrocentric and one submetacentric chromosome (Fig. 2a). Individuals from northern Mendoza (four males) and Catamarca (three females and one male) provinces showed karyotypes composed entirely of biarmed chromosomes, with FNa=72 (Fig. 2b). In Jujuy province, two individuals (two males) had FNa=71, with one heteromorphic pair (number 3 when arranged by size) composed of one acrocentric and one submetacentric chromosome (Fig. 2c). The remaining two specimens (one

female and one male) had FNa=70, with two acrocrocentric chromosomes (pair 3). In one of these acrocentric chromosomes, it was possible to distinguish a small chromosome arm (Fig. 2d).

C-banding

In the specimens studied, positive C-bands were observed in the pericentromeric regions of all chromosomes (Fig.3). Additionally, very small telomeric C-bands can be detected in some autosomes (Fig. 3d). The X chromosome was indistinguishable from the autosomal complement with respect to the amount of CH, while the Y chromosome was completely C-positive (Fig. 3b).

Different amounts of CH were observed in the acrocentric chromosome of individuals from the south and north of the country. In Malargüe (south of Mendoza province), the acrocentric chromosome was almost completely heterochromatic (Fig. 3a, d). In the north, in Jujuy, we observed two heterochromatic variants of the acrocentrics. One had very tiny pericentromeric C-bands (Fig. 3b) and the other one had a large C-band around the centromere. Specimens with only one acrocentric had the variant with small CH (Fig. 3b), while those with two acrocentrics carried both variants (Fig. 3c).

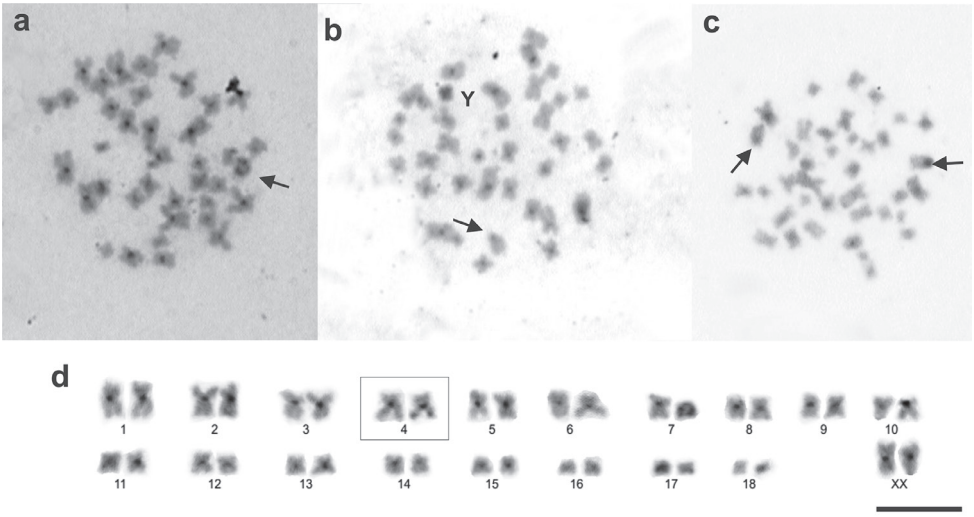

Figure 3. C-banding pattern in metaphase chromosomes of *Phyllotis xanthopygus*. **a** Female from Malargüe, the presence a large block of heterochromatin in acrocentric chromosome (arrow) **b** Small pericentromeric C-band in an acrocentric autosome (arrow), and the entirely heterochromatic Y chromosome, Jujuy, male **c** Small C-band in one acrocentric and a prominent pericentromeric C-heterochromatin in the second acrocentric, Jujuy, female (arrows) **d** C-banded karyotype, with small telomeric C-bands in one autosome pair (in box) and the heteromorphic metacentric/acrocentric pair No.7. Bar = 10 μm.

