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Repetitive sequences: the hidden diversity of heterochromatin in prochilodontid fish

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Abstract

The structure and organization of repetitive elements in fish genomes are still relatively poorly understood, although most of these elements are believed to be located in heterochromatic regions. Repetitive elements are considered essential in evolutionary processes as hotspots for mutations and chromosomal rearrangements, among other functions – thus providing new genomic alternatives and regulatory sites for gene expression. The present study sought to characterize repetitive DNA sequences in the genomes of *Semaprochilodus insignis* (Jardine & Schomburgk, 1841) and *Semaprochilodus taeniurus* (Valenciennes, 1817) and identify regions of conserved syntenic blocks in this genome fraction of three species of Prochilodontidae (*S. insignis*, *S. taeniurus*, and *Prochilodus lineatus* (Valenciennes, 1836) by cross-FISH using *Cot-1* DNA (renaturation kinetics) probes. We found that the repetitive fractions of the genomes of *S. insignis*

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and *S. taeniurus* have significant amounts of conserved syntenic blocks in hybridization sites, but with low degrees of similarity between them and the genome of *P. lineatus*, especially in relation to B chromosomes. The cloning and sequencing of the repetitive genomic elements of *S. insignis* and *S. taeniurus* using *Cot-1* DNA identified 48 fragments that displayed high similarity with repetitive sequences deposited in public DNA databases and classified as microsatellites, transposons, and retrotransposons. The repetitive fractions of the *S. insignis* and *S. taeniurus* genomes exhibited high degrees of conserved syntenic blocks in terms of both the structures and locations of hybridization sites, but a low degree of similarity with the syntenic blocks of the *P. lineatus* genome. Future comparative analyses of other prochilodontidae species will be needed to advance our understanding of the organization and evolution of the genomes in this group of fish.

Keywords
Chromosomal painting, Fish, microsatellites, repetitive sequences, sex chromosome, transposable elements

Introduction

Multiple copies of DNA sequences, known as "repetitive DNA", compose large portions of eukaryotic genomes. Repetitive DNA is generally divided into two groups: (1) tandem repeats, which include DNA satellites, minisatellites, and microsatellites; and (2) dispersed interspersed repeats composed of transposable elements (TEs) (Timberlake 1978, Charlesworth 1994, Jurka 2005), but there are other gene families with sequence repetitions also known as repetitive DNA, such as the genes encoding for ribosomal RNA (rRNA) (Long 1980). While the structure and organization of this genome fraction is still poorly understood in fish, most of these non-coding repetitive sequences appear to be located in heterochromatic regions (Fishcher et al. 2004, Martins et al. 2011).

The repetitive sequences were largely considered to be "junk", "selfish", or "parasitic" DNA (Doolittle and Sapienza 1980, Orgel and Crick 1980, Nowak 1994) due to the lack of any known functions in the genome for these sequences. With ever-increasing volumes of genomic information, however, these repetitive sequences are now known to play larger roles in the structural and functional evolution of the genome (Shapiro and Vonsternberg 2005, Biémont and Vieira 2006). Indeed, repetitive sequences are now known to be involved in chromosomal rearrangements and responsible for significant proportions of the karyotypic variations observed in many groups (Kidwell 2002, Schneider et al. 2013).

In Prochilodontidae, centromeric heterochromatin regions have been observed in all 54 chromosomes in all of the species analyzed, as well as in the B chromosomes of *Prochilodus lineatus* (Valenciennes, 1836) (Pauls and Bertollo 1980, Oliveira et al. 2003, Hatanaka et al. 2002, Terencio et al. 2012). However, *Semaprochilodus insignis* (Jardine & Schomburgk, 1841) has additional heterochromatic blocks in the terminal regions of the first metacentric pair, while *Semaprochilodus taeniurus* (Valenciennes, 1817) has large bitelomeric markings in metacentric pairs 2 and 3.

The ZZ/ZW sex chromosome system may have originated through an *in cis* process of heterochromatin accumulation that differentiated into the W chromosome – with consequent recombination restrictions starting with the first chromosome pair (Terencio et al. 2012a).

The phylogenetic biogeography of the Prochilodontidae indicates that the family dates back minimally to approximately 12 million years ago, with higher level intrafamilial cladogenic events also dating to at least that time period; these dates are congruent with data from the fossil record for more encompassing groups within the Characiformes (Sivasundar and Bermingham 2001, Castro and Vari 2004). Phylogenies constructed based on morphological (information from osteological and soft anatomical systems) and molecular (ATPase, D-loop, ND4 and COI) characters demonstrates that *Prochilodus* is the sister group to the clade formed by *Ichthyoelephas* plus *Semaprochilodus* (Turner et al. 2004). It is believed that heterochromatic regions play important roles in the differentiation of this fish group, despite the relatively stable karyotypic macrostructures of the Prochilodontidae. The genetic composition of these regions is still only poorly understood, however, and the only firm information available concerns the presence of large amounts of repetitive DNA sequences in the B chromosomes of *P. lineatus* (Camacho and Beukeboom 2000) and in the W sex chromosome of *S. taeniurus* (Terencio et al. 2012b). These sequences were identified and classified as microsatellites, transposons, and retrotransposons in the latter species.

Heterochromatic regions are essential to evolutionary processes because of their ability to propagate and influence genes (Grewal and Jia 2007), and the present study therefore sought to characterize the moderate to highly repetitive DNA sequences in *S. insignis* and *S. taeniurus* by cloning and sequencing them and identifying conserved syntenic blocks of this fraction in three species of the family Prochilodontidae (*S. insignis*, *S. taeniurus*, and *P. lineatus*) using cross-FISH techniques with *Cot*-1 DNA probes.

Materials and methods

Ten specimens of *S. insignis* (six females and four males) and 12 *S. taeniurus* (seven females and five males) were examined cytogenetically. These fish were captured with the authorization of ICMBio SISBIO 10609-1/2007 at the confluence of the Negro and Solimões Rivers (AM) and at the Amazonas and Tapajós (PA) Rivers. Five *P. lineatus* (two females and three males) were captured from the Tibagi River (PR). The fish were anesthetized in ice-cold water and were sacrificed. Voucher specimens were deposited in the INPA Animal Genetics Laboratory fish collection (10034, 10037, 10047 and 10696). Chromosome preparations were obtained from anterior kidney cells using an *in vivo* colchicine treatment (Bertollo et al. 1978). Institutional abbrevoations: UFAM, Federal University of Amazonas; INPA, National Institute of Amazonian Research; UEPG, State University of Ponta Grossa.

Isolation of repetitive DNA via re-association kinetics

Enriched samples containing repetitive DNA sequences from *S. insignis* and *S. taeniurus* were constructed based on the renaturation kinetics of *Cot-1* DNA (DNA enriched for highly and moderately repetitive DNA sequences) according to the protocol described by Zwick et al. 2010) and recently adapted by Ferreira and Martins (2008). DNA samples (50 µl of 100-500 ng/µl of DNA in 0.3 M NaCl) were autoclaved (121 °C) for 5 minutes (min) to obtain fragments ranging from 100 to 2000 base pairs. Next, the DNA was denatured at 95 °C for 10 min, placed on ice for 10 seconds (s) and subsequently placed at 65 °C for 1 min for re-annealing. The samples were incubated at 37 °C for 8 min with 1 U of S1 nuclease to permit the digestion of single-stranded DNA. The repetitive portion of this DNA was recovered by freezing in liquid nitrogen, and the DNA was extracted using phenol-chloroform. The resulting DNA fragments were used as probes for fluorescence *in situ* hybridization, cloned and sequenced.

Fluorescence *in situ* hybridization (FISH)

The repetitive *S. taeniurus* and *S. insignis* sequence probes isolated using *Cot-1* DNA were labeled with digoxigenin-11-dUTP and biotin-16-dUTP (Dig-Nick Translation mix and Biotin-Nick Translation mix; Roche), respectively, by nick translation reactions following the manufacturer's instructions. Two antibodies, namely, anti-digoxigenin-rhodamine and streptavidin (Life Technologies), were used for signal detection. Fluorescence *in situ* hybridization (FISH) was performed on mitotic chromosome spreads (Pinkel et al. 1986). Homologous and heterologous *in situ* fluorescent hybridizations were performed using 77% stringency (2.5 ng/µl of DNA, 50% deionized formamide, 10% dextran sulfate and 2 × SSC at 37 °C for 18 hours). The chromosomes were counterstained with DAPI (2 µg/ml) in Vectashield mounting medium (Vector).

Microscopy/Image Processing

Hybridized chromosomes were analyzed using an Olympus BX51 epifluorescence microscope, and the images were captured with a digital camera (Olympus DP71) using the Image-Pro MC 6.3 software.

Cloning and sequencing of repetitive sequence

One microgram of the *Cot-1* DNA products was cloned using a pMOS Blunt-ended PCR Cloning Kit (GE Healthcare), purified using the GFX PCR Purification Kit (GE

Healthcare) and sequenced using the Big Dye Kit (Applied Biosystems) in an ABI 3130 genetic analyzer. Sequence alignment was performed using Clustal W (Thompson et al. 1994), which is included in the BioEdit 7.0 software program (Hall 1999). Each clone was used as a query in BLASTN (Basic Local Alignment Search Tool nucleotide) searches against the NCBI nucleotide collection (http://www.ncbi.nlm.nih.gov) and in searches against the Repbase database (Jurka et al. 2005) at the Genetic Information Research Institute (Giri) (http://www.girinst.org/repbase/) using CENSOR software (Kohany et al. 2006).

Results

Hybridization of the *S. insignis Cot-1* DNA probe to its own chromosomes demonstrated that the repetitive elements of its genome were located in the centromeric regions of all chromosomes, as well as in the terminal region of several chromosomes (Fig. 1a and b). The cross-hybridization of the *S. taeniurus Cot-1* DNA probe to the chromosomes of *S. insignis* revealed markers in the centromeric region, although they were smaller than those observed using species-specific probes (Fig. 1c). Additionally, no terminal markers were observed in *S. insignis* using the heterologous probe, indicating that this species has chromosome pairs (Fig. 1d, arrowheads) that carry species-specific repetitive sequences not shared with *S. taeniurus* (Fig. 1a, b, c and d).

Hybridization of the *S. taeniurus Cot-1* DNA probe to its own chromosomes likewise revealed that repetitive sequences were abundant in the genome of this species and located in various regions (e.g., centromeric, interstitial, and terminal) of the entire chromosome complement (Fig. 1e and g). Cross-FISH reactions were performed using the *S. insignis Cot-1* DNA probe and demonstrated the presence of conserved syntenic blocks in several chromosomal regions (Fig. 1f). *S. taeniurus* also displayed species-specific repetitive DNA sites located in the centromeric and terminal regions of 14 chromosomes (Fig. 1h, arrows), with no observed hybridizations of the *S. insignis Cot-1* DNA probe to these same regions (Fig. 1e, f, g, and h).

Both *Cot-1* DNA probes of *S. insignis* and *S. taeniurus* displayed positive hybridization signals in the terminal regions of the entire complement of *P. lineatus* chromosomes. The supernumerary (i.e., B) chromosomes (Fig. l, arrowheads) revealed hybridization signals only with the *S. taeniurus Cot-1* DNA probe. The same marker pattern seen on one of the B chromosomes was also observed on the autosomal chromosomes, while only one of the chromosome arms exhibited hybridization signals shared with the other B chromosome (Fig. 1 i, j, k and l).

Cloning and sequencing the repetitive genome elements obtained from *S. insignis* and *S. taeniurus Cot-1* DNA identified 48 DNA fragments of varying sizes (GenBank: JX848379–JX848393). 71% of repetitive DNA diversity sampled (*Cot*-1 DNA) of *S. insignis* displayed high similarity to microsatellites, 17% to DNA transposons, and

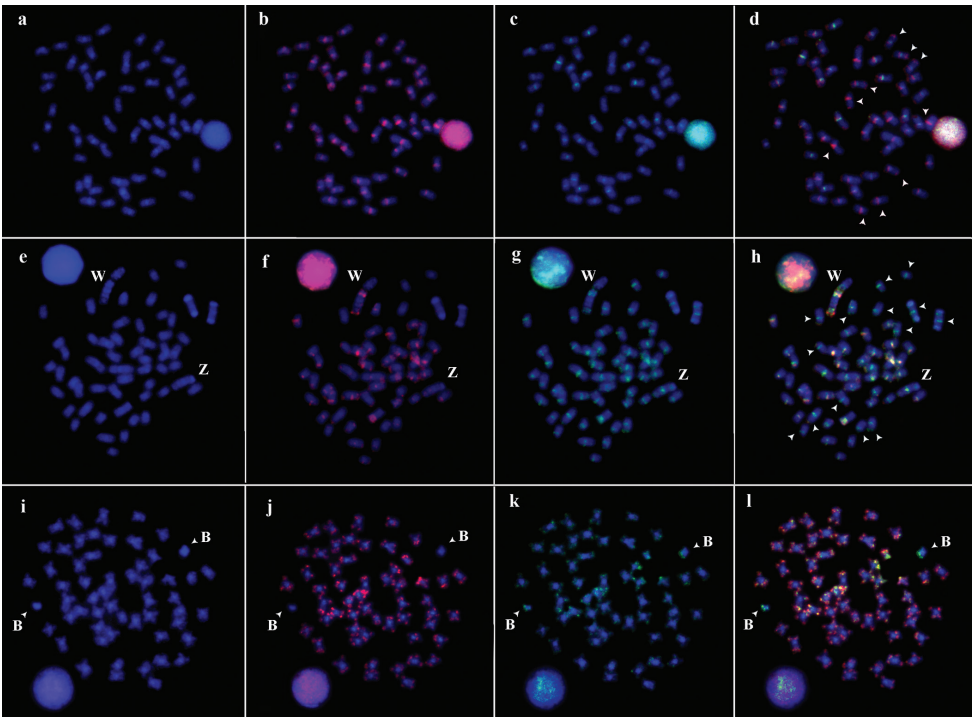

Figure 1. *Cot-1* DNA fraction hybridization in three species of Prochilodontidae. **a** *S. insignis* chromosomes counterstained with DAPI **b** *Cot-1* DNA from the *S. insignis* genome hybridized to its own chromosomes **c** *Cot-1* DNA from the *S. taeniurus* genome hybridized to *S. insignis* chromosomes **d** Double-FISH of the *Cot-1* DNA fraction **e** *S. taeniurus* chromosomes counterstained with DAPI **f** *Cot-1* DNA from the *S. taeniurus* genome hybridized to its own chromosomes **g** *Cot-1* DNA from the *S. insignis* genome hybridized to the *S. taeniurus* chromosomes **h** Double-FISH of the *Cot-1* DNA fraction **i** *P. lineatus* chromosomes counterstained with DAPI **j** *Cot-1* DNA from the *S. insiginis* genome hybridized to *P. lineatus* chromosomes **k** *Cot-1* DNA from the *S. taeniurus* genome hybridized to *P. lineatus* chromosomes **l** Double-FISH of the *Cot-1* DNA fraction.

10% to retrotransposons (Table 1); 75% of the repetitive sequences sampled of *S. taeniurus* displayed high similarity to microsatellites, 5% to transposons, and 15% to retrotransposons (Table 2).

Discussion

Diversity of repetitive DNAs in the genomes of *S. insignis* and *S. taeniurus*

Recent studies have indicated that repetitive sequences have definitely influenced genome evolution by controlling gene activity and by their involvement in chromosomal rearrangements (Valente et al. 2011). The cloning and sequencing the repetitive ge-

Table 1. Nucleotide homology of the *Cot-1* DNA fraction clones of *Semaprochilodus insignis* to known sequences in public databases. BLASTN results and their respective identities are displayed.

Isolate clone	Repetitive sequences	Similarity	Identities
Sin1	DNA transposon	EnSpm-3_DR (RepBase/GIRI*)	70%
Sin2	Microsatellite	*Cyprinus carpio* (GenBank JN756399.1)	92%
Sin3	Microsatellite	*Hypostomus gymnorhynchus* (GenBank HM545164.1)	83%
Sin4	Non-LTR retrotransposon	HERO-2_DR (RepBase/GIRI*)	78%
Sin5	Microsatellite/Retrotransposon	*Colossoma macropomum* (HM579956.1) SINE_2 (RepBase/GIRI*)	79%–75%
Sin6	DNA transposon	Mariner/Tc1 (RepBase/GIRI*)	76%
Sin7	DNA transposon	ERV2 Endogenous Retrovirus (RepBase/GIRI*)	77%
Sin8	Microsatellite	*Cyprinus carpio* (GenBank JN733372.1)	100%
Sin9	Non-LTR retrotransposon	Rex1 (RepBase/GIRI*)	73%
Sin10	Microsatellite	*Cyprinus carpio* (GenBank JN771242.1)	91%
Sin11	DNA transposon	*Labeo rohita*Tc1-like (GenBank AY083617.1)	77%
Sin12	Microsatellite	*Cyprinus carpio* (GenBank JN761177.1)	100%
Sin13	Microsatellite	*Hippoglossus hippoglossus* (GenBank AJ270780.1)	89%
Sin14	DNA transposon	Helitron-2_DR (RepBase/GIRI*)	83%
Sin15	Microsatellite	*Cyprinus carpio* (GenBank JN785563.1)	83%
Sin16	Microsatellite	*Salmo salar* CAG-repeat (GenBank Y11457.1)	87%
Sin17	Microsatellite	*Eleutheronema tetradactylum* (GenBank AB697177.1)	80%
Sin18	Microsatellite	*Oncorhynchus mykiss* (GenBank AY039630.1)	86%
Sin20	Microsatellite	*Prochilodus lineatus* (GenBank AY285824.1)	84%
Sin21	Microsatellite	*Cyprinus carpio* (GenBank JN745523.1)	89%
Sin22	Microsatellite	*Cyprinus carpio* (GenBank JN757227.1)	90%
Sin23	Microsatellite	*Cyprinus carpio* (GenBank JN737559.1)	92%
Sin38	Microsatellite	*Cyprinus carpio* (GenBank JN755429.1)	100%
Sin39	Microsatellite	*Cyprinus carpio* (GenBank JN744936.1)	92%
Sin41	Microsatellite	*Cyprinus carpio* (GenBank JN746351.1)	95%
Sin42	Microsatellite	*Cyprinus carpio* (GenBank JN757934.1)	81%
Sin48	Microsatellite	*Cyprinus carpio* (GenBank JN731077.1)	70%

* Database Repbase (http://www.girinst.org)

nome elements obtained from *S. insignis* and *S. taeniurus Cot-1* DNA displayed high similarities to repetitive sequences deposited in public DNA databases and classified as microsatellites, transposons, and retrotransposons.

Although some repetitive sequences are shared between the two *Semaprochilodus* species analyzed here, DNA sequencing indicated that the genomes of *S. insignis* and *S. taeniurus* were composed of different classes of repetitive sequences. Most of the clones displayed high similarity to microsatellites known from fish species in the order Characiformes (*Colossoma macropomum* Cuvier, 1816) and the family Prochilodontidae (*Prochilodus mariae* Eigenmann, 1922). We believed that the microsatellites were more abundant in this analysis because the method used to obtain the repetitive fraction of

Table 2. Nucleotide homology of the *Cot-1* DNA fraction clones of *Semaprochilodus taeniurus* to known sequences in public databases. BLASTN results along with their respective identities are displayed.

Isolate clone	Repetitive sequences	Similarity	Identities
Ste1	Microsatellite	*Prochilodus mariae* (GenBank JF832400.1)	87%
Ste2	Microsatellite	*Epinephelus fuscoguttatus* (GenBank GU799242.1)	82%
Ste3	Microsatellite	*Cyprinus carpio* (GenBank JN779618.1)	96%
Ste4	DNA transposon	Tc1_FR2(RepBase/GIRI*)	82%
Ste5	Microsatellite	*Cyprinus carpio* (GenBank JN756719.1)	87%
Ste6	Microsatellite	*Cynoglossus semilaevis* (GenBank EU907150.1)	96%
Ste7	Microsatellite	*Prochilodus mariae* (GenBank JF832400.1)	84%
Ste8	Non-LTR retrotransposon	L2-2_DRe (RepBase/GIRI*)	86%
Ste9	Microsatellite	*Cyprinus carpio* (GenBank JN21488.1)	96%
Ste10	Microsatellite	*Cyprinus carpio* (GenBank JN731879.1)	100%
Ste11	Microsatellite	*Cyprinus carpio* (GenBank JN28181.1)	87%
Ste12	Microsatellite	*Prochilodus mariae* (GenBank JF832400.1)	80%
Ste13	Microsatellite	*Salmo salar* (GenBank AJ402727.1)	100%
Ste14	Microsatellite	*Cyprinus carpio* (GenBank JN80674.1)	95%
Ste15	Microsatellite	*Cyprinus carpio* (GenBank JN721488.1)	96%
Ste16	Adeovirus	Bovine Adenovirus type2	99%
Ste17	Non-LTR retrotransposon	SINE3/ 5S (RepBase/GIRI*)	82%
Ste18	Microsatellite	*Brycon amazonicus* (GenBank JQ993450.1)	89%
Ste19	Microsatellite	*Cyprinus carpio* (GenBank JN759566.1)	87%
Ste20	Non-LTR retrotransposon	Rex1-9_XT (RepBase/GIRI*)	75%
Ste21	Non-LTR retrotransposon	L2	82%

* Database Repbase (http://www.girinst.org)

the genome (renaturation kinetics) generates short fragments of DNA (200–300bp) enabling the identification of microsatellites with full homology.

Microsatellites have been observed in a wide range of organisms and are common and widespread in both prokaryote and eukaryote genomes. Among the functions assigned to microsatellites are their participation in chromatin organization, DNA replication, recombination, and the regulation of gene activities (Martins et al. 2011, Li et al. 2011). In fish species such as *Steindachneridion scripta* (Miranda Ribeiro, 1918) *Rineloricaria latirostris* (Boulenger, 1900), and *Danio rerio* (Hamilton, 1822) these repetitive sequences tend to be clustered in the centromeric and telomeric regions (Shimoda et al. 1999, Vanzela et al. 2002). Future studies aimed at mapping microsatellites within the chromosomes of Prochilodontidae will further our understanding of the roles of those sequences in chromosomal evolution in that group.

A certain proportion of these DNA fragments displayed high degrees of similarity to transposable elements (i.e., both transposons and retrotransposons) (Tables 1 and 2) found in the genomes of fish species such as *Xiphophorus maculates* Gunther, 1866, *Leporinus elongatus* Valenciennes, 1850 and *Oryzias hubbsi* Roberts, 1998 (Volff et al. 2000, Bohne et al. 2012, Marreta et al. 2012, Takehana et al. 2012).