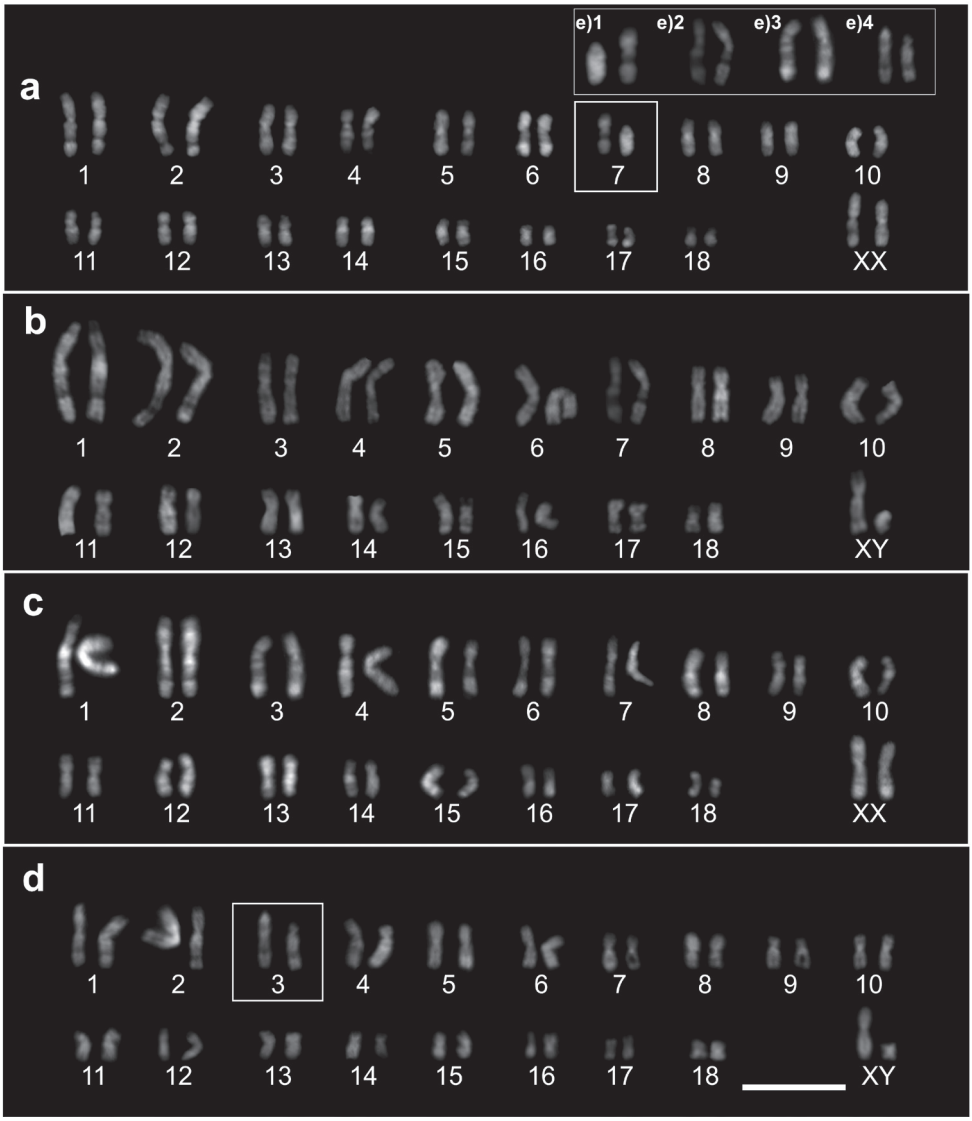

Figure 4. The DAPI staining of chromosomes of *P. xanthopygus* revealing: **a** Heteromorphic pair 7, female from Malargüe, the south of Mendoza **b** Homozygous metacentrics of male from the north of Mendoza **c** Homozygous metacentrics of female from Catamarca Province **d** Heterozygous pair 3 in male from Jujuy. In boxes are the heteromorphic pairs **e** Details of pairs involved in the chromosome polymorphisms described in this work: **e)1** pair 7 from south Mendoza population, **e)2** pair 7 from north of Mendoza, **e)3** pair 3 from Catamarca, **e)4** pair 3 from Jujuy, the size of chromosomes was modified for a better comparison of DAPI bands. Bar = 10 μm.