The sequences described in the present study may play an evolutionary role in the genomes of *S. insignis* and *S. taeniurus* as one of the sequences identified in the genome of *S. taeniurus* (Ste17) displayed 82% homology with a retrotransposon called SINE3 identified by (Kapitonov and Jurka 2003) as originating from 5S rRNA. As other species of the Prochilodontidae family have only one pair of chromosomes carrying these ribosomal sites, this information strengthens the hypothesis that the multiple 5S rDNA sites observed in *S. insignis* and *S. taeniurus* are pseudogenes (or repetitive sequences) derived from 5S rDNA (Terencio et al. 2012a). In *Gymnotus paraguensis* (Albert & Crampton, 2003) the multiplication of 5S rDNA gene clusters might has be caused by the involvement of transposable elements because the NTS has high identity (90%) with a Tc1-like transposon (Silva et al. 2011).

We were also able to identify sequences in *S. insignis* that exhibited high similarity with the transposable element Helitron. In maize, this TE seems to continually produce new non autonomous elements responsible for the duplicative insertion of gene segments at new locations and for the unprecedented genomic diversity of this species (Morgante et al. 2005). Intact Helitron elements were identified in the sex-determining region of the sex chromosomes of the platyfish *X. maculatus*, suggesting that TE are still active in the genome of platyfish and related species – where they may have roles in the evolution of sex chromosomes and other genomic regions (Zhou et al. 2003).

Tc1/mariner (isolated from the genomes of *S. insignis* and *S. taeniurus*) are the most widespread superfamily of DNA transposons and can be found in fungi, plants, ciliates, and animals (including nematodes, arthropods, fish, frogs, and humans). Most of the transposon copies isolated from vertebrates are clearly inactive remnants of once active transposons that were inactivated by mutations, but only after successfully colonizing their genomes (Plasterk et al. 2009, Ivics et al. 2006).

The retroelement Rex1 was also detected in the repetitive fraction of the genomes of *S. insignis* and *S. taeniurus*. The Rex family has been widely studied in fish, and a number of different lineages have been described in this group (Volff et al. 2000) where they are known to be scattered or grouped into conspicuous clusters in the chromosomes of Neotropical cichlids (Mazzuchelli and Martins 2009, Gross et al. 2009, Teixeira et al. 2009, Oliveira et al. 1999). These elements display compartmentalized distributions in some autosomes and show clear signals along the full lengths of W chromosomes in *S. taeniurus* (Terencio et al. 2012).

The Line2 element was detected only in the repetitive fraction of the *S. taeniurus* genome. This repetitive sequence may be present in the *S. insignis* genome but simply not sampled in our study, or alternatively, it may have been eliminated from the genome of this species. FISH showed that Line2 sequences are organized in small clusters dispersed over all of the chromosomes of *Oreochromis niloticus* (Linnaeus, 1758), but with higher concentrations near chromosome ends (Oliveira et al. 1999). Line elements in mammals appear clustered in the G-banding regions of the chromosomes, and on the sex chromosomes in some cases (Wichman et al. 1992, Fishcher et al. 2004).

Repetitive DNA organization in chromosomes

Repetitive DNA sequences comprising mostly the heterochromatic portions of the genome were observed using the C-banding technique. Previous studies (Feldberg et al. 1987, Vicari et al. 2006, Terencio et al. 2012b) revealed that this technique revealed that repetitive DNA sequence fractions in the genomes of fish of the family Prochilodontidae are not abundant and are located mainly in the centromeric regions of the chromosomes and, less frequently, in the terminal regions of the long arms of some chromosome pairs. Large heterochromatic blocks can be observed, however, in the supernumerary chromosomes (i.e., the B chromosomes) of *Prochilodus spp*. (Vicari et al. 2006, Voltolin et al. 2011) and in the W sex chromosome of *S. taeniurus* (Feldberg et al. 1987, Terencio et al. 2012a). Fluorescence *in situ* hybridization using species-specific probes of the repetitive fractions of the genomes partially confirmed the heterochromatic pattern demonstrated with the C-banding technique in both *S. insignis* and *S. taeniurus* – and positive signals were detected in the centromeric regions of all of their chromosomes. Markers were also observed in the terminal regions of some chromosomes, confirming that repetitive DNAs are also present in this area, although heterochromatin was not observed. Repetitive sequences located outside of heterochromatic regions are believed to significantly influence genome evolution, particularly by controlling and regulating gene activities, and genome sequencing has frequently revealed short and truncated copies of repetitive sequences in euchromatic genomic regions (Fischer et al. 2002, Biémont and Vieira 2006, Timberlake 1978, Yuan and Wessler 2011, Torres et al. 2011). This observation does not necessarily indicate that these repetitive sequences are constitutively expressed, however, since they tend to be silenced and undergo subsequent molecular deterioration. In other words, these sequences becomes inactive and progressively accumulate mutations, insertions, and deletions at neutral rates until completely losing their identities or become lost in the host genome (Fernández-Medina et al. 2012). The presence of repetitive DNAs in euchromatic regions has been observed in many groups, such as insects (Cabral-de-Mello et al. 2011), fish (Teixeira et al. 2009, Valente et al. 2011), and lizards (Pokorná et al. 2011), and these TEs have acquired structural/regulatory functions so that their accumulation in euchromatic regions may lend advantages to the host genome.

Cross-hybridizations of *S. insignis* and *S. taeniurus* showed patterns similar to those observed in homologous hybridization – which suggests that this portion of the genome has been conserved throughout evolution, perhaps due to a functional role. However, revealed that these species have species-specific centromeric and terminal sites not identified by heterologous hybridization.

Cross-FISH using *S. insignis* and *S. taeniurus Cot-1* DNA probes revealed hybridization signals in the subterminal regions of *P. lineatus*, in contrast to the heterochromatic pattern revealed by the C-banding technique with heterochromatin blocks being primarily observed in the centromeric region (Pauls and Bertollo 1990, Venere et al. 1990, Cavallaro et al. 2000, Artoni et al. 2006, Vicari et al. 2006, Voltolin et al. 2011). These data indicate that the repetitive fraction of centromeric heterochromatin of *P.*

lineatus is different from the other two species examined (*S. insignis* and *S. taeniurus*) and that shared repetitive sequences are located on the subtelomeric portions of their chromosomes. This same pattern was observed in three species of *Prochilodus* using (AATTT)n microsatellite (Hatanaka et al. 2002) and W-specific probes (Terencio et al. 2012b).

A common karyotypic feature of species belonging to the genus *Prochilodus* is the presence of supernumerary chromosomes (B chromosomes). Many studies of B chromosomes have indicated that these supernumerary chromosomes are rich in repetitive sequences and, in certain cases, may contain a number of functional genes (Camacho 2005, Ruiz-Estévez et al. 2012). The *P. lineatus* population analyzed in the present study displayed two B chromosomes with distinct hybridization sites. The *S. insignis Cot-1* DNA probe did not hybridize to the B chromosomes, possibly because the repetitive fraction of the *S. insignis* genome is not shared with the B chromosomes of *P. lineatus*. One possible hypothesis explaining this result would be that these repetitive sequences have undergone rapid differentiation and evolution in the genome of *S. insignis*, resulting in a loss of homology with sequences on the B chromosomes of *P. lineatus*. Another explanation could be due to the fact it is different genera, and therefore the Bs found in each species could have different origins. The *S. taeniurus Cot-1* DNA probe was positive, revealing sequence sharing with *P. lineatus* B chromosomes. A number of studies have suggested that B chromosomes can influence sex determination in fish (Noleto et al. 2012, Yoshida et al. 2012), although no relationship between the occurrence of B chromosomes and sex determination has been observed in *P. lineatus*. The B chromosomes detected in *P. lineatus* were recently shown to demonstrate positive signals when hybridized with the W-specific probe, indicating the sharing of repetitive DNA families between these two species (Terencio et al. 2012a).

Conclusions

Results from DNA sequencing indicated that the genomes of *S. insignis* and *S. taeniurus* comprise different classes of repetitive sequences that may have played important roles in their evolution. The repetitive fractions of the *S. insignis* and *S. taeniurus* genomes also exhibit high degrees of conserved syntenic blocks in terms of both the structure and location of hybridization sites. However, the genomes of both *S. insignis* and *S. taeniurus* displayed a low degree of syntenic blocks with the *P. lineatus* genome, especially with regard to the B chromosome, and the origin of this situation has not yet been elucidated.

Authors' contributions

MLT, CHS and MCG collected the samples, collaborated on all cytogenetic procedures, undertook the bibliographic review and coordinated the writing of the manuscript.

EJC, VN, RFA, MCA and MRV participated in the development of the laboratory techniques, performed the specific W-probe for *Semaprochilodus* and reviewed the manuscript. EF coordinated the study and reviewed the manuscript. All authors read and approved of the final manuscript.

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RESEARCH ARTICLE

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Intragenomic variations of multicopy ITS2 marker in *Agrodiaetus* blue butterflies (Lepidoptera, Lycaenidae)

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Abstract

The eukaryotic ribosomal DNA cluster consists of multiple copies of three genes, *18S*, 5.*8S* and *28S rRNAs*, separated by multiple copies of two internal transcribed spacers, *ITS1* and *ITS2*. It is an important, frequently used marker in both molecular cytogenetic and molecular phylogenetic studies. Despite this, little is known about intragenomic variations within the copies of eukaryotic ribosomal DNA genes and spacers. Here we present data on intraindividual variations of *ITS2* spacer in three species of *Agrodiaetus* Hübner, 1822 blue butterflies revealed by cloning technique. We demonstrate that a distinctly different intragenomic *ITS2* pattern exists for every individual analysed. *ITS2* sequences of these species show significant intragenomic variation (up to 3.68% divergence), setting them apart from each other on inferred phylogenetic tree. This variation is enough to obscure phylogenetic relationships at the species level.

Keywords

Agrodiaetus, Lycaenidae, *Polyommatus*, *ITS2*, ribosomal DNA, intraindividual variability, cloning

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Introduction

The eukaryotic ribosomal DNA (rDNA) cluster consists of three genes, *18S*, 5.*8S* and *28S rRNAs*, separated by two internal transcribed spacers, *ITS1* and *ITS2*. This array forms a transcription unit, which is are typically represented in a genome by several hundred tandemly repeated copies (Long and David 1980, Gerbi 1985). The number of rDNA sequence variants can vary within a wide range both at the species and individual level. For example, different species of *Drosophila* Linnaeus, 1758 are estimated to have three to 18 variants of rDNA sequences (Stage and Eickbush 2007). The genome of sea sponge *Amphimedon queenslandica* Hooper & van Soest, 2006 was found to contain approximately 14.5 copies of rDNA sequences per haploid complement (Srivastava et al. 2010). Furthermore, individuals of the same species can have very different numbers of rDNA copies because the clusters display both meiotic rearrangements and somatic mosaicism. It has been shown that in humans for example, the number of rDNA sequences even within a single cluster can vary in an enormous extent, from one repeat unit up to 140 repeats (Stults et al. 2008).

Ribosomal RNA genes have been widely used in taxonomy, biogeographic, phylogenetic analyses, and molecular cytogenetic studies (Hillis and Davis 1986, Mindell and Honeycutt 1990, Wesson et al. 1993, Vogler and DeSalle 1994). In particular, more detailed and precise karyotypes studies became available since fluorescence *in situ* hybridization (FISH) technology was applied to the chromosomal physical mapping. FISH mapping identifies useful chromosomal markers that can be applied to studies of genome organization and species evolution and can also identify specific chromosomes, homologous chromosomes, chromosome rearrangements and sex chromosomes, among others (Nakajima et al. 2012). Ribosomal RNA genes are among the most mapped sequences in chromosomes in many animal groups including insects (Cabrero and Camacho 2008, Grozeva et al. 2010, 2011, Nguyen et al. 2010, Kuznetsova et al. 2012, Maryańska-Nadachowska et al. 2013, Gokhman et al. 2014, Kuznetsova et al. 2015, Vershinina et al. 2015).

Accordingly, rDNA can be excellent source of cytogenetic markers for comparative genomic studies, evolutionary studies as well as the genetic identification of species (Mantovani et al. 2005, Pedrosa-Harand et al. 2006, Cabral-de-Mello et al. 2011).

At the nucleotide sequence level coding regions and spacers can reveal phylogenetic relationships ranging from the level of major phyla of living organisms to the population level, because they differ widely in their rate of evolution (Hillis and Dixon 1991, Wesson et al. 1992, Kuperus and Chapco 1994, Muccio et al. 2000, Wiegmann et al. 2000). *18S* and *28S* rDNA genes are reported to be highly informative to reconstruct higher-level phylogenies in plants and animals (see e.g. Soltis et al. 2000, Mukha et al. 2002).

Unlike highly conserved rRNA genes, non-coding fast evolving transcribed spacers have high level of interspecific variability. Therefore, the internal transcribed spacers are considered to be useful phylogenetic markers, specifically for low-level phylogenetic analyses. *ITS1* and *ITS2* have been used extensively in phylogenetic reconstruc-

tion of closely related species and cryptic species complexes (Wilkerson et al. 2004). For instance, *ITS* have become the standard barcode of choice in most investigations for plants and fungi (Stoeckle 2003, Kress et al. 2005, Sass et al. 2007, Bellemain et al. 2010, Hollingsworth et al. 2011, Schocha et al. 2012, Li et al. 2015).

During PCR all variants of *ITS* sequences presented in genome are amplified, therefore, direct sequencing could lead to inaccurate or erroneous phylogenetic reconstructions. Accordingly identifying and examination levels of intragenomic and intraspecific variation among *ITS* sequences are of real importance.

Agrodiaetus is a species-rich subgenus within the Palearctic genus *Polyommatus* (Talavera et al. 2013). The subgenus includes ca. 130 described species (see Vila et al. 2010, Lukhtanov et al. 2008, 2015a, Vershinina and Lukhtanov 2010, Przybyłowicz et al. 2014, Lukhtanov and Tikhonov 2015). The subgenus was estimated to have originated only about three million years ago (Kandul et al. 2004). Nowadays this rapidly radiated group of butterflies is a model system in studies of speciation (Lukhtanov et al. 2005, Lukhtanov et al. 2015b), and rapid karyotype evolution (Kandul et al. 2007). Several molecular phylogenetic studies have been conducted on *Agrodiaetus*, also based on *ITS2* molecular marker (Wiemers 2003, Wiemers et al. 2009, Wiemers et al. 2010, Lukhtanov et al. 2015a). However, until now rate of *ITS2* intragenomic variations in this rapidly evolved group have never been analyzed.

This paper addresses a more detailed analysis of intraindividual variability of *ITS2* region in three *Polyommatus* (*Agrodiaetus*) species: *P.*(*A.*) *peilei* Bethune-Baker, 1921, *P.*(*A.*) *karindus* (Reiley, 1921) and *P.*(*A.*) *morgani* (Le Cerf, 1909). These three species are closely related to each other (Lukhtanov et al. 2015b), but have clear differences in male wing color and karyotypes (haploid chromosome number are n= 38-39 in *P.*(*A.*) *peilei*; n=68 and n=73 in different populations of *P.*(*A.*) *karindus*; and n=25-27 in *P.*(*A.*) *morgani*) (Lukhtanov et al. 2015b). Direct sequencing of *ITS2* give ambiguous results; thus, we sought to clone and sequence *ITS2* from these species to quantify the prevalence of intragenomic *ITS2* variation and determine its effect on phylogenetic reconstructions.

Material and methods

Butterflies (only males) were collected in NW Iran (Zagros mt., Kordestan provience) in 2007–2014. Bodies were placed in 2 ml plastic vials with 100% ethanol for DNA analysis. Wings were stored in glassine envelopes for morphological study. All samples are stored at Zoological Institute, St Petersburg, Russia.

ITS2 region was amplified using the primer pair: ITS-3 and ITS-4 (White et al. 1990). When ITS-3 and ITS-4 primers failed to amplify a sufficient product, self-designed lepidopteran primers were used:

ILYC2F 5`- GAGAAACATCCAGGACCACT - 3` and
ILYC2RB 5` - CTGATCTGAGGCCA ACG - 3`.

The PCR amplifications were performed either in 50 μl reaction volume containing ca. 10–20 ng genomic DNA and 0.5 mM of each primer, using 26 PCR Master Mix (Fermentas, Lithuania). The temperature profile was as follows: initial denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 1 min with a final extension at 72 °C for 10 min.

Amplified fragments were purified using GeneJET Gel Extraction Kit (Fermentas, Lithuania). Purification was carried out according to the manufacturer's protocol. The success of PCR amplification and purification was evaluated by electrophoresis of the products in 1% agarose gel. Purified PCR product was used for direct sequencing or subsequent cloning.

ITS2 PCR products were cloned into blunt-end cloning vector pJET1.2 (Fermentas, Lithuania) according to the manufacturer's protocol for 10 minutes at room temperature. The pJet1.2 plasmid selects successful ligations through the disruption of an otherwise lethal gene, eco47IR, which enables positive selection of the recombinants. Before ligation, a 3'-A overhang were removed from the PCR products by treating the PCR product with a proofreading DNA polymerase. For transformation 5 μl of the ligation mixture reaction were added to 50 μl of chemo-competent *E. coli* DH101B cells an incubated for 10 min. on ice. After incubation transformation mixture were pipetted onto pre-warmed LB Anp IPTG agar plate and spread by using inoculation loop. Agar plates with competent *E. coli* were incubated overnight at 37 °C.

For each cloning, more than 500 clones were obtained. To check if the cloning procedures were successful, PCR with *ITS2*-speciffic primers were conducted for 20 colonies per cloning reaction. GeneJET Plasmid Miniprep Kit (Fermentas, Lithuania) was used for preparation of plasmid DNA from recombinant *E. coli* culture. A single colony from a freshly streaked selective plate were picked to inoculate 1–5 mL of LB medium supplemented with ampicillin and incubated for 12–16 hours at 37 °C while shaking at 200–250 rpm. The bacterial culture was harvested by centrifugation at 8000 rpm (6800 × g) in a microcentrifuge for 2 min at room temperature. The supernatant was decanted and all remaining medium was removed. The pelleted cells were resuspended and subjected to SDS/alkaline lysis to liberate the plasmid DNA. The resulting lysate was neutralized to create appropriate conditions for binding of plasmid DNA on the silica membrane in the spin column. Cell debris and SDS precipitate were pelleted by centrifugation, and the plasmid DNA were washed to remove contaminants and eluted.

Sequencing was carried out using 3500xL analyzer (Applied Biosystems). Not less than 300 ng of plasmid DNA template was used for sequencing procedure. Cloned fragments were analyzed edited and aligned in Bioedit Software.

A Bayesian approach for estimating phylogeny was used. Bayesian trees were inferred using partitioned models: GTR for nucleotide substitutions and standard model for indels as implemented in MRBAYES v. 3.2 (Ronquist and Huelsenbeck 2012). Each gap (indel) was treated as a single character regardless of the length of the gap, under the assumption that a given gap is a result from one mutational event (Simmons and Ochoterena 2000).

Results

Sequenced region contained 3` end of *5.8S* gene, *ITS2*, and the 5` end of the *28S* gene. Direct sequencing of amplicons of 30 individuals (10 individuals per each species) displayed intra-individual heterogeneities in all specimens analyzed. There are two kinds of heterogeneities: single nucleotide substitutions and mono, bi- and multi-nucleotide insertions/deletions. The presence of heterogeneities was indicated by double peaks in substitution positions, and by a series of mixed peaks in case of indel events, both positioned after a sequence of good quality. The examples of heterogeneities revealed by direct sequencing are displayed in Figure 1.

To elucidate the visible heterogeneity, the amplicons for 2 specimens of *P.*(*A.*) *peilei*, 2 specimens of *P.*(*A.*) *karindus* and one specimen of *P.*(*A.*) *morgani* were cloned and 10 clones per specimen were sequenced. The summary of the heterogeneities in the *ITS* region displayed by the clones is depicted in Table 1. Partial sequences of *5,8S* and *28S* genes were cropped from further analysis. Total length of *ITS2* varied from 477 bp up to 512 bp depending on the presence of insertions\deletions. Uncorrected "p" pairwise distances for all clones are given in Table 2.

There were 11 single-base substitutions, 3 mono and 4 multi-nucleotide indels, in clones of specimen W136 (*P.*(*A.*) *peilei*). Interestingly, that clone "W136_#08" differed significantly from all others in having 16-nycleotide polyT deletion at positions "329-344" and 3 base indel at position "171-173". Clones of second specimen *P.*(*A.*) *peilei* (W202) had 8 sites with single nucleotide substitutions and 8 positions, where mono multi-nucleotide indels occurred. Three clones had large polymorphic 17-nucleotide indel at positions "184-200". Variation among clones was significant, with

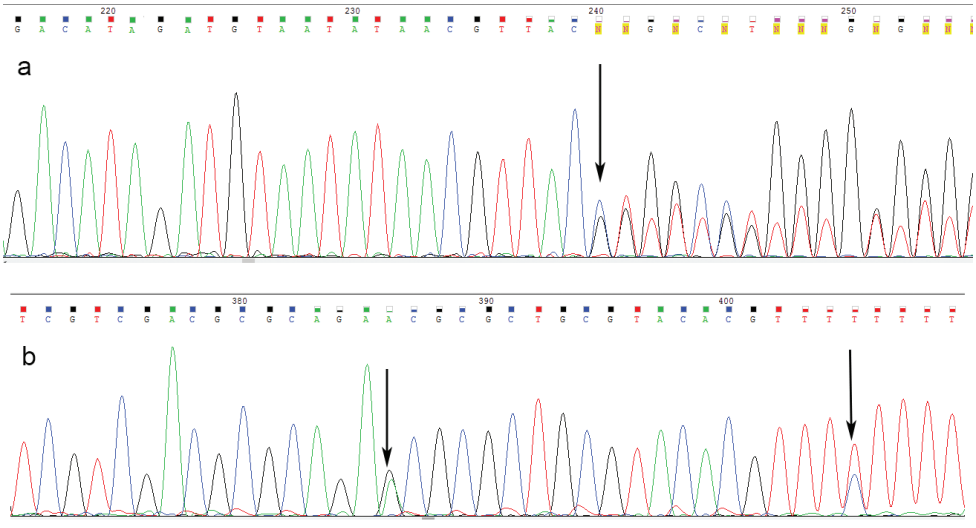

Figure 1. Examples of results from direct sequencing of *ITS2*. **a** Example of polymorphism caused by an indel (black arrow indicate the beginning position of an indel) **b** Example of single nucleotide substitutions (indicated by black arrows).

Table 1. Variable positions among sequenced clones.

Specimen	Clone number	Position
		128 130 131 171 172 173 235 316 326 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 356 400 414 465
W136 *P.(A.) peilei*	#01	T G - C G C A A T T T T T T T T - - C G T T T T T - C G G G C
W136 *P.(A.) peilei*	#02	T G - C G C A A T T T T T T T T - - C G T T T T T - C G G G T
W136 *P.(A.) peilei*	#03	T A A C A C G G C T T T T T T T T T T G T T T T T - C A G A C
W136 *P.(A.) peilei*	#04	T G - C G C A A T T T T T T T T - - T G T T T T T - C G G G C
W136 *P.(A.) peilei*	#05	T A A C A C G G C T T T T T T T T T T G T T T T T T - A G G C
W136 *P.(A.) peilei*	#06	T G - C G C A A T T T T T T - - - - T G T T T T T - C G G G C
W136 *P.(A.) peilei*	#07	T G - C G C A A C T T T T T T T T T T G T T T T T T - A G G C
W136 *P.(A.) peilei*	#08	A A - - - - A A T - - - - - - - - - - - - - - - - - - A A G C
W136 *P.(A.) peilei*	#09	T A A C A C G G C T T T T T T T T T T T G T T T T T T - A G G C
W136 *P.(A.) peilei*	#10	T G - C G C A A T T T T T T T T - - T G T T T T T - C G G G C

		14 41 128 131 169 170 171 176 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 235 236 326 336 337 338 345 346 356
W202 *P.(A.) peilei*	#01	T C T - A C C T - A - T T T - T - A
W202 *P.(A.) peilei*	#02	T T T A A C C T T C G C G T C G G C G A C G T G C G G C T T T T - A
W202 *P.(A.) peilei*	#03	T T G - - - - T T C G C G T C G G C G A C G T G C G G T T T C - C G
W202 *P.(A.) peilei*	#04	T T T - A C C C - A - T T - C - C G
W202 *P.(A.) peilei*	#05	C C T A A C C T T C G C G T C G G C G A C G T G C G G C T T - T - A
W202 *P.(A.) peilei*	#06	T T T A A C C T T C G C G T C G G C G A C G T G C G G C T T T T - A
W202 *P.(A.) peilei*	#07	T T T A A C C T T C G C G T C G G C G A C G T G C G G C T T T T - A
W202 *P.(A.) peilei*	#08	T C T - A C C T - A - T - - C - C G
W202 *P.(A.) peilei*	#09	T T T A A C C T T C G C G T C G G C G A C G T G C G G T T T T T - A
W202 *P.(A.) peilei*	#10	T C T - A C C T T C G C G T C G G C G A C G T G C A - T T T C - C G

		20 150 154 155 166 239 340 341 342
V145 *P.(A.) karindus*	#01	A C - - T T T T -
V145 *P.(A.) karindus*	#02	A C - - T T T - -
V145 *P.(A.) karindus*	#03	A T C G T T - - -
V145 *P.(A.) karindus*	#04	A T C G T T - - -
V145 *P.(A.) karindus*	#05	A T C G T T T - -
V145 *P.(A.) karindus*	#06	A C - - T C - - -
V145 *P.(A.) karindus*	#07	A T C G C T T T T
V145 *P.(A.) karindus*	#08	A C - - T T T - -
V145 *P.(A.) karindus*	#09	A C - - T T - - -
V145 *P.(A.) karindus*	#10	G C - - T T - - -

		27 84 128 136 169 170 171 331 335 336 337 337 338 339 340 346 351 352 353 354
Z04 *P.(A.) karindus*	#01	C G T T C C A C T T T T T T T C - A A A
Z04 *P.(A.) karindus*	#02	C G T T C C A T T T T T T T T C - A A A
Z04 *P.(A.) karindus*	#03	C G T T C C A T T T T T T T T C - A A A
Z04 *P.(A.) karindus*	#04	C G T T C C A T T T T T T T T C - A A A
Z04 *P.(A.) karindus*	#05	T G T - C C A T T T T T T T - C A A A A
Z04 *P.(A.) karindus*	#06	T G T - C C A T T T T T T T - C A A A A
Z04 *P.(A.) karindus*	#07	T A T - C C A T T T T T T T - C A A A A
Z04 *P.(A.) karindus*	#08	C G T T C C A T T T T T T T - C - A A A
Z04 *P.(A.) karindus*	#09	C G T T C C A T T T T T T T - C - A A A
Z04 *P.(A.) karindus*	#10	T G G - - - - C - - - - - - - - - - - - -

		7 38 43 79 127 128 148 171 172 173 229 301 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 347 348 349 350 352 353 354 355 357 358 391 400 472
W127 *P.(A.) morgani*	#01	A A C - C G T - - - T C A C A C G T T T T T T T T - - - - A A C G - - - A A A A G G
W127 *P.(A.) morgani*	#02	A A C - C G T - - - C C A C A C G T T T T T T T T T T T A A C G - - - A A T A G G
W127 *P.(A.) morgani*	#03	A A C - A T C C G C T G - G C A A G A G G T
W127 *P.(A.) morgani*	#04	A G C G A T T C G C T G - G C A A G A G G G
W127 *P.(A.) morgani*	#05	G G C G C G T - - - T C A C A C G T T C T T T T T T T T - A A C G - - - A A A A G G
W127 *P.(A.) morgani*	#06	A A C - C G T - - - T C A C A C G T T T T T T T T T T - - A A C G - - - A A A A A G
W127 *P.(A.) morgani*	#07	A A C - C G T C G C T G - G C A A G A G G G
W127 *P.(A.) morgani*	#08	A A C G A T T C G C T C - G C A - A A A G T
W127 *P.(A.) morgani*	#09	A A T G A T T C G C T G - G C A A G A G G G
W127 *P.(A.) morgani*	#10	A A C - C G T - - - T G - G C A A G A G G G

Table 2. Uncorrected "p" distance matrix of clones.

P. (A.) peilei	W136_#01	W136_#02	W136_#03	W136_#04	W136_#05	W136_#06	W136_#07	W136_#08	W136_#09	W136_#10
W136_#01	-									
W136_#02	0.0019	-								
W136_#03	0.0196	0.0216	-							
W136_#04	0.0019	0.0039	0.0177	-						
W136_#05	0.0216	0.0236	0.0058	0.0196	-					
W136_#06	0.0058	0.0078	0.0176	0.0039	0.0196	-				
W136_#07	0.0117	0.0137	0.0157	0.0098	0.0098	0.0098	-			
W136_#08	0.0182	0.0202	0.0223	0.0162	0.0202	0.0162	0.0141	-		
W136_#09	0.0216	0.0236	0.0058	0.0196	0	0.0196	0.0098	0.0202	-	
W136_#10	0.0019	0.0039	0.0177	0	0.0196	0.0039	0.0098	0.0162	0.0196	-
Average	**0,0134**									
P. (A.) peilei	W202_#01	W202_#02	W202_#03	W202_#04	W202_#05	W202_#06	W202_#07	W202_#08	W202_#09	W202_#10
W202_#01	-									
W202_#02	0.0139	-								
W202_#03	0.0200	0.0157	-							
W202_#04	0.0142	0.022	0.0140	-						
W202_#05	0.0119	0.0059	0.0119	0.0259	-					
W202_#06	0.0139	0	0.0157	0.0219	0.0059	-				
W202_#07	0.0139	0	0.0157	0.0219	0.0059	0	-			
W202_#08	0.0122	0.0239	0.0160	0.0061	0.0239	0.0239	0.0239	-		
W202_#09	0.0119	0.0019	0.0137	0.0199	0.0078	0.0019	0.0019	0.0219	-	
W202_#10	0.0100	0.0176	0.0177	0.0080	0.0176	0.0176	0.0176	0.0060	0.0157	-
Average	**0,0135**									
P. (A.) karindus	V145_#01	V145_#02	V145_#03	V145_#04	V145_#05	V145_#06	V145_#07	V145_#08	V145_#09	V145_#10
V145_#01	-									
V145_#02	0.0019	-								
V145_#03	0.0078	0.0059	-							
V145_#04	0.0078	0.0059	0	-						
V145_#05	0.0059	0.0039	0.0019	0.0019	-					
V145_#06	0.0059	0.0059	0.0078	0.0059	0.0078	-				
V145_#07	0.0078	0.0098	0.0078	0.0078	0.0078	0.0137	-			
V145_#08	0.0019	0	0.0059	0.0059	0.0039	0.0059	0.0098	-		
V145_#09	0.0039	0.0019	0.0039	0.0039	0.0059	0.0019	0.0117	0.0019	-	
V145_#10	0.0059	0.0039	0.0078	0.0059	0.0078	0.0039	0.0137	0.0039	0.0019	-
Average	**0,0056**									
P. (A.) karindus	Z704_#01	Z704_#02	Z704_#03	Z704_#04	Z704_#05	Z704_#06	Z704_#07	Z704_#08	Z704_#09	Z704_#10
Z704_#01	-									
Z704_#02	0.0019	-								
Z704_#03	0.0019	0	-							
Z704_#04	0.0019	0	0	-						
Z704_#05	0.0098	0.0078	0.0078	0.0078	-					
Z704_#06	0.0098	0.0078	0.0078	0.0078	0	-				
Z704_#07	0.0117	0.0098	0.0098	0.0098	0.0019	0.0019	-			
Z704_#08	0.0039	0.0019	0.0019	0.0019	0.0059	0.0059	0.0078	-		
Z704_#09	0.0039	0.0019	0.0019	0.0019	0.0059	0.0059	0.0078	0	-	
Z704_#10	0.0141	0.0162	0.0162	0.0162	0.0162	0.0162	0.0182	0.0182	0.0182	-
Average	**0,0072**									
P. (A.) morgani	V127_#01	V127_#02	V127_#03	V127_#04	V127_#05	V127_#06	V127_#07	V127_#08	V127_#09	V127_#10
V127_#01	-									
V127_#02	0.0101	-								
V127_#03	0.0289	0.0307	-							
V127_#04	0.0267	0.0328	0.0083	-						
V127_#05	0.0122	0.0141	0.0368	0.0246	-					
V127_#06	0.0041	0.0101	0.0267	0.0205	0.0121	-				
V127_#07	0.0144	0.0246	0.0083	0.0083	0.0266	0.0185	-			
V127_#08	0.0165	0.0266	0.0104	0.0104	0.0246	0.0246	0.0146	-		
V127_#09	0.0226	0.0328	0.0083	0.0041	0.0287	0.0267	0.0083	0.0104	-	
V127_#10	0.0124	0.0226	0.0104	0.0104	0.0267	0.0165	0.0021	0.0167	0.0104	-
Average	**0,0177**									

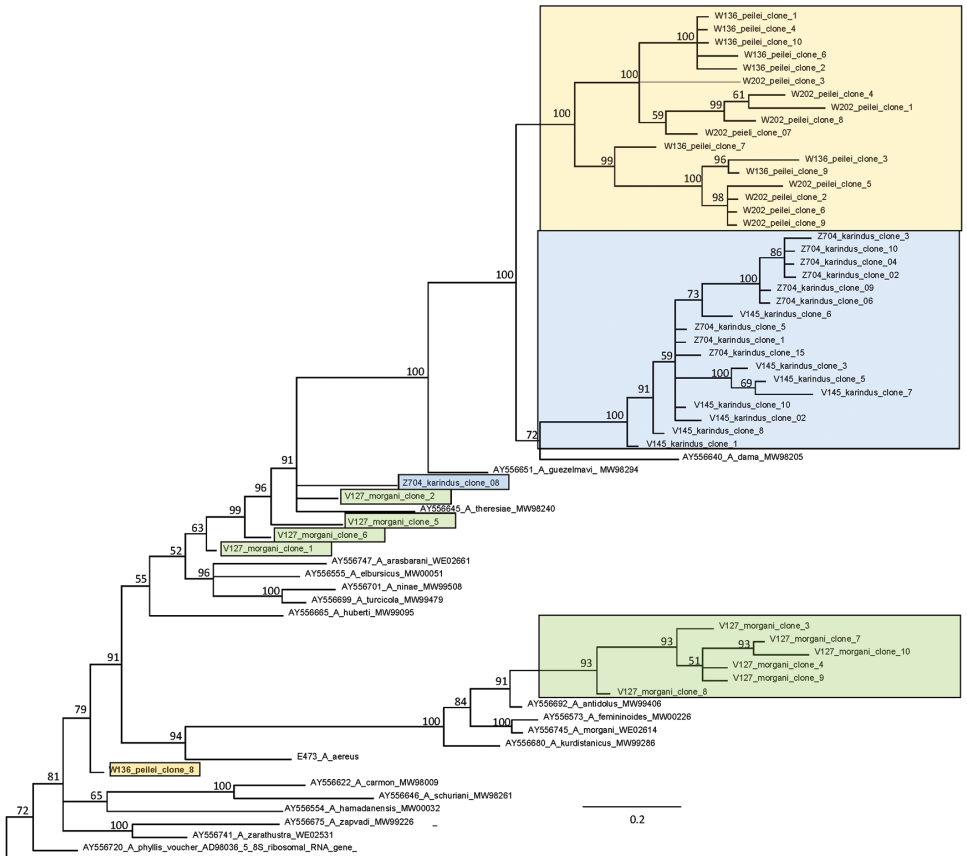

Figure 2. Fragment of consensus Bayesian tree of the subgenus *Agrodiaetus* inferred from *ITS2* sequences. Posterior probability values >50% are shown. The complete tree is given online in the Suppl. material 1. Cloned sequences of three studied species are highlighted: *P.*(*A.*) *peilei* – orange colour, *P.*(*A.*) *karindus* – blue colour, *P.*(*A.*) *morgani* – green colour.

intragenomic differences ranging from 0.0% to 2.39%. The average intragenomic genetic distances for two specimens of *P.*(*A.*) *peilei* (W136 and W202) were very similar: 1.34% and 1.35% respectively.

P.(*A.*) *karindus* had significantly lower rate of intragenomic variability. Specimens V145 and Z704 had 9 and 10 polymorphic positions, respectively. Furthermore, majority number of indels and base substitutions of Z704 specimen is accounted for by one clone (Z704#10). It has one single substitution and 3 multi-nucleotide deletions, which never occurred in other clones. The average intragenomic genetic distances for two specimens of *P.*(*A.*) *karindus* (V145 and Z704) were: 0.56% and 0.72%, respectively. The highest value was 1.82%.

Clones of *P.*(*A.*) *morgani ITS2* showed greater diversity than the other 2 species. For instance, the genetic distance between V127#05 clone and V127#03 was 3.68%. The average intragenomic genetic distance was also significantly higher for this species – 1.77%

In Bayesian analysis 50 cloned amplicons from *P.*(*A.*) *peilei*, *P.*(*A.*) *karindus*, *P.*(*A.*) *morgani* and *ITS2* sequences from all *Agrodiaetus* species available in the GenBank were included, giving a total of 127 sequences. Since *Polyommatus icarus* (Rottemburg, 1775) was earlier inferred as sister clade to the subgenus *Agrodiaetus* (Talavera et al. 2013), we used one specimen (GenBank accession number AY556732) as outgroup to root the phylogeny. Fragment of consensus Bayesian tree, showing clusterization of cloned sequences is given in Figure 2. The complete tree is given online in the Suppl. material 1.

Discussion

Despite the popularity of the *ITS2* nuclear rDNA marker in systematics of different groups of animals and plants, its variability on intraspecific and intraindividual level is still poorly known. The occurrence of multiple *ITS2* copies within a single genome should be accounted for before rDNA is used for phylogenetic or population studies. Furthermore, investigation of rates of intra-individual polymorphism can lighten addressing questions regarding speciation, species hybridization end evolutionary history. It is generally considered that multigene families, such as rDNA maintain homogeneity of all copies as a result of concerted evolution (processes of gene conversion and unequal crossing over) (Zimmer et al. 1980, Dover 1982). Mutations rapidly spread to all members of the gene family even if there are arrays located on different chromosomes (Dover 1982, Arnheim1983, Gerbi 1985, Tautz et al. 1988). The efficiency of homogenization of rDNA is usually high (Liao 1999). Concerted evolution of noncoding sequences, such as internal transcribed spacers, can result in fixed interspecific differences and intraspecific homogeneity. Despite this assumption, our results show, that intraindividual variability can be maintained, when mutation rates are higher than rates of homogenization. This can lead to erroneous phylogenetic reconstructions and species misidentification.

Here we contribute with the first insight into the intraspecific *ITS2* diversity in the blue butterflies of subgenus *Agrodiaetus.*

The *ITS2* of all specimens of three *Agrodiaetus* species - (*P.*(*A.*) *peilei*, *P.*(*A.*) *karindus* and *P.*(*A.*) *morgani*) were intragenomically variable. There were a number of indels and base substitutions accounting for both the length and sequence variabilities. Numerous indels lead to length variation (477-512 bp) of studied sequences. Bayesian phylogenetic reconstruction revealed that cloned sequences of certain individuals did not form a monophyletic unanimity, but the majority of clones clustered together within species borders. In particular, clones of *P.*(*A.*) *peilei* and *P.*(*A.*) *karindus* individuals are recovered as two distinct separated clusters, both with a Bayesian posterior probability of 1.00. The position of 6 clones of *P.*(*A.*) *morgani* specimen on the *ITS2* tree support the conclusion that abovementioned species belong to "*antidolus*" species-group which comprise 5 allopatric in distribution, closely related taxa: *P.*(*A.*) *femininoides* (Eckweiler, 1987), *P.*(*A.*) *antidolus* (Rebel, 1901), *P.*(*A.*) *aereus* (Eckweiler, 1998), *P.*(*A.*) *kurdistanicus* (Forster, 1961) and *P.*(*A.*) *morgani*. "*Antidolus*" clade revealed with a high level of

posterior probability. However, when considering all cloning data, in some cases differences between cloned sequences of the same individual were greater than that between species. For instance, the remainder of *P.*(*A.*) *morgani* clones are placed as the basal taxa to clade, consist of *P.*(*A.*) *guezelmavi* (Olivier, Puplesiene, van der Poorten, De Prins & Wiemers, 1999), *P.*(*A.*) *dama* (Staudinger, 1892) and majority of *P.*(*A.*) *peilei* and *P.*(*A.*) *karindus* clones. One clone of *P.*(*A.*) *karindus* (Z704_#08) also was recovered as sister taxa to abovementioned clade. Finally, W136_#08 clone of *P.*(*A.*) *peilei* is found to be more genetically distant from other clones of this individual than the great number of other species of the subgenus *Agrodiaetus* (Figure 2).

Recent works showed that tandem arrays of rRNA genes in most Lepidoteran species form one or two so-called rDNA clusters, although some exceptions in cluster number exist (Nguen et al. 2010). Data on the number and distribution of rDNA clusters in genomes of lycaenid butterflies are very scarce. Previous investigation by Vershinina et. al. (2015) examined ribosomal clusters in seven blue butterflies of the genus *Polyommatus* and showed the presence of two different variants of the location of major rDNA clusters in *Polyommatus* species: with one or two rDNA-carrying chromosomes in haploid karyotype (Vershinina et al. 2015). *P.*(*A.*) *peilei*, *P.*(*A.*) *karindus* and *P.*(*A.*) *morgani* were among studied species, which bear a single rDNA cluster. Thus, all intragenomic *ITS2* patterns for every individual analysed, belong to a single rDNA cluster, which means that examined level of intragenomic variability not caused by sequencing *ITS2* copies located on different chromosomes.

To conclude, our study demonstrates that the results of direct sequencing may not describe the actual and entire set of sequence variants. Level of divergence between clones of one individual can be comparable to interspecific genetic differences variations or even exceed them. Hence, cloning and subsequent intraindividual haplotypes handling are required for reliable phylogenetic reconstructions.

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Supplementary material 1

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

Authors: Nazar A. Shapoval, Vladimir A. Lukhtanov

Data type: TIFF image

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

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RESEARCH ARTICLE

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Identification of new cytotypes of *Valeriana jatamansi* Jones, 1970 (Valerianaceae) from North-Western Himalayan region of India

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Abstract

Valeriana jatamansi, a medicinally important species of the family Valerianaceae, has been cytologically studied in different geographical areas of North-Western Himalayan region of India. The tetraploid cytotype with chromosome numbers 2n=32 is in conformity with the earlier reports of the species from different parts of the world. An octoploid cytotype (2n=64) makes a new addition for the species on a worldwide basis, whereas the diploid cytotype (2n=16) is new to India have been reported for the first time in India. These cytotypes (2n=16, 32, 64) show significant variations with respect to morphology as well as geographical distribution in the Western Indian Himalayas. Further, anomalous populations have been marked with meiotic abnormalities in the form of cytomixis, chromosomal stickiness, unoriented bivalents, formation of laggards and bridges resulting in abnormal microsporogenesis, and production of heterogeneous-sized fertile pollen grains along with reduced pollen fertility.

Keywords

Valeriana jatamansi, cytotypes, meiotic abnormalities, morphovariants, western Himalayas

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Introduction

The genus *Valeriana* Linnaeus, 1753 belongs to the family Valerianaceae which comprises 250 species of perennial herbs (Chen et al. 1999) distributed throughout temperate and cold regions of the Northern hemisphere (Bell 2004). In India, 16 species and two subspecies of the genus have been reported; of these 5 species inhabit the high-altitude ranges of the Kumaon and Garhwal regions of central Himalayas (Prakash 1999).

Valeriana jatamansi Jones (= *Valeriana wallichii* de Candolle, 1830) popularly known as Indian valeriana (English), Mushkibala (Kashmiri/ Hindi), Sugandhwala or Tagar (Sanskrit), grows wild in the temperate Himalayan region between 1000 and 3000 m altitude. The species generally grows on sloppy, moist places, damp woods, ditches and along the streams. The species grows particularly in the understory of *Quercus leucotrichophora* Camus, 1938 – *Pinus roxburghii* Sargent, 1897 mixed forests and on grassy habitats of Himalayas. Its occurrence can be observed in different geographical areas and the species possesses diverse morphological and genetic features, affecting its levels of active ingredients. *V. jatamansi* is a perennial herb with thick root stocks, horizontal thick descending fibers with pubescent stem, radical leaves often 1-3 cm in diameter, deeply cordate and usually acute toothed margins. Flowers white, stamens- 3, ovary -3 celled, stigma shortly 2-3 fid and fruits oblong lanceolate and compressed crowned by persistent pappus calyx. The flowering and fruiting time for the species is March–June. The mode of propagation is both sexual through seeds and asexual through rhizome.

The species is being exploited for its roots and rhizomes which contain valepotriates (Chopra 1956) highly effective against leprosy (Kour et al.1999) and curing Lewybody dementia (Bagchi and Hooper 2011). The reported annual collection of *Valeriana jatamansi* from the North-Western Himalayas is about 100 quintals whereas hundreds of quintals *Valeriana* roots are smuggled and go unnoticed. Due to over-exploitation of rhizomes for its medicinal value, it is labeled as endangered plant species in the Himalayan region. In spite of its importance as a medicinal plant, there is a lack of information regarding genetic diversity and chromosome number of different cytotypes present in the area, which is prerequisite for initiation of any systematic breeding programme.