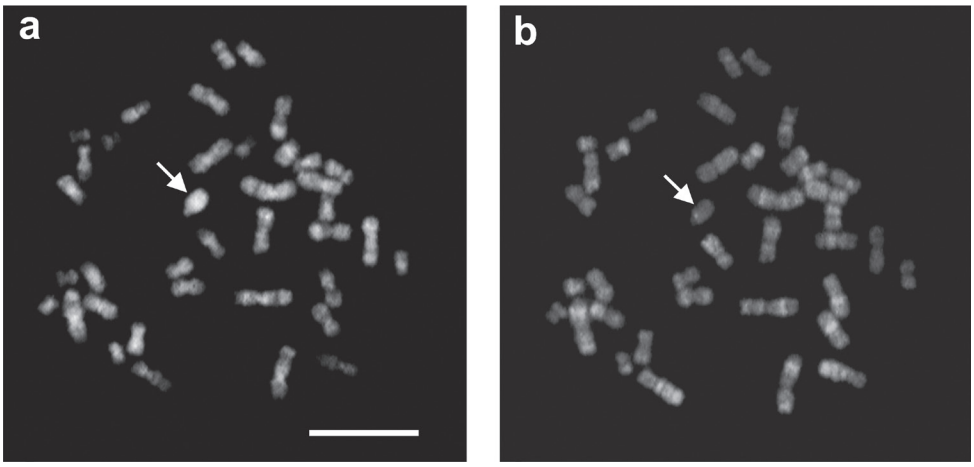

Figure 5. An acrocentric of a heteromorphic pair No. 7 from a metaphase plate treated with DAPI (**a**) and CMA_3 (**b**). Bar = 10 μm.

Fluorochromes

The DAPI bands of *P. xanthopygus* revealed similar localization among the karyotypes of specimens from different geographic regions (Fig. 4a–d). We found homology in most autosomal pairs. However, pairs such as 12, 14 and 15, have not been seen completely homologous particularly in karyotypes from Jujuy. This can be due to differences in chromosome condensation, or to small chromosome rearrangement not detected with the cytogenetic techniques used in this work. The pericentromeric regions of nearly all chromosomes appeared positive with DAPI and negative or neutral with CMA_3 (Fig. 5).

On the other hand, within the heteromorphic pair 7 from Malargüe, the acrocentric chromosome presented a large DAPI-positive/CMA_3-neutral block, which covered almost the entire chromosome length, and a small DAPI-negative/CMA_3-positive block in the pericentromeric region (Fig. 5). The biarmed chromosome of this pair showed DAPI-negative blocks in the interstitial region of the long arm (Fig. 4). We found great homology between the biarmed chromosomes of pair 7 from southern (Malargüe) and northern Mendoza (Fig. 4). Among the chromosomes of specimens from Catamarca, pair 3 appears to be homologous to the biarmed chromosome of the heteromorphic pair 7 from Malargüe (Fig. 4). In Jujuy, the acrocentric homologs of pair 3 contained an interstitial DAPI-negative/CMA_3-neutral block in their long arm. Besides, while one acrocentric chromosome showed a DAPI-positive band in the centromeric region, this band was absent in the other one. Therefore, the homology between both acrocentric chromosomes is only partial (Fig. 4).

With respect to sex chromosomes, the X chromosome revealed the pericentromeric region as DAPI positive/CMA_3-neutral. Additionally, a large DAPI-positive/CMA_3-neutral band was evidenced in the telomeric region of its long arm. The Y chromosome was found to be almost completely fluorescent with DAPI staining in all specimens analyzed (Fig.4).

Discussion

Although *Phyllotis xanthopygus* is one of the widely distributed rodents across the countries situated along the Andean landscapes, the range of the karyotype variation in this species is rather partially known. In our work, samples studied in Argentina add new cytogenetic information on this species. The chromosome complement of 2n=38 is found all over its distribution area, including the territories of the anew involved in chromosome examination provinces of Jujuy, Catamarca and Mendoza by western boundary of the country. It is confirmed that the species karyotype is composed almost exclusively of biarmed-metacentric and submetacentric-chromosomes that corresponds to previous reports for this taxon and for some other related species (Pearson and Patton 1976, Walker et al. 1991, 1999, Kramer et al. 1999).

Studies of chromosome homologies in Argentine specimens of *P. xanthopygus* have not yet been performed and might be of interest due to the commonly expected cytogenetic input in the establishment of taxonomic identity and chromosome relations of geographic populations. The chromosome banding pattern obtained in this work using DAPI staining is largely comparable to the G-band pattern published for the three subspecies *P. x. vaccarum*, *P. x. rupestris* and *P. x. xanthopygus* (Walker et al. 1991). G-banding patterns were similar among the chromosomes of these three subspecies of *P. xanthopygus* analyzed by Walker et al. (1991), with the exception of the sex chromosome from *P. x. xanthopygus*. The G-banding pattern for the X chromosome in the latter subspecies had correspondence to that observed for the same chromosome in our work with DAPI banding. These results indicate that our specimens are chromosomally more similar to *P. x. xanthopygus* than to any other subspecies, at least in euchromatic regions.