Therefore, keeping in view the economic importance, threatened status of the species, and cytological variability within the species, at present cytomorphological studies have been carried out on a population basis from different areas of the North-Western Himalayas. The present study also discusses the impact of cytomixis on meiotic behavior and reduced pollen fertility and formation of heterogeneous-sized pollen grains in the species.

Material and methods

Material. Material for cytological studies in the form of buds was collected from different parts of the North-Western Himalayas. The propagating material of these plants

was also collected and planted in the experimental fields of Chaudhary Sarwan Kumar Himachal Pradesh Agricultural University, Palampur, India in a Randomized Complete Block design with two replications. The plant to plant distance was kept at 5 cm while row to row distance was kept at 50 cm.

A) Morphological study

Different qualitative morphometric characters were studied for each cytotype to have proper insight on morphological variation present in these cytotypes. For stomatal studies, mature leaves were treated with 10% aqueous solution of potassium hydroxide (KOH) at room temperature for 10–15 min and then epidermal peels so obtained were stained with 10% saffanin in 90% ethanol. In order to reveal the significant difference in the stomata and pollen grain size of diploid, tetraploid and octaploid cytotypes, the t-test was been performed.

B) Cytological studies

For meiotic studies, flower buds were collected from plants growing under natural conditions from selected areas of the Western Himalayas. These flower buds were collected from 15 randomly selected plants of each population and fixed in Carnoy's fixative (6:3:1 ethanol/chloroform/acetic acid v/v/v) for 24 h. Flower buds were washed and preserved in 70% ethanol at 4 °C until used. Smears of appropriate-sized flower buds were made, using the tandard acetocarmine technique (Marks 1954). About 20–50 fresh slides in each case were prepared from different anthers/flowers for different individuals of a particular population and were analyzed in each case. To confirm the chromosome number in case of normal meiosis, around 50 pollen mother cells (PMCs) were observed at different stages of meiosis, preferably at diakinesis/ metaphase-I/ anaphase-I, II. In case of abnormal meiosis, however, more than 300 PMCs were considered to ascertain the type and frequency of various abnormalities per plant. Pollen fertility was estimated by mounting mature pollen grains in glycero–acetocarmine (1:1) mixture (Belling 1921). Nearly 400–500 pollen grains were analyzed in each case for ascertaining pollen fertility and pollen size. Well-filled pollen grains with stained nuclei were taken as apparently fertile, while shrivelled and unstained pollen grains were counted as sterile. Photomicrographs of pollen mother cells and pollen grains were made from freshly prepared slides using Optika Digital Imaging System. The data regarding the number of cytologically worked out species, number of cytotypes, and frequency of polyploids of a particular genus have been compiled on worldwide and India basis from various Chromosomal Atlases and Indexes to Plant Chromosome Numbers by Darlington and Wylie (1955), Fedorov (1974), Moore (1973, 1974, 1977), Kumar and Subramanian (1986), and Khatoon and Ali (1993), various journals, Internet resources, as well as presently studied plants.

Results and discussion

Detailed meiotic studies were carried out on 22 populations of *V. jatamansi* collected from different localities with altitude ranging from 764 to 3647 m above mean sea level in the North-Western Himalayan region of India. The data regarding locality with altitude, latitude and longitude, present meiotic chromosome number, ploidy level and meiotic course of the presently worked out populations have been presented in Table 1.

A) Morphological observations

Morphological variation was assessed on 24 vegetative and reproductive characters of different cytotypes of *V. jatamansi* (Table 2). All the three cytotypes (2x, 4x and 8x) at

Table 1. Information about, locality, latitude and longitude, altitude, meiotic chromosome number, ploidy level and meiotic course of *Valeriana jatamansi*.

S. No.	Locality with latitude and longitude, altitude	Present meiotic chromosome number (2n)	Ploidy level	Meiotic in meters
District Chamba (Himachal Pradesh)				
P-1	Bhali mata/ 32°37'N, 76°0'E/1,900	32	4*x*	Abnormal
P-2	Salooni/32°43'N,76°03'E/1,900	16	2*x*	Normal
P-3	Chamba/ 32°33'N, 76°07'E/ 1,200	32	4*x*	Abnormal
P-4	Leg Valley/31°58'N, 77°06'E/ 1,720	32	4*x*	Normal
P-5	Tisa/32°32'N, 76°08'E/ 1,220	32	4*x*	Normal
P-6	Dehgram/32°41'N, 76°08'E/ 2,165	32	4*x*	Abnormal
District Shimla (Himachal Pradesh)				
P-7	Kandi/32°36'N, 76°02'E/ 854	32	4*x*	Normal
P-8	Rampur/ 31°58'N,77°06'E/ 1,350	16	2*x*	Normal
P-9	Pander/ 31°26'N,77°03'E/ 2,236	32	4*x*	Normal
P-10	Shimla I/ 31°6'N, 77°10'E/ 2,202	32	4*x*	Abnormal
P-11	Shimla II/31°18'N, 77°20'E/ 1,820	32	4*x*	Normal
District Mandi (Himachal Pradesh)				
P-12	Rewalsar/31°38'N ,76°50'E/ 1,360	32	4*x*	Normal
P-13	Rakni/ 31°24'N, 77°07'E/1,649	32	4*x*	Normal
P-14	Prashar / 31°45'N, 77°6'E / 2,200	32	4*x*	Abnormal
P-15	Mandi I/ 31°42'N, 76°51'E/ 764	32	4*x*	Normal
P-16	Mandi II/31°31'N, 76°59'E/ 945	32	4*x*	Abnormal
District Kullu (Himachal Pradesh)				
P-17	Kullu I/ 32°09'N,77°02'E/ 3,647	64	8*x*	Abnormal
P-18	Sojha/31°42'N, 77°54'E/ 2,692	32	4*x*	Normal
P-19	Jalori Pass/ 31°32'N, 77°22'E/ 3,134	64	8*x*	Normal
P-20	Kullu II/ 32°08'N, 77°04'E/ 2,734	32	4*x*	Abnormal
P-21	Parvati valley/32°01'N, 77°20'E/1,640	32	4x	Normal
P-22	Manikaran /32°08'N, 77°26'E/ 1,820	32	4x	Normal

intraspecific level revealed significant variations for some of the qualitative characters as is evident from increased stomatal size, guard, and subsidiary cells, stomatal frequency and index in polyploids as compared to diploids (Table 2). Along with these characters, some significant differences were noticed for leaf size in all the three cytotypes (Fig. 1). The size of pollen grain in the octaploid was also found to be significantly larger than their diploid equivalent ($p<0.05$, Table 2). Such comparable results have been previously reported for many angiosperms such as in *Rorippa amphibia* Besser, 1822 (Luttikhuizen et al. 2007), *Nicotiana alata* Link & Otto, 1840 (El-Morsy et al. 2009), *Ocimum basilicum* Linnaeus, 1753 (Omidbaigi et al. 2010), etc. These morphological variations may be attributed to the variation in chromosome number as has been reported earlier in *Centaurea stoebe* Ledebur, 1833 (Nakagawa 2006 and Španiel et al. 2008), *Chamerion angustifolium* (Linnaeus, 1753), *Heuchera grossulariifolia* Rydberg, 1900, *Vaccinium corymbosum* Linnaeus, 1753 (Soltis et al. 2007), *Ranunculus parnassifolius* Linnaeus, 1753 (Cires et al. 2009), *Ranunculus hirtellus* Royle, 1753 (Kumar 2011), etc. Overall, increase in ploidy level is correlated with gigantism for some of the vegetative and floral characters.

B) Cytological observations

Based on x=8 (Darlington and Wyile 1955), the 22 different populations of *V. jatamansi* examined for cytological variations revealed the existence of diploid (2n=16), tetraploid (2n=32) and octaploid (2n=64) cytotypes. Out of 22 populations, two diploid (2n=16) populations showing 8:8 distribution of chromosomes at anaphase-I (Fig. 2a, b) are chromosomally reported for the first time from India. However, diploid (2n=16) cytotypes have been previously reported from Germany and Pakistan by Engel (1974), Khatoon and Ali (1993), respectively. The occurrence of eighteen tetraploid cytotypes with 16:16 distribution of chromosomes at anaphase-I (Fig. 2c, d) and is in conformity with the earlier reports from Kashmir Himalayas (Jee et al. 1983). Two other populations with 2n=64 (32: 32 distribution of chromosomes at anaphase-I) (Fig. 2e) have been cytologically worked out for the first time on worldwide basis.

A perusal of cytological literature reveals that majority of the species in the genus have been worked out showing 2n=14, 16, 28, 32, 42, 48, 56, 60, 64, 72, 80, 90 and 96. The genus is dibasic with x=7, 8 as has been suggested by Darlington and Wylie (1955). The highest level of ploidy of the genus has been reported to be 12x (Engel 1976).

C) Geographical distribution

In the North-Western Himalayas, the distribution pattern of euploid cytotypes shows definite relation to altitudinal variations (Table 1). The distribution of diploids in Himachal Pradesh is uncommon but some accessions are available from lower altitudes of 1350–1900 m. Tetraploids are the most common and are widely distributed within

Table 2. Detailed morphological comparison of three cytotypes of *Valeriana jatamansi*.

No.	Morphological character	**Diploid** (2n=16)	**Tetraploid** (2n=32)	**Octaploid** (2n=64)
1	Distribution	Uncommon	Most Common	Uncommon
2	Habit	Small sized herb	Medium sized herb	Medium sized herb
3	Habitat	Found under shade of *Pinus roxburghii*	Found in the moist, shady and humus rich places	Found on the slopes in forests
4	Plant height (cm)	14.42±2.84	19.46±2.34	28.70±1.69
Stem				
5	Surface	Non-glabrous	Non-glabrous	Glabrous
6	No. of hairs/ mm^2	-		12.6±1.3
7	Length of hairs (cm)	-	-	2.3±0.38
Basal Leaves				
8	Number of leaves / plant	22.66±7.76	20.77±4.55	47.50±10.60
9	Shape	Ovate	Lanceolate	Lanceolate
10	Size (cm)	4.53±0.95×5.83±1.05	5.56±0.86×6.98±1.01	7.65±0.63×7.26±1.36
11	Surface	Non-glabrous	Non-glabrous	Glabrous
12	Leaves Margin	Toothed	Entire	Wavy
Cauline Leaves				
13	Number of leaves / plant	4.00±1.00	11.41±2.18	21±2.41
14	Size (cm)	4.23±0.70×4.63±0.64	6.53±0.59×7.40±0.74	7.41±2.18×21.00±1.41
15	Surface	Non-glabrous	Non-glabrous	Glabrous
16	Leaves Margin	Toothed	Entire	Entire
Stomata				
17	Size (µm)	21.66±1.56×18.3±0.36	23.82±0.76×19.58±0.67	26.74±0.35×20.55±0.48
18	Stomatal frequency on upper/ lower surface of leaf (mm^2)	5.41±0.45/3.25±0.36	5.48±0.48/7.45±0.45	3.25±0.36/3.78±0.11
19	Stomatal index of upper/ lower surface of leaf (µm)	22.28/12.45	23.45/13.24	24.50/14.32
Inflorescence				
20	Length (cm)	9.9±1.8	10.2±3.5	10.7±1.8
21	Diameter (cm)	2.69±0.23	2.39±0.28	2.34±0.30
22	Number of flowers/plant	9.5±1.58	11±2.16	12.2±1.0
Flower				
23	Petal size (mm)	4.77±0.59	4.06±0.8	4.15±0.91
24	Sepal size (mm)	3.01±0.39	2.99±0.40	3.38±0.5
Pollen size				
25	Pollen size (µm)	37.24±1.15×39.06±0.79	41.05 ± 0.52×44.55 ± 0.59	45.24±0.57×46.19±0.38

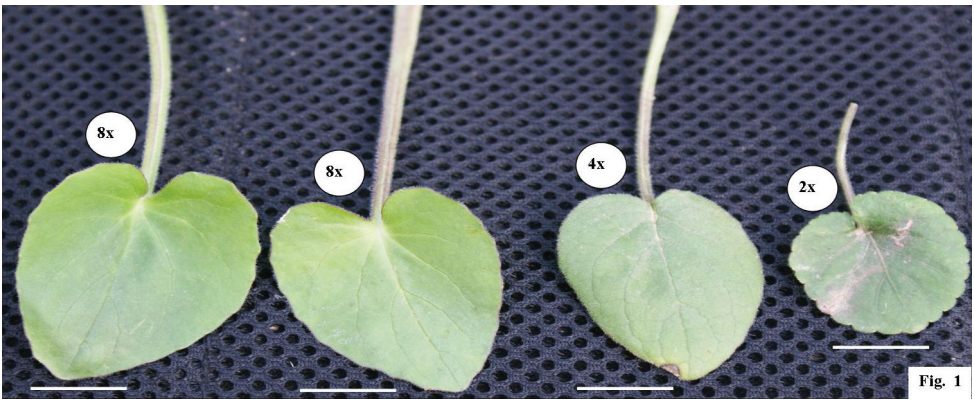

Figure 1. Leaves showing variations among 2x, 4x and 8x cytotypes of *Valeriana jatamansi*.

the altitude of 764–2236 m in the state. The octaploid cytotypes are restricted only to higher altitude localities (3,164–3,647 m) in Kullu district of Himachal Pradesh. Thus it is clear that north-western Himalayan region harbours maximum genetic diversity for the species (Fig. 3).

Meiotic abnormalities

The meiotic course was found to be abnormal in eight populations (Table 3). In such populations, abnormalities in the form of cytomixis, unoriented bivalents, chromatin stickiness, chromatin bridges and laggards, or multipolarity have been observed at different stages of meiosis (Fig. 2f–s; Table 3). Thus in the presently studied populations indicate the existence of intraspecific genetic diversities in the species. Such genetic differences have earlier been reported in different plant species (Sheidai et al. 2003, Rani et al. 2013, Jeelani et al. 2013). The phenomenon of chromatin transfer from early prophase to the pollen formation stage (Fig. 2f–h) has been observed in most of these populations with the highest percentage recorded in populations P-20 from Kullu II (Table 3).

Cytomixis in these populations resulted in the production of hyper- and hypoploid PMCs. According to Sarvella (1958), cytomixis results in some genetic consequences and it is a mechanism to explain the origin of aneuploid gametes. Some others considered it to be of great importance as the most probable consequence of cytomixis is the formation of hypo-, hyperploid and enucleated PMCs, aberrant microspore tetrads and pollen sterility (Sheidai and Fadaei 2005, Jeelani et al. 2011). Cytomixis results in the production of unreduced gametes in several angiosperms and leads to the production of aneuploid or polyploids plants (Fadaei et al. 2010, Mandal et al. 2013). The formation of unreduced gametes is of evolutionary significance in a way that it can lead to the production of plants with higher ploidy level through polyploidization (Villeux 1985). Chromatin stickiness involving few bivalents or whole complement

Table 3. Data on cytomixis, abnormal meiotic course and pollen fertility in *Valeriana jatamansi*.

	Cytomixis at Meiosis-I/ Meiosis II		**Meiotic course showing PMCs with**				**Pollen grains**
Population number	% of PMCs involved	Number of PMCs Involved	Chromosomal stickiness at M-I (%)	Bridges at Meiosis-I/ Meiosis-II (%)	Laggards at Meiosis-I/ Meiosis-II (%)	Micronuclei at T-II (%)	Fertility (%)
PP-1	8.33 (10/120)/	2-3	4.00 (4/100)	5.88 (6/102)/	2.86 (2/70)/	17.47 (18/103)	66.23
	7.07 (7/99)			-	2.63 (2/76)		
PP-3	5.78 (7/121)/	2-3	-	3.80 (4/105)/	2.70 (2/74)/	18.18 (16/88)	65.07
				-	2.66 (2/75)		
PP-6	4.34 (5/115)/	2-3	-	2.40 (3/125)/	3.30 (4/121)/	5.78 (7/121)	75.78
	4.59 (4/87)			1.73 (2/115)	-		
PP-10	-	-	4.87 (6/123)	2.27 (3/132)/	-	7.57 (10/132)	70.32
				2.60 (3/115)			
PP-14	5.88 (6/102)/	2-4	9.75 (12/123)	5.35 (6/112)/	5.69 (7/123)/	4.46 (5/112)	71.65
	6.25 (7/112)			3.92 (4/102)	4.83 (6/124)		
PP-16	-	-	7.33 (8/109)	-	3.53 (4/113)/	-	77.87
					-		
PP-17	-	-	8.33 (11/132)	2.52 (3/119)/	-	5.60(7/125)	79.67
				-			
PP-20	9.00 (9/100)/	2-4	-	-	5.73 (7/122)/	4.50(5/111)	79.65
	8.00 (10/125)				-		

was seen from prophase-I to metaphase-I (Fig. 2i–j). Cytomixis and chromatin stickiness are considered to be the result of genetic factors (Bellucci et al. 2003, Ghaffari 2006, Fadaei et al. 2010) and environmental factors (Nirmala and Rao 1996) as well as genomic–environmental interaction (Baptista-Giacomelli et al. 2000) and seems to be equally applicable to the presently investigated populations. Chromatin stickiness also results in the formation of fragmented chromatin (Kumar and Singhal 2011) and at present very low percentage of such fragments was observed.

During present investigations, single or multiple bridges (Fig. 2k) have been recorded in six populations with highest percentage in Population -14 from Prashar. According to Rothfels and Mason (1975) bridges may originate from chiasma formation in heterozygous inversions. Bridges and fragments are the results of spontaneous breakage and fusion of the chromosomes. Early disjunction of bivalents normally does

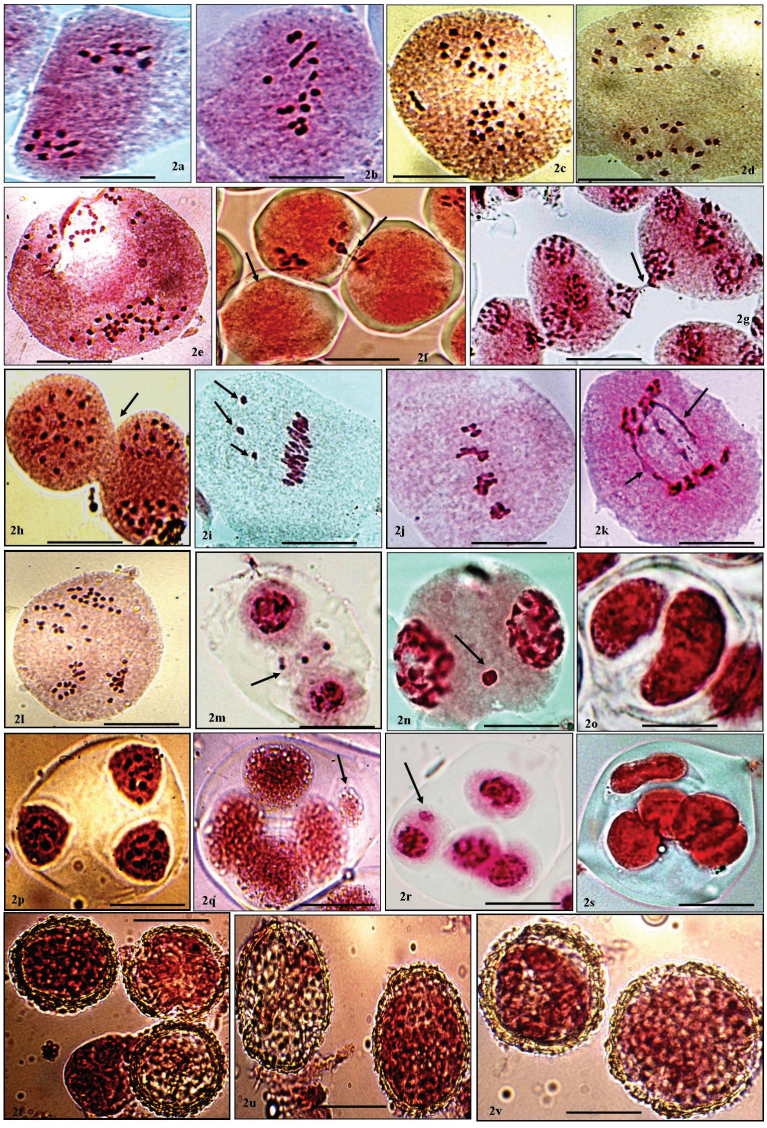

Figure 2. a (Diploid cytotype) PMC at Anaphase-I showing 8:8 distribution of chromosomes **b** (Diploid cytotype) PMC at Metaphase-I showing 8II bivalents **c** (Tetraploid cytotype) PMC at Anaphase-I showing 16:16 distribution of chromosomes **d** PMC at Metaphase-II showing 16:16 distribution of chromosome **e** (Octaploid cytotype) PMC at Anaphase-I showing 32:32 distribution of chromosomes **f** (Diploid cytotype) Three PMCs showing transfer of chromatin material (arrowed) **g** A group of PMCs (arrowed) involved in cytomixis at telophase-I **h** (Tetraploid cytotype) Size difference and direct connection between two PMCs during chromatin material (arrowed) **i** PMC at metaphase-I showing unoriented bivalent (arrowed) **j** PMC at metaphase-I showing chromatin stickiness **k** PMC at anaphase-I showing chromatin bridges (arrowed) **l** PMC at anaphase-II showing unequal distribution of chromosomes (arrowed) **m–n** PMC at telophase-I showing chromatin chromosomal laggards (arrowed) **o** Diad **p** Triad **q–r** Tetrad with micronuclei (arrowed) **s** Polyad **t–v** Fertile, sterile and Heterogeneous sized fertile pollen grains. Bar = 10 μm.

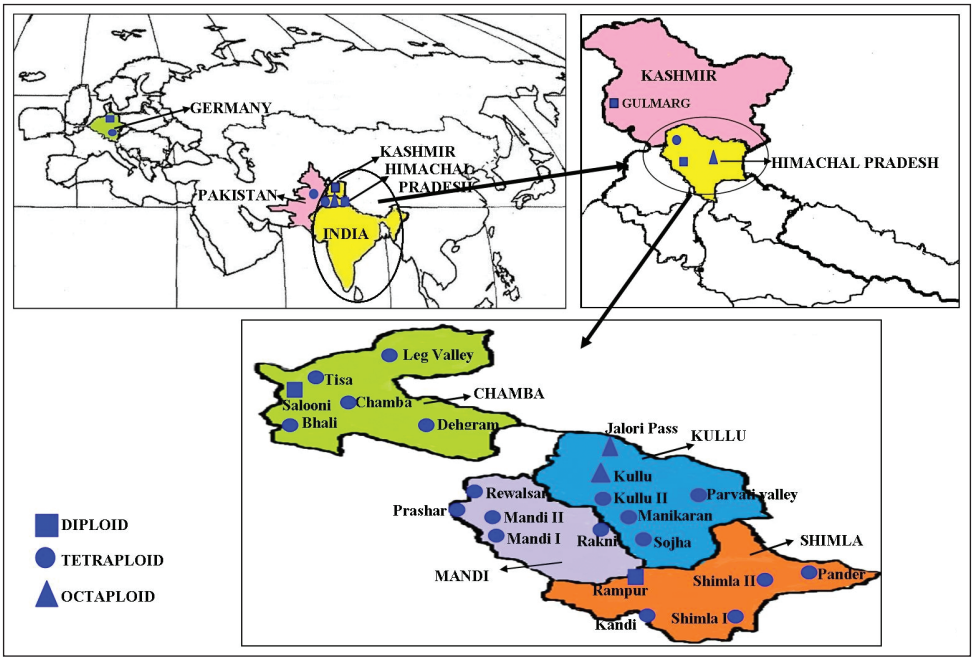

Figure 3. Geographical distribution of 2x, 4x and 8x cytotypes of *Valeriana jatamansi*.

not affect the normal distribution of chromosomes at anaphase-I, but late separation of bivalents which normally exists in hybrids and cytologically abnormal diploids causes some meiotic disturbances (chromatin bridges and laggards) and consequently pollen malformation (Wang et al. 2004). Bivalents and chromosomes that lag behind and are unable to reach at poles during anaphase-I, telophase-I, anaphase- II and telophase-II stages of meiosis form laggards. In our study, chromosomal laggards were noticed in six populations (Fig. 2l–n). There are different explanations for the formation of chromosomal laggards such as interlocking of bivalents and paracentric inversions (Sinha and Godward 1972, Tarar and Dyansagar 1980). One of the most acceptable reasons for the formation of chromosomal laggards is lack of synapsis at early prophase stages or precocious separation and delayed terminalization of chiasmata (Pagliarini 1990, Kumar and Tripathi 2007). All these meiotic abnormalities result in abnormal microsporogenesis, leading to the formation of monads, dyads, triads, or polyads (Fig. 2o–s; Table 4). Furthermore, micronuclei have also been observed in most of these species (Fig. 2q–r; Table 4). These meiotic abnormalities along with abnormal microsporogenesis lead to the formation of heterogeneous sized (large and small) fertile pollen grains and reduced pollen fertility (Fig. 2t–v). The frequency of large-sized pollen grains ranges from 3% to 4% in different populations. The occurrences of large pollen grains conforms to previous information about possibility of such pollen grains to be resulting from unreduced 2n gametes as has been seen in several angiosperms (Bertagnolle and Thomson 1995, Sheidai et al. 2008, Fadaei et al. 2010, Jeelani et al. 2011). Presence of genetic diversity within the populations of *V. jatamansi* stresses the need for further cytological analysis

Table 4. Data on abnormal microsporogenesis in different cytotypes of Valeriana jatamansi marked with abnormal meiosis.

	Microsporogenesis Accessions							
	Dyads		Triads		Tetrads		Polyads	
	WMN	WN	WMN	WN	WMN	WN	WMN	WN
PP-1	0.99 (1/101)		0.99 (1/101)	1.98 (2/101)	71.28 (72/101)	21.78 (22/101)	1.98 (2/101)	-
PP-3	-	-	2.04 (2/98)	-	76.53 (75/98)	21.42 (21/98)	-	-
PP-6	0.96 (1/104)	-	0.96 (1/104)	-	70.19 (73/104)	26.92 (28/104)	0.96 (1/104)	-
PP-10	2.04 (2/98)	1.02 (1/98)	2.04 (2/98)	-	88.77 (87/98)	6.12 (6/98)	-	-
PP-14	2.97 (3/101)	0.99 (1/101)	3.96 (4/101)	0.99 (1/101)	79.20 (80/101)	9.90 (10/101)	0.99 (1/101)	0.99 (1/101)
PP-16	-	0.94 (1/106)	2.83 (3/106)	0.94 (1/106)	84.90 (90/106)	10.37 (11/106)	-	-
PP-17	-	-	0.98 (1/102)	0.98 (1/102)	89.21 (91/102)	8.82 (9/102)	-	-
PP-20	1.86 (2/107)	0.93 (1/107)	3.73 (4/107)	0.93 (1/107)	76.63 (82/107)	14.01 (15/107)	0.93 (1/107)	0.93 (1/107)

WMN = without micronuclei; WM = with micronuclei.

from different geographical areas. Intraspecific variability at population level has been brought to the fore, to be conserved and/or utilized for further plant improvement programme. The genetic diversity in *V. jatamansi* points towards the need of studies for chemical and molecular characterization and biological activity of these variants to identify superiour chemotypes for further conservation and exploitation.

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RESEARCH ARTICLE

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Variability of 18rDNA loci in four lace bug species (Hemiptera, Tingidae) with the same chromosome number

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Abstract

Male karyotypes of *Elasmotropis testacea* (Herrich-Schaeffer, 1835), *Tingis cardui* (Linnaeus, 1758), *T. crispata* (Herrich-Schaeffer, 1838), and *Agramma femorale* Thomson, 1871 (Heteroptera, Cimicomorpha, Tingidae) were analyzed using conventional chromosome staining and FISH with 18S rDNA and $(TTAGG)_n$ telomeric probes. The FISH technique was applied for the first time in the Tingidae. In spite of the fact that all species showed the same chromosome number (2n = 12 + XY), they have significant differences in the number and position of rDNA loci. FISH with the classical insect $(TTAGG)_n$ probe produced no signals on chromosomes suggesting telomeres in lace bugs to be of some other molecular composition. Tingidae share absence of the $(TTAGG)_n$ telomeric sequence with all so far studied taxa of the advanced true bug infraorders Cimicomorpha and Pentatomomorpha.

Keywords

Karyotype, sex chromosomes, FISH, rDNA, $(TTAGG)_n$, Hemiptera, Heteroptera, Cimicomorpha, Tingidae

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Introduction

Tingidae (lace bugs) are a large widespread family of herbivorous bugs including 2200 species belonging to 280 genera. The family is subdivided into two, Tinginae and Cantacaderinae, or into three (Vianaidinae in addition) recent subfamilies; the subfamily Tinginae is the largest and the most diverse subfamily of lace bugs. Tingidae are placed in the Cimicomorpha, but their relationships within this large infraorder are not entirely clear (Golub and Popov 2012, Golub et al. 2012).

Many studies have proven that chromosome alterations are significant for species evolution and then, cytogenetics can be a useful tool for evolutionary, taxonomic, phylogenetic and speciation studies (White 1973, King 1993).

Cytogenetic data on members of the Tingidae are scarce and only involve species of the Tinginae. Currently, chromosome information of 29 species, belonging to 18 genera, i.e., approximately 1% and 6% respectively is known (Ueshima 1979, Nokkala and Nokkala 1984, Grozeva and Nokkala 2001). With one exception (see Discussion), the karyotypes of the species studied are similar in that they include six pairs of autosomes.

All previous investigations of lace bugs have been carried out using conventional chromosome staining techniques. Identification of individual chromosomes in karyotypes is a difficult task in the case of true bugs because of morphologically uniform holokinetic chromosomes. However, with the use of C-banding technique, Grozeva and Nokkala (2001) were successful in identifying separate chromosomes in 13 lace bugs species and revealing differences between them in C-band pattern. These findings showed that C-heterochromatin distribution has had a major role in the karyotype evolution of the family Tingidae.

In the past decades, fluorescence in situ hybridization (FISH) has increased the resolution of the true bugs' cytogenetics. Thanks to this technique, the analysis of the karyotypes has become more informative and comprehensive. In true bugs, ribosomal genes are commonly used as markers for the physical mapping of their chromosomes (reviewed in Grozeva et al. 2014).

Here, the first FISH-based study for the characterization of tingid karyotypes is presented. We describe the karyotypes of *Elasmotropis testacea* (Herrich-Schaeffer, 1835), *Tingis cardui* (Linnaeus, 1758), *T. crispata* (Herrich-Schaeffer, 1838), and *Agramma femorale* Thomson, 1871 after FISH with an 18S rDNA probe. Note that for two last species, the standard karyotype is reported for the first time.

Additionally, we used FISH with a $(TTAGG)_n$ probe to analyze whether the classical "insect" telomeric motif $(TTAGG)_n$ is present in the lace bug species. Previous studies on species of two cimicomorphan families (Miridae and Cimicidae) showed the absence of this telomeric repeat (Frydrychová et al. 2004, Grozeva et al. 2011).

Material and methods

The material studied is presented in Table 1.

Lace bug species were collected in 2014 by V. Golub in Republic of Bashkortostan, Russia. Only male adult specimens were analyzed. In field, the specimens were fixed im-

Table 1. Material used for chromosome analysis.

Species	Number of males/ chromosome plates studied	Locality and date of collection	Host plant
Elasmotropis testacea	2/37	Russia, Republic of Bashkortostan, South-Ural state natural reserve, env. of village Terekly, 12 km ENE of settl. Arhangelskoe, 54°26'N, 56°57'E, alt. 269 m, 5.08.2014	*Echinops* sp. (Asteraceae)
Tingis cardui	2/19	Russia, Republic of Bashkortostan, South-Ural state natural reserve, env. of settl. Inzer, 54°13'N, 57°34'E, alt. 349 m, 4.08.2014	*Carduus* sp. (Asteraceae)
T. crispata	3/143	Russia, Tolyatti, 53°31'N, 49°25'E, alt. 95 m, 13.08.2014	*Artemisia vulgaris* Linnaeus, 1753 (Asteraceae)
Agramma femorale	2/23	Russia, Republic of Bashkortostan, South-Ural state natural reserve, env. of village Revet', 54°11'N, 57°37'E, alt. 285 m, 10.08.2014	*Juncus* sp. (Juncaceae)

mediately after capturing in 3:1 fixative (96% ethanol: glacial acetic acid) and stored at 4 °C. In laboratory, testes were dissected in a drop of 45% acetic acid and squashed. The cover slips were removed using dry ice. Prior to staining, the preparations were examined by phase contrast microscopy. Chromosome staining techniques applied were a Feulgen-Giemsa method as described in Grozeva and Nokkala (1996) and fluorescence *in situ* hybridization (FISH) with 18S rDNA and $(TTAGG)_n$ telomeric probes. 18S rDNA and $(TTAGG)_n$ probe preparation and hybridization were carried out as described in Grozeva et al. (2010, 2014). In brief, chromosome preparations were treated with 100 μg/ml RNaseA and 5 mg/ml Pepsin solution to remove excess RNA and proteins. Chromosomes were denatured on a slide in hybridization mixture with biotinylated 18S rDNA probe from the genomic DNA of *Pyrrhocoris apterus* (Linneus, 1758) and rhodaminated $(TTAGG)_n$ probe with addition of salmon sperm DNA blockage and then hybridized for 36 h. Hybridization signals were detected with NeutrAvidin-FITC. Chromosomes were mounted in an antifade medium (ProLong Gold antifade reagent with DAPI, Invitrogen) and covered with a glass coverslip. 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.

Results

Conventional staining and FISH with an 18S rDNA probe

Tingis crispata, 2n = 14 (12A + XY)
Published data: absent

During the diffuse stage, the autosomes were de-condensed whilst the X and Y chromosomes appeared to be fused and heteropycnotic (Fig. 1). Early diplotene (Fig.

2) revealed six autosomal bivalents, each with one, rarely two chiasmata, and the X and Y chromosomes positioned close to each other. The bivalents gradually decreased in size, and sex chromosomes were of different size. At early metaphase I (MI), sex chromosomes were seen well apart from each other (Fig. 3) whilst at mature MI they formed a heteromorphic pseudobivalent (Fig. 4). At early anaphase I, sex chromosomes segregated ahead of the autosomal bivalents (Fig. 5). At MII, the two daughter nuclei, each with seven elements, namely, 6 autosomes and either the X or the Y chromosome, were present (Fig. 6).

The 18S rDNA FISH resulted in appearance of a comparatively small interstitial signal in the larger sex chromosome (presumably, the X) and a larger subterminal signal in the smaller sex chromosome (presumably the Y) (Fig. 7).

Tingis cardui, 2n = 14(12A + XY)
Published data: 2n = 14(12A + XY) (Southwood and Leston 1959)

At first prometaphase subjected to 18S rDNA FISH, eight elements were present, including six autosomal bivalents and X and Y chromosomes which lied separately from each other. The bivalents constituted a series decreasing in size, and sex chromosomes were of different size. The subterminally located 18S rDNA sites were revealed on both homologues of a medium-sized autosomal bivalent (Fig. 8).

Elasmotropis testacea, 2n = 14(12A + XY)
Published data: 2n = 14(12A + XY) (Grozeva and Nokkala 2001)

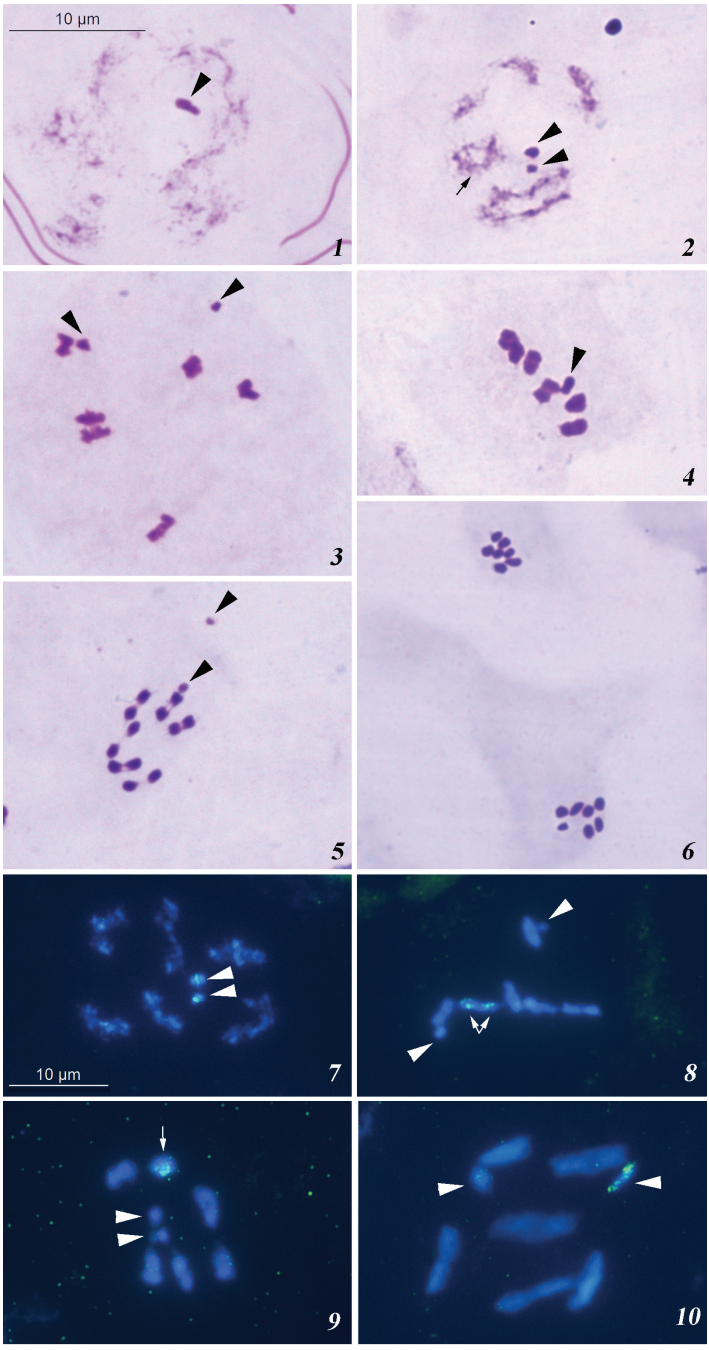
At first metaphase subjected to 18S rDNA FISH, eight elements were present, including six autosomal bivalents which formed a ring with a pseudobivalent of the X and Y chromosomes located in its center. The bivalents constituted a series decreasing in size, and sex chromosomes were of similar size. The subterminally located 18S rDNA sites were revealed in a medium-sized bivalent (Fig. 9).

Agramma femorale, 2n = 14(12A + XY)
Published data: absent

At first prometaphase subjected to 18S rDNA FISH, eight elements were present, including six autosomal bivalents and X and Y chromosomes which lied separately from each other. The bivalents constituted a series decreasing in size, sex chromosomes could not be told apart because of their similar size. The 18S rDNA signals were dispersed all over one of the two sex chromosomes (Fig. 10).

FISH with a $(TTAGG)_n$ telomeric probe

In none of the species studied, the $(TTAGG)_n$ telomeric probe produced fluorescent signals.



Figures 1–10. 1–6 Male meiosis in *Tingis crispata* (conventional staining): **1** diffuse stage **2** early diakinesis, two-chiasmate bivalent is indicated by arrow **3** early MI **4** mature MI **5** early AI **6** MII. Sex chromosomes are indicated by arrowheads **7–10** Meiotic chromosomes in Tingidae species after FISH with an 18S rDNA probe: **7** diakinesis in *T. crispata* **8** first prometaphase in *T. cardui* **9** MI in *Elasmotropis testacea* **10** first prometaphase in *A. femorale*. Sex chromosomes are indicated by arrowheads; autosomally located signals are indicated by arrows.

Discussion

Like other true bugs, Tingidae have holokinetic chromosomes (Ueshima 1979, Kuznetsova et al. 2011). These chromosomes possess diffuse or non-localized centromeres and can therefore display a unique capability for karyotype evolution via occasional fusion/fission events (White 1973). In spite of this, both previous cytogenetic investigations (Ueshima 1979, Nokkala and Nokkala 1984, Grozeva and Nokkala 2001) and our new data suggest that Tingidae are characterized by a stable number of autosomes, 12 in diploid complements. The only exception seems to be *Acalypta parvula* (Fallén, 1807) which has, according to Southwood and Leston (1959), 2n = 12(10A + XY) in a population from British Isles. However males of this species from Finland were reported to have 2n = 12A + X (Grozeva and Nokkala 2001). Assuming these chromosome data are correct, one can suggest the existence of two species hidden under one species name. The majority of hitherto studied lace bug species, namely 25 of the 29, possess a XY/XX type of sex determination. This sex chromosome system was suggested to represent a plesiomorphic state in the Heteroptera, and the sporadic occurrence of X(0) bed bug species to be due to repeated loss of the Y chromosome, i.e. a result of convergent evolution (homoplasy) (Nokkala and Nokkala 1983, 1984, Kuznetsova et al. 2011, Grozeva et al. 2014). Such a loss has also occurred at least twice within the Tingidae: in the genera *Acalypta* Westwood, 1840 and *Kalama* Puton, 1876. All the three studied *Acalypta* species, namely, *A. carinata* (Panzer, 1806), *A. nigrina* (Fallén, 1807), and most likely also *A. parvula* (Grozeva & Nokkala, 2001), and a single studied *Kalama* species, namely *K. tricornis* Schrank, 1801 (Nokkala and Nokkala 1984: as *Dictyonota tricornis* (Schrank, 1801), Grozeva and Nokkala 2001), were found to have a derived system X(0).

For insects with holokinetic chromosomes the low number of chiasmata is characteristic and is considered as a result of a specific structure of holokinetic bivalents (Nokkala et al. 2004). In Tingidae, one or occasionally two chiasmata in every bivalent were described (Ueshima 1979, Grozeva and Nokkala 2001). This pattern is also revealed in the four species here examined. Within Cimicomorpha, Tingidae share male chiasmate meiosis with Reduviidae (Ueshima 1979), whereas other families for which such evidence is available, namely, Microphysidae, Nabidae s.str., Anthocoridae s.str., Cimicidae, and Miridae, seem to have achiasmate meiosis in males (Kuznetsova et al. 2011).

In “standard” meiosis, during the first division all the chromosomes reduce in number (reductional division), whereas during the second division the chromatids separate (equational division), and this pattern is named “pre-reduction” (White 1973). However Heteroptera show an inverted sequence of meiotic divisions for sex chromosomes in males, the so-called “sex chromosome post-reduction”. It means that, unlike autosomes, the sex chromosome(s) divide equationally at anaphase I and reductionally at anaphase II. On very rare occasion, in individual bug species, a pre-reductional division of sex chromosomes was observed, and such species have also been reported within cimicomorphan families Miridae (Grozeva et al. 2006, 2007) and Reduviidae (Manna and Deb-Mallick 1981). Importantly, lace bugs are the only heteropteran

family showing pre-reduction of sex chromosomes in spermatogenesis of all the studied species (Ueshima 1979, Grozeva and Nokkala 2003, present study). Since all other members of the Hemiptera invariably display pre-reduction, the sex chromosomes' post-reduction can be considered as an autapomorphy of true bugs without Tingidae.

In groups with holokinetic chromosomes, the main problem is to identify individual chromosomes and chromosomal regions in karyotypes. Different cytogenetic techniques, e.g. C-banding, DNA-specific fluorochrome staining, $AgNO_3$ staining, make possible only a few markers to be revealed in true bugs' karyotypes (Papeschi and Bressa 2006, Kuznetsova et al. 2011). Regarding the Tingidae, a single work aimed to reveal differences between species in C-banding pattern was published by Grozeva and Nokkala (2001). The 13 studied species belonging to 10 genera were found to differ in the number (from one to eight per haploid complement) and location (terminal, interstitial or both) of bands on both autosomes and sex chromosomes. The data obtained showed that a quite substantial redistribution of chromosome material within chromosomes occurred during the evolution of this group without chromosome fragmentation or fusions (Grozeva and Nokkala 2001). Thus, the species-specific organization of the constitutive heterochromatin can be used as an additional cytogenetic marker for the lace bug species differentiation.