The specific separation of *P. darwini* and *P. xanthopygus* is well supported (Spotorno and Walker 1983, Walker et al. 1984). Accordingly, when we compared the pattern obtained for our DAPI-stained sample with the G-banding pattern of *P. darwini*, we observed chromosomal differentiation in several chromosome pairs, similar to that previously described by Walker et al. (1984).

Constitutive heterochromatin (CH) is a feature that is often variable among the karyotypes of mammals showing different patterns in members of the genus *Phyllotis* as in different taxa of mammals (Walker et al. 1991, Graphodatsky et al. 2011). The pericentromeric location of CH is a predominant characteristic in *Phyllotis* and in other rodents (Patton and Sherwood 1983). However, variations in the amount of CH have been demonstrated for the subspecies of *P. xanthopygus*. The pattern of CH obtained in this study is not completely consistent with those described for the subspecies of *P. xanthopygus* analyzed in other studies. Most autosomes of *P. x. xanthopygus* exhibited very tiny pericentromeric C-bands or a few small ones, with exception of pair 15, which showed a larger one. On the other hand, *P. x. rupestris* and *P. x. vaccarum* presented large pericentromeric C-bands in all autosomes (Walker et al. 1984, 1991, 1999).

The Y chromosome was completely C-positive in all populations analyzed. The same pattern was identified in the subspecies *P. x. rupestris* and in *Phyllotis darwini*.

However, as mentioned above, the autosomal C-band pattern in *P. x. rupestris* subspecies does not correspond to those obtained in this study. In subspecies *P. x. vaccarum* and *P. x. xanthopygus* the whole Y chromosome was faintly heterochromatic (Walker et al. 1991).

Despite the uniformity in most chromosomes of the complement, we found intra and inter-population variations, which resulted in modifications of the FNa from 70 to 72. In Malargüe we observed high frequency of individuals with a heteromorphic pair (FNa=71). Also in Jujuy province we observed chromosome heteromorphisms. In this last region, Pearson and Patton (1976) described specimens with a single acrocentric chromosome. In our sample from Jujuy, we also observed specimens with two different acrocentric chromosomes (FNa=70). In the north of Mendoza and in Catamarca we found no acrocentric chromosomes, but this could be because of the small size of the samples from these geographically intermediate areas (Fig. 1).

Additionally, we found differences in the quantity and distribution of CH when comparing the acrocentric chromosomes within and among localities. In Malargüe, this chromosome is almost completely heterochromatic. The absence of homozygous individuals with acrocentric chromosomes in this locality could be due small sample size. Alternatively this chromosomal condition could be negatively heterotic, since large additions of heterochromatin are probability related to loss of gene function and genetic degeneration (John 1988, King 1993, Waters et al. 2007).

A geographic variation of heterochromatin is shown in this work. In Jujuy province, in the north of the country, acrocentric chromosomes showed much less amount of CH than in the south in Malargüe. At the same time, two different acrocentric chromosomes varying in morphology and in the amount of heterochromatin were detected in Jujuy specimens. It can be suggested that this variation in amount of CH is due to a gradual process of heterochromatin addition or deletion in these chromosomes. But additional evidence in the sequences involved is necessary to confirm this hypothesis.

Application of fluorochromes also allowed us to study the possible structural rearrangements that generated the south to north variation in FNa. The acrocentric chromosome from Malargüe showed no homology with any other chromosome of complement, but the biarmed chromosome of the pair showed high homology with pair 7 from northern Mendoza and with pair 3 from Catamarca and Jujuy. In addition, the two different acrocentric chromosomes of pair 3 from Jujuy showed partial homology via fluorochromes (Fig. 4). These results can only be explained by a complex sequence of rearrangements, possibly involving amplifications and/or deletions of heterochromatic segments. Karyotype variation in the amount of heterochromatin within and among populations are common in some rodents species as *Mus musculus*, *Perognathus baileyi*, etc. (Patton and Sherwood 1983, Graphodatsky et al. 2011).

The role of chromosomal changes in the differentiation of populations and speciation has been the subject of continued interest and controversy (Patton and Sherwood 1983, Faria and Navarro 2010, Romanenko and Volobouev 2012, Romanenko et al. 2012). Cytogenetic data showed moderate chromosome variability and differentiation within and between populations. These results are consistent with previous works on

other Sigmodontinae species that also show great chromosomal variability at intra- and inter-population level (Patton and Sherwood 1983, Lanzone et al. 2011). However, considerable additional data will be required to clarify the taxonomic status of *P. xanthopygus* and its subspecies, as well as to understand the evolutionary process that generates this diversity.

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