In order to reveal additional chromosomal markers and gain deeper insights into the evolution of the Tingidae, we have applied FISH with 18S rDNA and telomeric $(TTAGG)_n$ probes to the four species from the present study. This is the first time that the lace bugs have been the subject of a molecular cytogenetic study. Physical location of genes remains very poorly studied in true bugs. Out of approximately 40.000 described species (Weirauch and Schuh 2011), only 94 species have been investigated in this respect and only the rRNA genes and telomeric sequences were mapped (Grozeva et al. 2014). The species studied belong to 38 genera, 10 families, and three (out of 8) infraorders including Nepomorpha (Belostomatidae), Pentatomomorpha (Coreidae, Lygaeidae, Pentatomidae, and Pyrrhocoridae), and Cimicomorpha (Cimicidae, Largidae, Miridae, Reduviidae, and Rhopalidae). The sites for rRNA at a rate of one to four (per diploid genome) were found to locate variously in different species: either on autosomes (the largest or one of the medium-sized pairs), or on m-chromosomes, or on sex chromosomes (X or both X and Y) or on both a pair of autosomes and the X-chromosome. The autosomal location seems to predominate being found in half of the species studied. The majority of rDNA sites show a terminal localization, however in rare cases they are positioned interstitially in chromosomes. The most impressive variation regarding the number and the type of chromosomes (autosomes and/or sex chromosomes) that carried the rRNA genes is described in the kissing bug subfamily Triatominae (Cimicomorpha: Reduviidae) even though it demonstrates a highly conserved karyotype including 20 autosomes in the great majority of studied species (Panzera et al. 2012, 2014, Pita et al. 2013).

A very similar variation holds for the four tingid species possessing the same karyotype, 2n = 12 + XY, including two closely related species of the genus *Tingis* Fabricius, 1803. Our findings suggest that chromosomal divergence can occur among seemingly

conserved karyotypes and may play a role in reproductive isolation and speciation of the family Tingidae. Males of *T. crispata* were found to have rDNA sites on both sex chromosomes, interstitial on the larger and subterminal on the smaller. Since in the XY true bugs species the larger of the two sex chromosomes is conventionally taken as the X (e.g. Ueshima 1979, Grozeva et al. 2014), we suggested that this is also the case in *T. crispata.* In contrast, males of *T. cardui* showed subterminally located sites on one medium-sized pair of autosomes. In the two remaining species, *E. testacea* and *A. femorale*, ribosomal genes were found on a medium-sized autosomal pair (located subterminally) and on one of the two homomorphic sex chromosomes (multiple sites), respectively.

Changes in the number and location of rDNA loci are a well-known phenomenon in eukaryotic organisms, including true bugs (e.g. Panzera et al. 2012, Grozeva et al. 2014). As regards the ability of rDNA clusters to move and vary in number among the closely related species with the same chromosome number, different mechanisms have been suggested, including structural chromosome rearrangements (inversions and translocations), transposition, ectopic recombination, transposable elements (Panzera et al. 2012, Pita et al. 2013, Grozeva et al. 2014) and even a homoploid hybrid speciation, i.e. hybridization without a change in chromosome number (referenced in Vershinina et al. 2015). In Triatominae bugs, the occurrence of heterologous associations among non-homologous autosomes and heterochromosomes seems to favor the transposition and ectopic recombination hypotheses (Panzera et al. 2012). However, much more work is needed to identify mechanisms responsible for the ribosomal loci variation in lace bugs.

The majority of insect species is known to share the telomeres composed of the pentanucleotide TTAGG repeat which is considered as an ancestral telomeric motif in this large group of Arthropoda (Frydrychová et al. 2004, Vitková et al. 2006). Many higher level insect groups preserved this telomeric sequence, but some of them have lost it during the evolution. Recently, it has been shown that in Heteroptera, the classical insect $(TTAGG)_n$ telomeric sequence is absent in the evolutionarily advanced families Miridae, Cimicidae (Cimicomorpha), Pyrrhocoridae and Pentatomidae (Pentatomomorpha) (Frydrychová et al. 2004. Grozeva et al. 2011) but is present in the family Belostomatidae from a more basal infraorder Nepomorpha (Kuznetsova et al. 2012). According to our data, this telomeric sequence is absent in all the four examined lace bug species and probably in the family Tingidae as a whole. This new finding reinforces the hypothesis that the $(TTAGG)_n$ telomeric motif was lost during the evolution of the Heteroptera, at least in the common ancestor of large infraorders Pentatomomorpha and Cimicomorpha (Kuznetsova et al. 2012).

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RESEARCH ARTICLE

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First evidence for (TTAGG)n telomeric sequence and sex chromosome post-reduction in Coleorrhyncha (Insecta, Hemiptera)

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Abstract

Telomeric repeats are general and significant structures of eukaryotic chromosomes. However, nothing is known about the molecular structure of telomeres in the enigmatic hemipteran suborder Coleorrhyncha (moss bugs) commonly considered as the sister group to the suborder Heteroptera (true bugs). The true bugs are known to differ from the rest of the Hemiptera in that they display an inverted sequence of sex chromosome divisions in male meiosis, the so-called sex chromosome post-reduction. To date, there has been no information about meiosis in Coleorrhyncha. Here we report a cytogenetic observation of *Peloridium pomponorum*, a representative of the single extant coleorrhynchan family Peloridiidae, using the standard chromosome staining and fluorescence *in situ* hybridization (FISH) with a $(TTAGG)_n$ telomeric probe. We show that *P. pomponorum* displays 2n = 31 (30A + X) in males, the classical insect $(TTAGG)_n$ telomere organization and sex chromosome post-reduction during spermatocyte meiosis. The plesiomorphic insect-type $(TTAGG)_n$ telomeric sequence is suggested to be preserved in Coleorrhyncha and in a basal heteropteran infraorder Nepomorpha, but absent (lost) in the advanced heteropteran lineages Cimicomorpha and Pentatomomorpha. The telomere structure in other true bug infraorders is currently unknown. We consider here the inverted sequence of sex chromosome divisions as a synapomorphy of the group Coleorrhyncha + Heteroptera.

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Keywords
Karyotype, sex chromosome post-reduction, $(TTAGG)_n$ telomeric sequence, Hemiptera, Coleorrhyncha, Peloridiidae, *Peloridium pomponorum*

Introduction

Coleorrhyncha (moss bugs) are little-known insects believed to be relict members (survivors) of an ancient evolutionary lineage which split off from the rest of Hemiptera during the late Palaeozoic. The suborder includes the sole extant family Peloridiidae with 17 genera and 37 species in South America (Argentina and Chile), Australia, New Zealand, New Caledonia, and Lord Howe Island. The phylogenetic relationships of peloridiids within Hemiptera, the largest nonholometabolan insect assemblage, have been a matter of contentious debates for a long time. In the past, they have been variously assigned to Heteroptera or Homoptera but today, the peloridiids are generally put to their own suborder Coleorrhyncha and are commonly considered as the sister group to the suborder Heteroptera (Wootton 1965, Schlee 1969, Wheeler et al. 1993, Campbell et al. 1995, Ouvrard et al. 2000, Burckhardt 2009, Cryan and Urban 2012), whereas there is also some support for other opinions (Cobben 1978, Popov and Shcherbakov 1996, Cui et al. 2013).

Recently, the first cytogenetic data on Coleorrhyncha were published (Grozeva et al. 2014b). Males of *Xenophyes cascus* Bergroth, 1924 from New Zealand were reported to display paired testes composed each of a single follicle, holokinetic chromosomes, a karyotype of 2n = 27 (26A + X), sex chromosome system of an X(0) type, and one chiasma in every bivalent in meiosis.

The ends of chromosomes are known to be cupped by specific nucleoprotein structures, the telomeres, which are responsible for their stability. DNA of the telomeric regions consists of tandemly repeated short nucleotide motifs. Comparative analysis of these motifs in different groups of organisms showed that they tend to be conserved in particular groups, for example, TTTAGGG in plants, TTAGGC in nematodes, TTAGG in arthropods, and TTAGGG in vertebrates (Traut et al. 2007, Lukhtanov and Kuznetsova 2010). Information on the telomere structure is presently available for many groups of insects (Sahara et al. 1999, Frydrychová et al. 2004, Vitková et al. 2005, Grozeva et al. 2010, 2011, Kuznetsova et al. 2012, 2015, Maryańska-Nadachowska et al. 2013, Golub et al. 2014, 2015, Gokhman et al. 2014, Vershinina et al. 2015, Lachowska-Cierlik et al. 2015). Among insects, the $(TTAGG)_n$ sequence has been detected in most tested orders and is considered as the ancestral telomeric motif not only for insects but also for all arthropods (Vitková et al. 2005). Despite of this, in several insect groups, e.g. in Diptera, in some Coleoptera and Hymenoptera, the classical insect-type $(TTAGG)_n$ motif is absent (Frydrychová and Marec 2002, Frydrychová et al. 2004, Vitková et al. 2005, Gokhman et al. 2014). Among Hemiptera Heteroptera, the advanced infraorders Cimicomorpha and Pentatomomorpha also appear to have lost this telomeric motif (Grozeva et al. 2011 and references therein, Golub et al. 2015).

Although data on other infraorders were absent, there was a general belief that all members of the Heteroptera lost the $(TTAGG)_n$ motif (Frydrychová et al. 2004, Lukhtanov and Kuznetsova 2010, Grozeva et al. 2010, 2011). However, the recent finding of this motif in a more basal true bug infraorder Nepomorpha (Kuznetsova et al. 2012) showed that the Heteroptera are heterogeneous for the telomere organization.

The Heteroptera are known to differ from the rest of the Hemiptera in that they display an inverted sequence of sex chromosome divisions during spermatocyte meiosis, the so-called sex chromosome post-reduction. To date, there has been no information about meiosis in Coleorrhyncha.

In this paper we present first data on telomere structure and male meiosis in Coleorrhyncha. We report the karyotype, meiosis with special reference to the behavior of sex chromosomes, and molecular composition of telomeres in males of *Peloridium pomponorum* Shcherbakov, 2014.

Material and methods

Specimens of *Peloridium pomponorum* were collected at the Biological Station Senda Darwin (Chile, Region X, Isla Grande de Chiloé, Ancud) in January-February 2014 from three different species of *Sphagnum* Linnaeus, 1753 (*S. fimbriatum* Wilson, 1847, *S. magellanicum* Bridel, 1798 and *S. falcatulum* Bescherelle, 1885) and *Hypnum chrysogaster* Müller, 1851; fixed alive in 3:1 ethanol/acetic acid and shipped in the fixative a couple of weeks later to the lab, where further analyses were undertaken.

Preparations were made from testes, which were dissected in a drop of 45% acetic acid and squashed under a coverslip on a glass microscope slide. The slides were frozen using dry ice, the coverslips were removed with a razor blade, and the preparations were air dried.

Spread chromosome plates were found in testes of 19 males (a total of 32 adults and the last instar nymphs were examined). For the standard staining, the method described in Grozeva and Nokkala (1996) with minor modifications was used. In brief, the preparations were first subjected to hydrolysis in 1 N HCl at room temperature for 20 min, then at 60 °C for 8 min and stained in Schiff's reagent for 20 min. After rinsing thoroughly in distilled water, the preparations were additionally stained in 4% Giemsa in Sorensen's buffer, pH 6.8 for 20 min, rinsed with distilled water, air-dried, and mounted in Entellan.

The molecular structure of telomeres was investigated by fluorescence *in situ* hybridization of chromosomes (FISH) with a $(TTAGG)_n$ probe. The telomere probe was generated by non-template PCR and labelled with Rhodamine-5-dUTP (GeneCraft, Cologne, Germany). FISH was performed as described in Grozeva et al. (2011, 2014a). Chromosome preparations were treated with 100 μg/ml RNaseA, incubated in 5 mg/ml pepsin in 0.01 M HCl to remove excessive amounts of RNAs and proteins. After pretreatment, the chromosomes were hybridized with a hybridization mixture containing about 100 ng of labelled probe and 10 μg of sonicated salmon-sperm DNA (Sigma-Aldrich, St. Louis, MO, USA).

Chromosomes were mounted in an antifade medium (ProLong Gold antifade reagent with DAPI, Invitrogen) and covered with a glass coverslip. 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.

Results

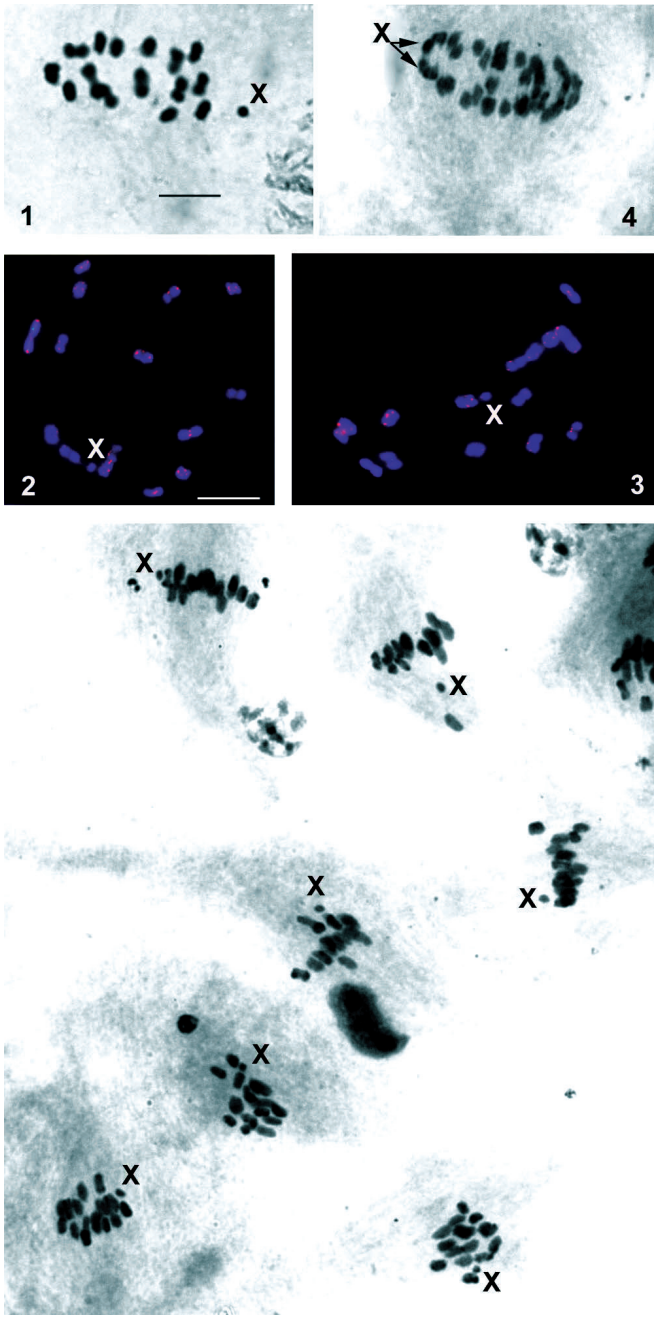
In *Peloridium pomponorum* males, the paired testes are composed each of a single follicle, and the meiotic karyotype comprises 16 elements, including 15 autosomal bivalents and a univalent X chromosome, at first metaphase (MI) (Figs 1–3). Thus, the male diploid karyotype of the species consists of 2n = 31 (30A + X). The chromosomes show no primary constrictions (the centromeres), thereby testifying that they are holokinetic and display, instead of localized, a diffuse kinetochore. The bivalents constitute a continuous series gradually decreasing in size and form each one, rarely two, chiasmata. The X chromosome appears as one of the smallest chromosomes in the karyotype. At the first anaphase (AI), the autosomes segregate reductionally, whereas the univalent sex chromosome undergoes the equational division (the separation of sister chromatids) (Fig. 4), so that all the second metaphases (MII) carry the X chromosome (Fig. 5). It is notable that the X chromosome tends to be situated outside the division plane both in MI and MII plates.

In all the preparations, a $(TTAGG)_n$ telomeric probe hybridized to the ends of the chromosomes (Fig. 2, 3) indicative of the presence of this telomeric nucleotide sequence in *P. pomponorum*.

Discussion

So far, the only coleorrhynchan species with known karyotype was *Xenophyes cascus* (Grozeva et al. 2014b). Males of this species originated from New Zealand were shown to have holokinetic chromosomes, as all other Hemiptera, and karyotype of 2n = 27 (26A + X). Despite difference in the number of autosomes, i.e. 26 in *X. cascus* while 30 in *P. pomponorum* from Chile analyzed here, these species appear similar in that they have paired testes consisting each of a single follicle (a pattern probably shared by all peloridiids; Grozeva et al. 2014b), holokinetic chromosomes (like in all other Hemiptera; White 1973), the formation of one, rarely two, chiasmata per bivalent (a characteristic property of holokinetic bivalents; Nokkala et al. 2004), the sex chromosome system of an X(0) type, decreasing size differences between chromosomes, and the X as one of the smallest chromosomes of the set.

The male diploid chromosome number in true bugs ranges from 2n=4 to 2n=80; however, the great majority of the studied species show 2n varying between 14 and 34 (Papeschi and Bressa 2006) and, thus, both chromosome numbers found to date in peloridiid species fall into this range.



Figures 1–5. Male meiotic chromosomes of *Peloridium pomponorum* subjected to standard staining (**1, 4, 5**) and FISH with a $(TTAGG)_n$ telomeric probe (**2, 3**). **1** MI, n = 16 (15AA + X) **2, 3** MI, n = 16 (15AA + X); hybridization signals (red) are located at the ends of chromosomes **4** AI, the sister chromatids (arrowed) of X chromosome are separated and oriented toward opposite spindle poles **5** part of a secondary spermatocyte cyst; X chromosome is present in every MII plate evidencing for the equational division during the first division. Bar = 10 μm.

The X(0) sex determination system is generally accepted as an ancestral one in Insecta (Blackman 1995). This system is prevailing in most Hemiptera, with the only exception of the Heteroptera. In this group, an XY system appears characteristic of the overwhelming majority of studied species whereas an X(0) system occurs only sporadically, being encountered in separate representatives of both primitive and advanced taxa (Ueshima 1979, Papeschi and Bressa 2006, Kuznetsova et al. 2011). Two contradictory hypotheses for the evolution of sex chromosomes in true bugs supported by different sources of evidence have been proposed. One of these holds that the XY system has evolved from an X(0) system (Ueshima 1979) while the other assumes that the XY mechanism is plesiomorphic, the existence of the X(0) true bug species being a result of the repeated loss of the Y chromosome during the evolution (Nokkala and Nokkala 1983, 1984, Grozeva and Nokkala 1996). Deducing the ancestral state of a character for the taxon requires knowledge on this character state in the basal taxa. Of the two most primitive true bug infraorders, Enicocephalomorpha and Dipsocoromorpha (Štys and Kerzhner 1975), the cytogenetic information is currently available for the six species of the latter (Cobben 1968, Grozeva and Nokkala 1996). Species of the genera *Alpagut* Kıyak, 1995, *Cryptostemma* Herrich-Schaeffer, 1835 and *Pachycoleus* Fieber, 1860 (the family Dipsocoridae) were shown to differ both in chromosome number and sex chromosome systems. Specifically, *A. castaneovitreus* (Linnavuori, 1951) displays 2n = 22 (18A + 2m? + XY) (Grozeva and Nokkala 1996, as *Cryptostemma* (*Harpago*) *castaneovitreus* Linnavuori, 1951); *C. hickmani* Hill, 1987 – 2n = 22 (18A + 2m + XY); *P. pusillimus* (J. Sahlberg, 1870) – 2n = 21 (16A + 2m + XY_1Y_2); while *P. waltli* Fieber, 1860 (Cobben 1968: as *Pachycoleus rufescens* J. Sahlberg, 1875) – 2n = 21 (20A + X). On the other hand, both studied representatives of the family Schizopteridae, namely, *Pateena elimata* Hill, 1980 and *Rectilamina australis* Hill, 1984, were found to share 2n = 33 (30A + 2m + X) (Grozeva and Nokkala 1996). The occurrence of an X(0) system both in dipsocorids and schizopterids as well as in peloridiids seems to favor the Ueshima's (1979) hypothesis, however, much more data from the primitive true bug taxa are needed to choose with certainty between the two alternatives.

With very rare exceptions (e.g. the family Tingidae; Ueshima 1979, Golub et al. 2015), true bugs show an inverted sequence of sex chromosome divisions in male meiosis, the so-called "sex chromosome post-reduction" (Ueshima 1979). It means that, in spermatocyte meiosis the first division is reductional for the autosomes and equational for the sex chromosomes, whereas the second division is, on the other hand, reductional for the sex chromosomes and equational for the autosomes. The significance of this unusual pattern is unknown. We observed that *P. pomponorum* males likewise share the sex chromosome post-reduction. Taking into account that all other members of the Hemiptera display the "normal" pre-reductional sequence of sex chromosome divisions (White 1973), we consider the inverted sequence as a synapomorphy of the group Coleorrhyncha + Heteroptera.

Recently, it has been shown that the classical insect $(TTAGG)_n$ telomeric sequence is absent in members of the evolutionarily advanced true bug infraorders Cimicomorpha

(at least in the families Miridae, Cimicidae and Tingidae for which data are available) and Pentatomomorpha (at least in the families Pyrrhocoridae and Pentatomidae for which data are available) (Frydrychová et al. 2004, Grozeva et al. 2010, 2011, Golub et al. 2015). Moreover, dot-blot hybridization of genomic DNA from the cimicomorphan species *Cimex lectularius* Linnaeus, 1758, *Oxycarenus lavaterae* (Fabricius, 1787), and *Nabis* sp. did not suggest any other candidate telomeric sequence, including, besides the insect TTAGG, also ciliate TTTTGGGG and TTGGGG, nematode TTAGGC, shrimp TAACC, vertebrate TTAGGG, and plant TTTAGGG (Grozeva et al. 2011) thus leaving the question of the telomeric motif(s) in these species open.

However in more recent times, the presence of the $(TTAGG)_n$ telomeric repeat was documented by FISH for the family Belostomatidae from a more basal true bug infraorder Nepomorpha (Kuznetsova et al. 2012) and now is confirmed for the peloridiid species *P. pomponorum*. These new findings reinforce the hypothesis (Kuznetsova et al. 2012) that the plesiomorphic insect-type $(TTAGG)_n$ telomere structure preserved in the basal true bug taxa was subsequently lost during the further evolution of the Heteroptera, at least in the last ancestor of a monophyletic lineage including Pentatomomorpha and Cimicomorpha.

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RESEARCH ARTICLE

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(GAA)n microsatellite as an indicator of the A genome reorganization during wheat evolution and domestication

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Abstract

Although the wheat A genomes have been intensively studied over past decades, many questions concerning the mechanisms of their divergence and evolution still remain unsolved. In the present study we performed comparative analysis of the A genome chromosomes in diploid (*Triticum urartu* Tumanian ex Gandilyan, 1972, *T. boeoticum* Boissier, 1874 and *T. monococcum* Linnaeus, 1753) and polyploid wheat species representing two evolutionary lineages, Timopheevi (*T. timopheevii* (Zhukovsky) Zhukovsky, 1934 and *T. zhukovskyi* Menabde & Ericzjan, 1960) and Emmer (*T. dicoccoides* (Körnicke ex Ascherson & Graebner) Schweinfurth, 1908, *T. durum* Desfontaines, 1798, and T. *aestivum* Linnaeus, 1753) using a new cytogenetic marker – the pTm30 probe cloned from *T. monococcum* genome and containing $(GAA)_{56}$ microsatellite sequence. Up to four pTm30 sites located on 1AS, 5AS, 2AS, and 4AL chromosomes have been revealed in the wild diploid species, although most accessions contained one–two (GAA)n sites. The domesticated diploid species *T. monococcum* differs from the wild diploid species by almost complete lack of polymorphism in the distribution of (GAA)n site. Only one (GAA)n site in the 4AL chromosome has been found in *T. monococcum*. Among three wild emmer (*T. dicoccoides*) accessions we detected 4 conserved and 9 polymorphic (GAA)n sites in the A genome. The (GAA)n loci on chromosomes 2AS, 4AL, and 5AL found in of *T. dicoccoides* were retained in *T. durum* and *T. aestivum*. In species of the Timopheevi lineage, the

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only one, large (GAA)n site has been detected in the short arm of 6A^t chromosome. (GAA)n site observed in *T. monococcum* are undetectable in the A^b genome of *T. zhukovskyi*, this site could be eliminated over the course of amphiploidization, while the species was established. We also demonstrated that changes in the distribution of (GAA)n sequence on the A-genome chromosomes of diploid and polyploid wheats are associated with chromosomal rearrangements/ modifications, involving mainly the NOR (nucleolus organizer region)-bearing chromosomes, that took place during the evolution of wild and domesticated species.

Keywords

Triticum monococcum, *T. boeoticum*, *T. urartu*, *T. zhukovskyi*, *T. dicoccoides*, (GAA)n microsatellite, FISH

Introduction

The genus *Triticum* Linnaeus, 1753 comprises species at different ploidy levels, from diploid to hexaploid. Common wheat *T. aestivum* L., 1753 is natural allopolyploid with the genome BBAADD, which emerged about 8–10 thousand years ago (TYA) *via* the cross of tetraploid Emmer species (BBAA genome) with *Aegilops tauschii* Cosson, 1850 (DD genome). Another hexaploid wheat, *T. zhukovskyi* Menabde & Ericzjan, 1960 (genome GGA^{t}A^{t}A^{b}A^b) was discovered in 1957, in the Zanduri region of Western Georgia and is regarded as natural allopolyploid of *T. timopheevii* (Zhukovsky) Zhukovsky, 1934 and *T. monococcum* L., 1753 growing in the same area (Jakubtsiner 1959, Tavrin 1964). As is currently assumed by the majority of researchers, tetraploid Emmer (*T. dicoccoides* ((Körnicke ex Ascherson & Graebner) Schweinfurth, 1908, *T. durum* Desfontaines, 1798, etc. genome BBAA) and Timopheevi (*T. araraticum* Jakubziner, 1947, *T. timopheevii*, and *T. militinae* Zhukovsky & Miguschova, 1969, genome GGA^{t}A^t) wheats occurred as a result of hybridization between the ancestral forms of *Ae. speltoides* Tausch, 1837 as a maternal parent and *T. urartu* Tumanian ex Gandilyan, 1972, as a paternal parent (Dvorak et al. 1988, Tsunewaki 1996, Huang et al. 2002). Although both evolutionary lineages of the tetraploid wheats originated *via* hybridization of closely related parental forms, their emergence occurred independently at different times and probably in different places. In particular, the origin of the tetraploid *T. dicoccoides* is dated back to over 500 TYA, versus *T. araraticum*, dated back to 50–300 TYA (Mori et al. 1995, Huang et al. 2002, Levy and Feldman 2002).

The diploid wheats are the most ancient members of the genus *Triticum*. Among them taxonomists recognize three species, namely, cultivated *T. monococcum* and two wild species, *T. boeoticum* Boissier, 1874 and *T. urartu* (Goncharov, 2012). Two different types of the A genome, A^u (*T. urartu*) and A^b (*T. boeoticum* and *T. monococcum* L., 1753), have been discriminated among the diploid wheats. According to the current concept, the A^u and A^b genomes diverged approximately one million years ago (Huang et al. 2002). Morphologically *T. urartu* and *T. boeoticum* are very similar (Filatenko et al. 2002), and differ distinctly only in the leaf pubescence pattern (velvety *vs*. bristly), controlled by allelic genes (Golovnina et al. 2009). These wild species have overlapping distribution ranges, and in some cases accessions belonging to either one of the species are identified incorrectly.

Despite morphological similarity, the level of genome divergence between *T. urartu* and *T. boeoticum* is very high. First of all it is indicated by the sterility of hybrids between *T. urartu* and *T. boeoticum* and/or *T. monococcum,* although in certain combinations of accessions and crossing direction hybrid fertility was elevated from zero to 4.5% (Fricano et al. 2014). Analysis of a broad sample of diploid A-genome species using multilocus markers, such as SSAP (sequence specific amplification polymorphism) and AFLP (amplified fragment length polymorphism) demonstrated considerable genetic differentiation among the accessions; and two super-clusters of diploid wheats have been discriminated among them (Konovalov et al. 2010, Fricano et al. 2014). The first super-cluster contains *T. urartu* and the second one, domesticated species *T. monococcum* and its wild progenitor, *T. boeoticum*. Importantly, intermediate forms between two super-clusters are detectable independently of the approach used for analysis; moreover, solitary accessions morphologically affiliated with *T. urartu* fall either within the opposite cluster or close to it.

Genome rearrangements, such as translocations, inversions, and the emergence of large blocks of repeats *via* amplification, are of considerable importance for the reproductive isolation of species. Such large-scale rearrangements are detectable by meiotic chromosome pairing analysis, comparative genome mapping, and FISH with repetitive probes. The data obtained so far suggest that the emergence of two evolutionary lineages of polyploid wheats, Emmer and Timopheevi, was accompanied by several species-specific translocations (Rodriguez et al. 2000, Salina et al. 2006a). Only one of these translocations, 4AL/5AL, which was inherited by polyploid wheat species from their diploid A genome progenitor, is characteristic of both *T. urartu* and *T. monococcum* (King et al. 1994, Devos et al. 1995). No information concerning the detection of other intraspecific and interspecific translocations in diploid wheats is available from literature.

One of the approaches for the identification of chromosomal rearrangements is cytogenetic analysis. Despite significant progress in sequencing and mapping of cereal genomes, this method is still most powerful for detection of chromosome aberrations; however, it needs a sufficient pool of cytogenetic markers. The number of cytogenetic markers used for the analysis of A genome chromosome is currently rather few. Single hybridization signals can the obtained with probes pSc119.2, pAs1, pTa71 (45S RNA genes), and pTa794 (5S rRNA genes) (Dubcovsky and Dvořák 1995, Schneider et al. 2003, Megyeri et al. 2012, Uhrin et al. 2012). Several (GAA)n sites have been detected on the A genome chromosomes of polyploid wheat, although signals are located predominantly on the B and G genome chromosomes of wheats and in the S-genome chromosomes of their diploid progenitor *Aegilops speltoides* (Gerlach and Dyer 1980). Hybridization with the (GAA)n probe not always produces stable signals on the A genome chromosomes, since either a synthetic probe or PCR fragments amplified from the wheat or rye genomic DNA were used (Kubaláková et al. 2005, Megyeri et al. 2012).

The goal of this work was to study the rearrangement of the A genome chromosomes of wheats during the evolution based on the distribution of (GAA)n microsatellite on the chromosomes.

Materials and methods

Plant material

The following diploid Triticum species were used in our work (see Table 1 for the complete list): *T. boeoticum* (2n = 2x = 14, A^bA^b) – six accessions; *T. monococcum* (2n = 2x = 14, A^bA^b) – six accessions, and *T. urartu* (2n = 2x = 14, A^uA^u) – seven accessions. For each species we selected accessions differing in the level of genomic divergence. In particular, according to SSAP analysis based on the *BARE-1* and *Jeli* retrotransposons (Konovalov et al. 2010), all accessions of diploid wheats were divided into groups and super-clusters (Table 1).

Table 1. Accessions of the diploid and polyploid Triticum species used in the work.

Accession/ *group	Species	Subspecies/variety (if available)	Centre of genetic resource	Accession number	Geographic origin
BO2/IG1	*T. boeoticum*	subsp. *thaoudar*	Kyoto Univ.	KU8120	Iraq
BO3/IG2	*T. boeoticum*	–	VIR	K-25811	Armenia
BO9/IG1	*T. boeoticum*	–	ICARDA	IG116198	Turkey
BO12/IG2	*T. boeoticum*	subsp. *boeoticum*	VIR	K-18424	Crimea
BO14/IG1	*T. boeoticum*	–	USDA	PI427328	Iraq
BO19/IG2	*T. boeoticum*	subsp. *boeoticum*	VIR	K-33869a	Armenia
MO1/IG3	*T. monococcum*	var. *macedonicum*	VIR	K-18140	Azerbaijan
MO3/IG3	*T. monococcum*	var. *monococcum*	VIR	K-20409	Spain
	T. monococcum	–	VIR	K-18105	Nagorno-Karabakh Autonomous Region
	T. monococcum	–	VIR	K-8555	Crimea
	T. monococcum	–	USDA	PI119423	Turkey
	T. monococcum	var. *hornemannii*, population Zanduri	VIR	K-46586	Georgia
UR1/IIG4	*T. urartu*	–	USDA	PI538736	Lebanon
UR2/IIG4	*T. urartu*	var. *albinigricans*	VIR	K-33869b	Armenia
UR3/IG3	*T. urartu*	–	USDA	PI428276	Lebanon
UR4/IG1	*T. urartu*	–	ICARDA	IG116196	Turkey
UR5/IG2	*T. urartu*	var. *albinigricans*	VIR	K-33871	Armenia
UR6/IIG4	*T. urartu*	–	ICARDA	IG45298	Syria
UR44/IIG4	*T. urartu*	–	USDA	PI428182	Armenia
	T. timopheevii	population Zanduri	VIR	K-38555	Georgia
	T. zhukovskyi	population Zanduri	VIR	K-43063	Georgia
	T. dicoccoides		ICARDA	IG46273	Israel
	T. dicoccoides		ICARDA	IG46288	Israel
	T. dicoccoides		ICARDA	IG139189	Jordan
	T. durum		VIR	K-1931	Russia
	T. aestivum		ICG	cv. Chinese Spring	China
	T. aestivum	var. *lutescens*	ICG	cv. Saratovskaya 29	Russia

* Designation of accessions and their clustering into groups (I/II, superclusters and G, groups) are according to Konovalov et al. (2010).

Current analysis included accessions from both well-differentiated super-clusters, *T. urartu* and *T. boeoticum*/*T. monococcum*, as well as three *T. urartu* accessions (UR3,UR4,UR5) for which the genome affinity determined by morphological traits was not confirmed by molecular analysis (Konovalov et al. 2010).

Polyploid wheat species belonging to either Timopheevi (*T. timopheevii*, 2n = 4x = 28, GGA^tA^t, and *T. zhukovskyi*, 2n = 6x = 42, $GGA^tA^tA^bA^b$), or Emmer evolutionary lineage (*T. dicoccoides*, 2n = 4x = 28, BBAA, *T. durum*, 2n = 4x = 28, BBAA, and *T. aestivum*, 2n = 6x= 42, BBAADD) were analyzed (Table 1).

The plants of all accessions used in our work were grown at the Joint Access Laboratory for Artificial Plant Cultivation for verification of species authenticity by morphological characters using a guide published by Goncharov (2012).

T. zhukovskyi authenticity was verified by electrophoresis of wheat storage proteins (gliadins) (Goncharov et al. 2007).

DNA isolation and cloning

The (GAA)n microsatellite sequence was cloned from einkorn wheat genome in order to increase the resolution of FISH analysis.

Total DNA was isolated from 5–7-day-old seedlings according to Plaschke et al. (1995). PCR for production of (GAA)n microsatellite was conducted according to Vrána et al. (2000) using $(CTT)_7$ and $(GAA)_7$ as primers and *T. urartu* (IG45298) and *T. monococcum* (PI119423) DNAs as templates. PCR comprised 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and synthesis at 72 °C for 1 min. The amplification products were cloned with a Qiagen kit. The clones differing in the length of the insert were selected and sequenced using ABI PRISM Dye Terminator Cycle Sequencing ready reaction kit (Perkin Elmer Cetus, USA). Sequencing was performed in an ABI PRISM 310 Genetic Analyzer (Perkin Elmer Cetus).

Giemsa C-banding

Giemsa C-banding was performed according to the protocol by Badaeva et al. (1994). The slides were examined with a Leitz Wetzlar microscope and recorded with a Leica DFC 280 CCD digital camera. The chromosomes were classified according to the standard nomenclature (Friebe and Gill 1996, Gill et al. 1991).

The work was performed at the Vavilov Institute of General Genetics, Russian Academy of Sciences.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was conducted as earlier described (Salina et al. 2006b). The probes were labeled with biotin or digoxigenin by nick translation.

Biotinylated probes were detected with fluorescein avidin D (Vector Laboratories). The digoxigenin-labeled probes were detected with antibodies to anti-digoxigenin-rhodamine, Fab fragments (Roche Applied Science).

The chromosomes were identified using the pSc119.2 (120 bp, rye repeats; Bedbrook et al. 1980) or pTa71 (45S RNA genes; Gerlach and Bedbrook 1979) probes.

The preparations were embedded into Vectashield mounting medium (Vector Laboratories), containing 0.5 μg/ml DAPI (4',6-diamidino-2-phenylindole, Sigma) for chromosome staining. The chromosomes were examined with an Axioskop 2 Plus (Zeiss) microscope and recorded with a VC-44 (PCO) CCD camera.

The work was performed at the Joint Access Center for Microscopic Analysis of Biological Objects with the Siberian Branch of the Russian Academy of Sciences.

Results

Cloning of (GAA)n microsatellite

Totally, four clones differing in the length of the insert were selected and sequenced. The clones obtained from *T. urartu* were designated pTu and from *T. monococcum*, pTm. All the clones contain (GAA)n microsatellite sequence, but differ in length: pTm30 has a length of 167 bp [$(GAA)_{56}$]; pTm17, 62 bp [$(GAA)_{21}$]; pTu33, 56 bp [$(GAA)_{19}$]; and pTu38, 36 bp [$(GAA)_{12}$]. The $(GAA)_{56}$ microsatellite variant pTm30 generating most distinct signals was selected for further work.

Localization of (GAA)n probe on einkorn wheat chromosomes

The new probe pTm30 containing $(GAA)_{56}$ sequence was hybridized to chromosomal preparations of *T. urartu*, *T. boeoticum* and *T. monococcum;* the pTa71 (45S rRNA genes) probe was used for chromosome identification. As was expected, the 45S ribosomal RNA genes in all species were localized to the nucleolar organizer region in the distal parts of the 1AS and 5AS chromosomes.

The accessions of wild species *T. boeoticum* and *T. urartu* displayed polymorphism in the distribution of (GAA)n microsatellite on the chromosomes. One to three pTm30 sites per haploid genome can be detected in these two species (Table 2, Fig. 1). As a whole, (GAA)n can be detected in four positions, on the 1AS, 2AS, 5AS, and 4AL chromosomes of *T. boeoticum* and *T. urartu*. The (GAA)n site on the 1AS is observed only in *T. urartu*, being detectable in four of the seven examined accessions. Both *T. boeoticum* and *T. urartu* carry (GAA)n sites on 2AS and 5AS chromosomes; however, the microsatellite is detectable at a higher rate on the 5AS of *T. urartu* and the 2AS of *T. boeoticum.* (GAA)n site on the 2AS in one accession of *T. boeoticum* was heteromorphic between homologous chromosomes (Fig. 1c). Some accessions of *T. boeoticum* and *T. urartu* carry a (GAA)n site on the 4AL chromosome.

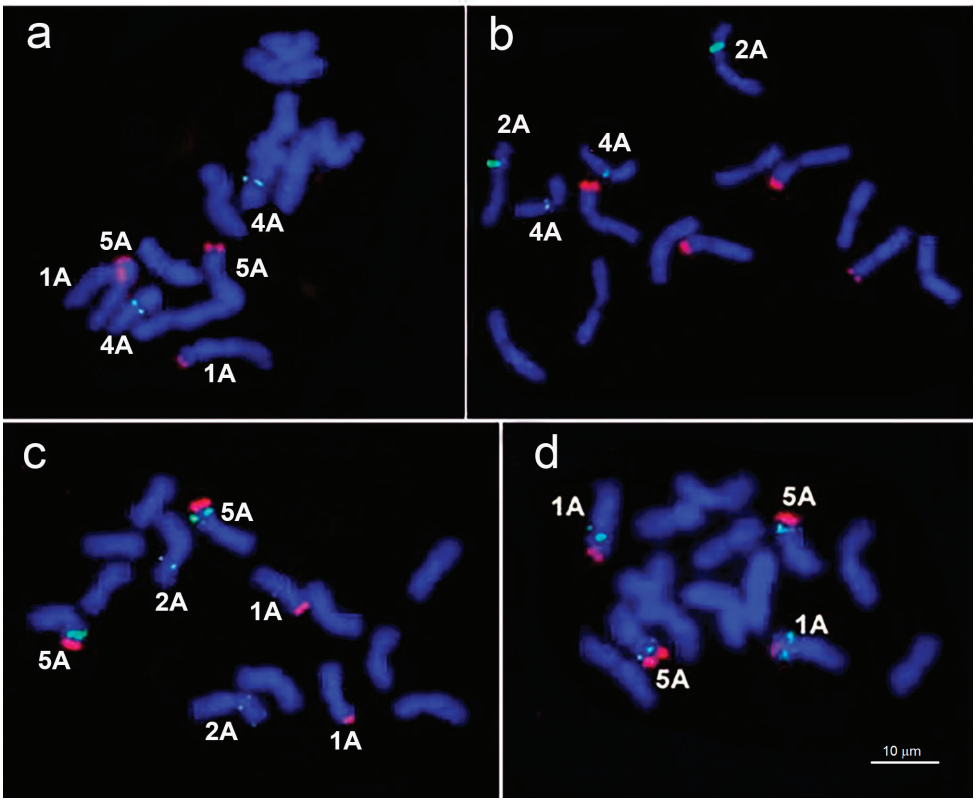

Figure 1. FISH with probes pTm30 (green signal) and pTa71 (red signal) on the chromosomes of diploid Triticum species: **a** *T. monococcum* MO1 **b** *T. boeoticum* BO3 **c** *T. boeoticum* BO14 and **d** *T. urartu* UR6.

Table 2. Localization of pTm30 probe on the chromosomes of diploid Triticum species.

Chromosome (arm)	*T. boeoticum*[1]						*T. urartu*[1]							*T. monococcum*[2]
	BO2 IG1	BO3 IG2	BO9 IG1	BO12 IG2	BO14 IG1	BO19 IG2	UR1 IIG4	UR2 IIG4	UR3 IG3	UR4 IG1	UR5 IG2	UR6 IIG4	UR4 IIG4	
1A(S)								+			+	+	+	
2A(S)		+		+	+	+		+		+				
5A(S)	+			+	+		+	+			+	+	+	
4A(L)		+	+						+					+

[1] Designation of accessions and their clustering into groups (I/II, superclusters and G, groups) are according to Konovalov et al. (2010).

[2] Characteristic of five examined *T. monococcum* accessions; no pTm30 hybridization sites are detected for PI119423.

The domesticated species *T. monococcum* differs from the wild species by an almost complete lack of polymorphism in the distribution of pTm30 probe. The (GAA)n site

in five of the six examined accessions is localized to the pericentromeric region of 4AL (Fig. 1a). No distinct hybridization sites of pTm30 have been found on chromosomes of accession PI119423.

FISH analysis of Timopheevi wheats

The examined accessions of *T. timopheevii* originated from the Zanduri population (Western Georgia), where the species *T. zhukovskyi* was first identified. *Triticum timopheevii* carries pSc119.2 signals predominantly on the G genome chromosomes and also on $1A^tL$ and $5A^tS$; the pTa71 signals are present on $6A^tS$ and 6GS chromosomes (Fig. 2). The pTm30 probe intensively hybridized to all G genome chromosomes and generates only one hybridization site on the chromosome $6A^tS$ of the A^t genome (Fig. 2).

Similar results were obtained for hexaploid *T. zhukovskyi* ($2n$ = 42, $GGA^tA^tA^bA^b$ genome), a natural amphiploid resulting from interspecific hybridization between *T. timopheevii* ($2n$ = 28, GGA^tA^t genome) and *T. monococcum* ($2n$ = 14, AA genome). *T. zhukovskyi,* like *T. timopheevii*, carries the pTm30 site in the short arm of the $6A^t$ chromosome and pSc119.2 on $1A^tL$ (Fig. 2). The remaining A^t genome chromosomes lack both pTm30 and pSc119.2 signals. The 45S RNA genes were detected on the $6A^tS$ and 6GS chromosomes, as in *T. timopheevii,* and additionally on $1A^bS$ and $5A^bS$, as in *T. monococcum* (Fig. 2). No pTm30 hybridization sites, which could have been donated by *T. monococcum*, have been detected.

C-banding and FISH analysis of Emmer wheats

Since karyotyping of the A genome of polyploid wheats by FISH alone is not precise, Giemsa C-banding was also used in order to identify all chromosomes of *T. dicoccoides, T. durum*, and *T. aestivum*. In addition, the distribution pattern of probe pSc119.2 was considered, when identifying the chromosomes.

Among three *T. dicoccoides* accessions we identified the conserved pSc119.2 sites in subtelomeric regions of 1AS and 4AL chromosomes (Fig.3), whereas polymorphic pSc119.2 sites were detected on chromosomes 5AS (subtelomeric localization), 5AL (intercalary localization), and 2AL (intercalary localization).

A comparative analysis of the A genome chromosomes of *T. dicoccoides* by FISH with pTm30 probe revealed both conserved and polymorphic (GAA)n signals (Fig. 3). The 1A lacked (GAA)n site during tetraploid formation, while up to two polymorphic (GAA)n loci were detectable on the 3A, 6A, and 7A chromosomes. The highest number of pTm30 hybridization sites was observed on the 2A and 4A chromosomes; of them, the pericentromeric site on 2AS and proximal and distal sites on 4AL were conserved. Chromosome 5AL carried a conserved (GAA)n site in the proximal region; no polymorphic blocks were detected. Note also that the localization of (GAA)n, as was

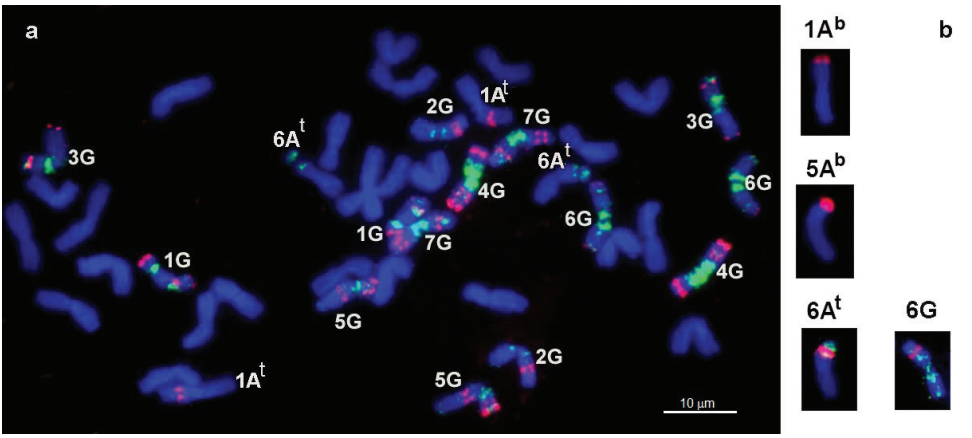

Figure 2. FISH with probe pTm30 (green signal) on the chromosomes of *T. zhukovskyi*: **a** red signal, pSc119.2 and **b** red signal, pTa71.

expected, always coincided with the position of C-bands; however, for approximately 20% of the C-bands no corresponding (GAA)n regions have been detected.

The distribution of pTm30 hybridization sites on the A genome chromosomes of two hexaploid wheat cultivars, Chinese Spring and Saratovskaya 29, was similar (Fig. 3).

The absence of (GAA)n microsatellite on the 1A chromosome was the common feature for all *T. dicoccoides*, *T. durum*, and *T. aestivum* accessions. In addition, the conserved (GAA)n sites on *T. dicoccoides* chromosomes 2AS, 4AL, and 5AL were remained in *T. durum* and *T. aestivum*.

Discussion

(GAA)n as a cytogenetic marker for einkorn wheat

The goal of the search for cytogenetic markers for the wheat A genome dates back to the very first application of cytogenetic methods to analysis of chromosome structure and phylogeny of *Triticum* species. This was due to both a small number of C-bands detectable by Giemsa staining and the difficulties in FISH-based distinguishing between the A genome chromosomes. In particular, two cytogenetic markers, pSc119.2 and pAs1, are able to discriminate all B and D genomes chromosomes of Emmer wheat, but only three A genome chromosomes (Schneider et al. 2003). The pSc119.2 probe hybridized mainly to the B genome chromosomes of polyploid wheats belonging to Emmer evolutionary lineage, as has been demonstrated for hexaploid *T. aestivum* and tetraploid *T. durum* (Schneider et al. 2003; Kubaláková et al. 2005). In the A-genome of hexaploid wheat, the pSc119.2 hybridization sites were detected only on chromosomes 2AL, 4AL (subtelomeric localization), 5AS (subtelomeric localization), and 5AL (intercalary site). Note that different wheat cultivars display polymorphism

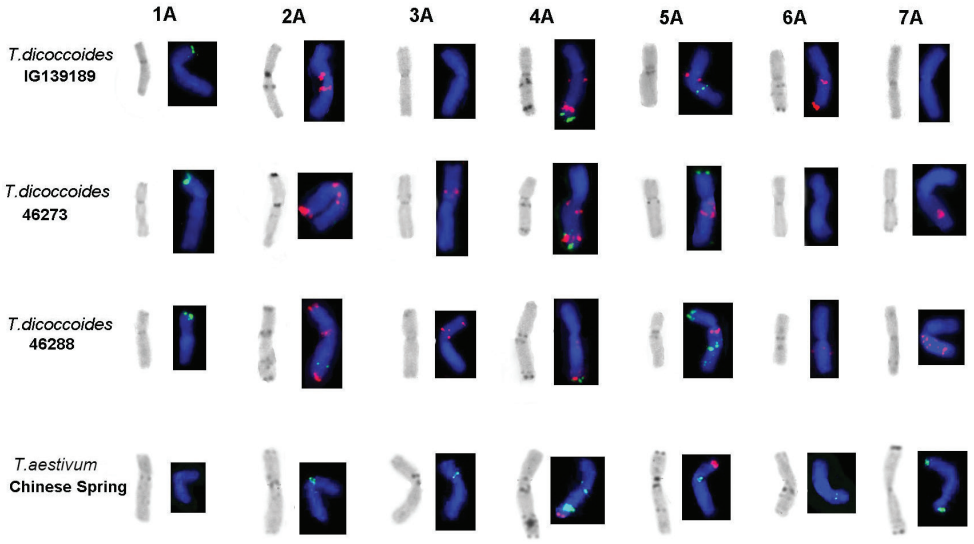

Figure 3. Localization of probe pTm30 on the chromosomes of Emmer wheats and C-banding. Accessions of *T. dicoccoides*: pTm30 (red) and pSc119.2 (green); accessions of *T. aestivum*: pTm30 (green) and pSc119.2 (red).

in distribution of pSc119.2 probe on the A-genome chromosomes (Schneider et al. 2003). Involvement of additional probes—pTa71 (45S RNA) and pTa794 (5S RNA genes), localized to 1A (pTa71 and pTa794) and 5A (pTa794) chromosomes—failed to improve the resolution of this assay. The (GAA)n microsatellite, detected in all A genome chromosomes of common and durum wheat except for 1A (Fig. 3), is mainly used for chromosome sorting in polyploid wheats (Pedersen and Langridge 1997; Vrána et al. 2000; Kubaláková et al. 2005). However the direct application of this probe to phylogenetic studies of the A genome of polyploid wheats is hardly possible, because it gives only few minor signals compared to numerous major hybridization sites on the B and G genome chromosomes (Fig. 2; Kubaláková et al. 2005; Cuadrado et al. 2008).

The situation with chromosome identification in einkorn wheat is even more complex. The probes that are frequently used in molecular cytogenetic analysis of polyploid wheats, such as pSc119.2 and pAs1, either do not hybridize to einkorn chromosomes at all, or give few fuzzy signals (Megyeri et al. 2012, Danilova et al. 2012, I.G. Adonina, unpublished data). The rDNA markers, pTa71 and pTa794, produce conserved hybridization sites on the 1AS and 5AS chromosomes and fail to distinguish the einkorn genomes. An *Afa* probe, PCR-amplified from the genomic DNA of common wheat (Megyeri et al. 2012), may be the most promising for the study of chromosome reorganization of the A genomes. The *Afa* probe produces numerous hybridization sites; however, so far this has been demonstrated for only one accession of *T. monococcum*.

The distribution of (GAA)n microsatellite on chromosomes of the A genome diploid species has not been studied until recently. Dvorak (2009) wrote in his review referring to the works of Peacock et al. (1981) and Pedersen (et al. 1996) that (GAA)n sequence is

absent in the diploid A genome donors of common wheat. However, karyotype analysis of individual *T. monococcum* and *T. urartu* accessions employing either $(GAA)_9$ oligonucleotide or GAA fragments amplified by PCR from wheat genomic DNA has been recently reported (Danilova et al. 2012, Megyeri et al. 2012). Megyeri et al. (2012) studied one accession of *T. monococcum* and discovered two chromosomes with major hybridization sites of the (GAA)n probe in their distal and pericentromeric regions, which were identified as 2AS and 6AL, respectively, based on the distribution of *Afa* family and pTa71 probe. Danilova et al. (2012) identified the chromosomes more precisely using FLcDNAs and defined the chromosomes carrying the major sites as 2AS and 4AL in *T. monococcum* and 1AS in *T. urartu.* In addition, one to three minor (GAA)n sites were detected in two studied accessions of diploid species. So far, no publications describing the (GAA)n distribution on *T. boeoticum* chromosomes has been reported.

As has been demonstrated here, the pTm30 produces up to four major hybridization sites on the A genome chromosomes of diploid wheats (1AS, 2AS, 5AS, and 4AL), while any minor hybridization sites are undetectable. All four major hybridization sites are present in *T. urartu* only, and the site on 1AL is absent in *T. monococcum* and *T. boeoticum*. Interestingly, *T. urartu* accessions belonging to super-cluster II (*urartu*) mainly display two (GAA)n sites, on the 1AS and 5AS chromosomes, also carrying the 45S RNA genes. The major (GAA)n site on 1AS and minor site on 5AS have been also detected in the *T. urartu* accession by Danilova et al. (2012). An interesting fact has been obtained by FISH analysis of the three *T. urartu* accessions (UR3, UR4, and UR5), which were regarded as intermediate forms according to comparison of morphological and SSAP data (Tables 1 and 2). All three accessions differ in the distribution of (GAA)n microsatellite. However, UR5 accession is attributed to super-cluster II (*urartu*) according to pTm30 [$(GAA)_{56}$] pattern, while UR3 and UR4 carry (GAA)n sites on the 2AS and 4AL chromosomes, which are mainly characteristic of *T. boeoticum* and *T. monococcum*. Cultivated einkorn *T. monococcum* displays the lowest polymorphism. We found pTm30 hybridization site on one chromosome pair only, designated 4AL.

Thus, it has been shown that the (GAA)n microsatellite can be used as marker for the 1AS, 2AS, 4AL, and 5AS chromosomes of einkorn wheat; however, it should be kept in mind that depending on species, the number of hybridization sites varies from zero to three in individual accessions. The (GAA)n site on chromosome 1AS is present only in *T. urartu*, while *T. boeoticum* and *T. monococcum* often carry (GAA)n site on the chromosome 4AL.

Evolutionary reorganization of the A genomes in diploid and polyploid wheat species

The evolution of diploid and polyploid wheat species is known to be accompanied by reorganization of the genomes. At the diploid level, genome divergence occurs *via* accumulation of DNA mutations, amplifications/deletions of tandem repeats, proliferation of mobile elements, and, in some cases, chromosomal rearrangements (Devos et

al. 1995, Salina et al. 2011, Fricano et al. 2014). Polyploid wheat displays a high level of chromosome rearrangements (Jiang and Gill 1994, Rodriguez et al. 2000, Salina et al. 2006a, Badaeva et al. 2007).

As any other tandem repeats, microsatellites frequently form large clusters on chromosomes, detectable with FISH. The polymorphism of satellite repeats most typically involve changes in the copy number, resulting in the appearance/elimination of large blocks of repeated sequences.

Study of the distribution of (GAA)n hybridization sites in diploid and polyploid wheats allows us to propose that several factors could have led to redistribution of regions housing this microsatellite. In particular, a decrease in the number of major microsatellite blocks in domesticated *T. monococcum* may only be a result genetic diversity shortage caused by bottleneck effect during domestication. Another important fact is that all studied accessions of polyploid wheat species *T. dicoccoides*, *T. durum*, *T. aestivum*, and *T. timopheevii* lack hybridization sites on the short arms of their 1A and 5A chromosomes (Fig. 3), which are characteristic of *T. urartu*, a putative donor of the A genome. The most likely reason for such event is the involvement of (GAA)n loci in reorganization of the nucleolus organizer region on the A genome chromosomes during formation and stabilization of primary allotetraploids which took place about 500 TYA. This resulted in total loss of 45S DNA locus and (GAA)n site on the 5AS as well as in the significant reduction in the number of 45S RNA gene copies (Jiang and Gill 1994) and the loss of (GAA)n site on the 1AS chromosome (Fig. 3). Hexaploid species *T. zhukovskyi* (genome $GGA^tA^tA^bA^b$) was formed about 60YA or more *via* the cross of *T. timopheevii* (genome GGA^tA^t) and *T. monococcum* (genome A^bA^b). According to our data, this species retained the 45S DNA loci on the $1A^bS$ and $5A^bS$ chromosomes, inherited from *T. monococcum*, however, the A^b genome chromosomes of *T. zhukovskyi* lacks (GAA)n sites observed in *T. monococcum*. This can be due to either the lack of such sites in the parental *T. monococcum* form, or elimination of (GAA)n loci over the course of amphiploidization, while the species was established.

As in the parental species *T. timopheevii*, *T. zhukovskyi* displays only one (GAA)n site in the short arm of chromosome $6A^t$, near the nucleolus organizer region. It is known that the T6AS/1GS translocation took place during *T. timopheevii* speciation (Jiang and Gill 1994). Thus, it is likely that the (GAA)n site on appeared a result of this translocation.

The (GAA)n sites of the einkorn wheat that are localized to more conserved chromosome regions, namely, pericentromeric regions of 2AS and 4AL, were inherited by all polyploid Emmer species (Fig. 3). No (GAA)n sites have been detected on the A^t genome homoeologous chromosomes of Timopheevi wheats, thereby confirming that their origin is independent from the Emmer group.

Thus, amplification and cloning of the long fragment of (GAA)n sequence from *T. monococcum* genome allowed us to obtain the new DNA probe for analysis of the A-genome chromosomes in diploid and polyploid wheat. An increased sequence length provides for higher probe stability, which enhances resolution of hybridization. Using a new probe we defined differences between A^b and A^u variants of the A-genomes, revealed variability of labeling patterns among *T. boeoticum* and *T. urartu* accessions,

and significant shortage of polymorphism in *T. monococcum*, probably due to domestication. We suppose that distribution of (GAA)n sites in diploid and polyploid species reflects the chromosome reorganizations, mainly including the nucleolus organize region, that have taken place during the evolution of wild and domesticated species.

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RESEARCH ARTICLE

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Variation of karyotype and nuclear DNA content among four species of *Plectranthus* L' Héritier, 1788 (Lamiaceae) from Brazil

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Abstract

Plectranthus is a genus which includes species of ornamental and medicinal potential. It faces taxonomic problems due to aggregating species previously belonging to the genus *Coleus*, a fact that has contributed to the existence of various synonymies. The species *Plectranthus amboinicus*, *Plectranthus barbatus*, *Plectranthus grandis* and *Plectranthus neochilus* are included in this context. Some authors consider *P. barbatus* and *P. grandis* as synonyms. The present work was carried out with the aim of comparing plants of the above-mentioned species, originating from different localities in Brazil, with regards to chromosome number and karyotypic morphology, correlated to the nuclear DNA content. There was no variation in chromosome number among plants of the same species. *P. amboinicus* was the only species to exhibit 2n=34, whereas the others had 2n=30. No karyotypic differences were found among the plants of each species, except for *P. barbatus*. The plants of the *Plectranthus* species revealed little coincidence between chromosome pairs. The nuclear DNA content allowed grouping *P. amboinicus* and *P. neochilus*, with the highest mean values, and *P. grandis* and *P. barbatus* with the lowest ones. Differences in DNA amount among the plants were identified only for *P. barbatus*. These results allow the inference that the populations of *P. amboinicus* and *P. neochilus* present coincident karyotypes among their plants, and *P. grandis* is probably a synonym of *P. barbatus*.

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Keywords
Cytogenetics, Cytotaxonomy, Flow cytometry, Karyotypic evolution

Introduction

The family Lamiaceae Martinov, 1820 contains approximately 250 genera and 6,500 species (Anon 2014). Its main area of distribution is the Mediterranean region and Mid-East, up to Central Asia (Chengyih and Hsiwen 1982). In Brazilian territory, 500 species of 34 genera can be found broadly distributed across the country (Harley et al. 2013).

Plectranthus L' Héritier, 1788 is one of the most common genera of this family, and comprises about 300 species (Richardson 1992) that generally serve medicinal and ornamental interest (Thoppil 1993). Species of this genus are native to tropical and subtropical regions of Africa, Australia, the East Indies, the Malay Archipelago, and the Philipines (Lebowitz 1985).

This genus, along with *Burnatastrum* Briquet, 1897, *Coleus* Loureiro, 1790 *Englerastrum* Briquet, 1894, *Isodictyophorus* Briquet, 1917 and *Neomullera* unrecorded, has already been placed in the genus *Ocimum* Linnaeus, 1753. *Coleus* and *Plectranthus* have been considered distinct genera only because of morphological differences of the stamen; however, this characteristic has later been considered insufficient for the separation of these taxa. This way, the *Coleus* species were aggregated to the genus *Plectranthus*, turning this grouping into a unique genus and independent of *Ocimum* Linnaeus, 1753 (Morton 1962). The taxonomic history of *Plectranthus* has contributed to some of its species being known by many different synonymies (Lukhoba et al. 2006).

In Brazil there are some important species of this genus used as herbal medicines. *Plectranthus amboinicus* (Loureiro, 1825) Sprengel, 1825 is native to East Asia, later introduced in Cuba and distributed in America (Castillo and Gonzalez 1999). *Plectranthus barbatus* Andrews, 1810 is native to Africa and it is one of the most cited species in ethnobotanical surveys in Brazil (Carriconde et al. 1996). *Plectranthus neochilus* Schlecher, 1896 is also native of Africa, later introduced in Brazil (Lorenzi and Matos 2002). *P. barbatus* and *P. grandis* (Cramer, 1979) Willemse, 1985 are very similar species and they are usually confused, since they are used for the same medicinal purposes. Pioneering cytogenetic works in this genus have revealed species with diversified chromosome numbers, from 2n=14 to 84, with 2n=28 being most frequent (Morton 1962).

Cytogenetic differences among plants of the same species may reflect in variation in amount, quality and type of secondary metabolites produced by the plant, as observed by Pierre et al. (2011) in plants of *Lippia* Linnaeus, 1753.

Knowledge of DNA content, along with cytogenetics and molecular genetics, contributes to the genetic characterization of related species. The correct definition of the taxon, associated to biochemical and pharmacological evaluations, is essential for the correct use of plants for medicinal purposes. This preoccupation becomes even more important considering the recognition by the World Health Organization that about

80% of the population in developing countries makes use of plants or preparations thereof as home and communitarian remedies (Brasil 2006).

Considering the variety of chromosome number descriptions and the lack of karyotypic information about *Plectranthus* species, an enhanced investigation of the chromosome complement is necessary with the purpose of supporting taxonomic studies and evolutionary inferences. In this sense, the present work aimed at the characterization and comparison of the karyotype and DNA content of plants, from distinct localities, of the species *P. amboinicus*, *P. barbatus*, *P. grandis* and *P. neochilus*.

Material and methods

Plants of *P. amboinicus*, *P. barbatus*, *P. grandis* and *P. neochilus* from Lavras-MG, Campinas-SP and Santa Maria-RS was cytogenetically compared. In each region three cuttings of a plant of each species were collected from plant clumps. Plants from Minas Gerais State were supplied by Medicinal Plant Garden of the University of Lavras (UFLA), the ones from Rio Grande do Sul State was provided by Medicinal Plant Garden of University of Santa Maria (UFSM) and plants from São Paulo State by the Campinas Agronomy Institute (IAC). Voucher specimens were deposited at the Research Center for Chemistry, Biology and Agriculture (CPQBA), State University of Campinas, and the State University of Campinas Herbarium (UEC), São Paulo, Brazil (Table 1). The cuttings were transplanted into vases and kept in a greenhouse. After root development, the root tips were collected and pre-treated with solution of 3 mM 8-hydroxyquinoline for 4 hours, at 4 °C, and fixed in Carnoy's solution (3:1 /ethanol:acetic acid). The material was then stored at -20 °C for at least 24 hours.

Slides were prepared by the squash technique, and chromosomes were stained with 1% acetic orcein after enzymatic maceration in pectinase-cellulase solution (100U:200U) for 15 min, at 37 °C.

Metaphases were digitized by means of a bright field microscope (Leica DMLS) equipped with microcamera (Nikon Digital Sight DS-Fil). The chromosomes were measured using the software Image Tool 3.0 from the UTHSCA (University of Texas Health Science Center in San Antonio).

For assembly of the karyograms and idiograms, at least four mitotic metaphases of each plant collected were used. Measurements of short and long arm length (SA/LA) were carried out for each chromosome pair, as well as of total length for each chromosome (TL_i = LA + SA), total length of haploid lot (TLHL = STL_i), and relative length (RL = TL_i/TLHL × 100).

Table 1. Voucher specimens numbers of *Plectranthus* plants collected.

Species	Voucher specimens
P. amboinicus	CPQBA 364
P. barbatus	UEC 121.403
P. grandis	CPQBA 1433
P. neochilus	CPQBA 1388

The data on relative length of chromosome pairs of *P. grandis* as well as of *P. barbatus* plants were compared by the least significant difference (LSD) at 5% probability, using the statistical program SAS.

Morphological classification of the chromosomes was based on centromere position, as proposed by Levan et al. (1964). Karyotypic asymmetry was evaluated according to criteria by Zarco (1986) using $A_{1,}$ (intrachromosomal asymmetry) and $A_{2,}$ (interchromosomal asymmetry) indices. These indices were compared to the asymmetry index (AI) proposed by Paszko (2006). Karyotypic categories were determined using the methods proposed by Stebbins (1958).

Estimation of nuclear DNA content by flow cytometry was obtained from leaf tissue according to the work of Doležel and Bartoš (2005). Nine samples of plants from each location were evaluated, total of 27 samples per specie. A total of nine samples of *P. grandis* was evaluated. Approximately 20-30 mg of young leaves of the *Plectranthus* species were used per sample, along with the same amount of *Pisum sativum* Linnaeus, 1753 leaves (internal reference standard, 2C=9.09 pg) (Doležel et al. 1992). The nuclei were released by dissociation of the plant material in Petri dish containing 1 mL of Marie and Brown (1993) cold buffer for yielding the nuclei suspension, to which were added 5 µL of RNase Type I. The suspension was stained with 25 µl of propidium iodide (1 mg/mL). For each sample, at least 10,000 nuclei were analyzed with a flow cytometer FACSCalibur (Becton Dickinson). The histograms with coefficients of variation below 0.8% were obtained using the software Cell Quest (Becton, Dickinson and Company, San Jose, CA, USA), and analyzed with the software WinMDI 2.8 (2009). The absolute DNA amount of the samples was calculated based on the values of the G1 peak means (Sample 2C DNA content = [(sample G1 peak mean)/(standard G1peak mean)] × standard 2C DNA content (pg DNA).

The data on DNA content of plants of each location, as well as the means of each species, were submitted to analysis of variance, and the mean values were compared with help of the statistical program SAS, using Tukey test at 5% probability to compare the plants and species.

Results

No variation was found as to the number of chromosomes among the plants of *Plectranthus* species. The somatic number of chromosomes was common (2n=30), except for *P. amboinicus*, which presented 2n=34 chromosomes (Fig. 1).

Differences were observed in chromosome morphology among the karyotypes of the species. *P. amboinicus* follows the karyotypic formula 13m+4sm, *P. grandis* 7m+8sm, and *P. neochilus* 9m+6sm (Table 2, Fig. 2). *P. barbatus* presented intraspecific variation: 8m+7sm, 9m+6sm and 10m+5sm, respectively corresponding to the specimens originated from the regions of Lavras/MG, Campinas/SP and Santa Maria/RS (Table 2, Fig. 3).

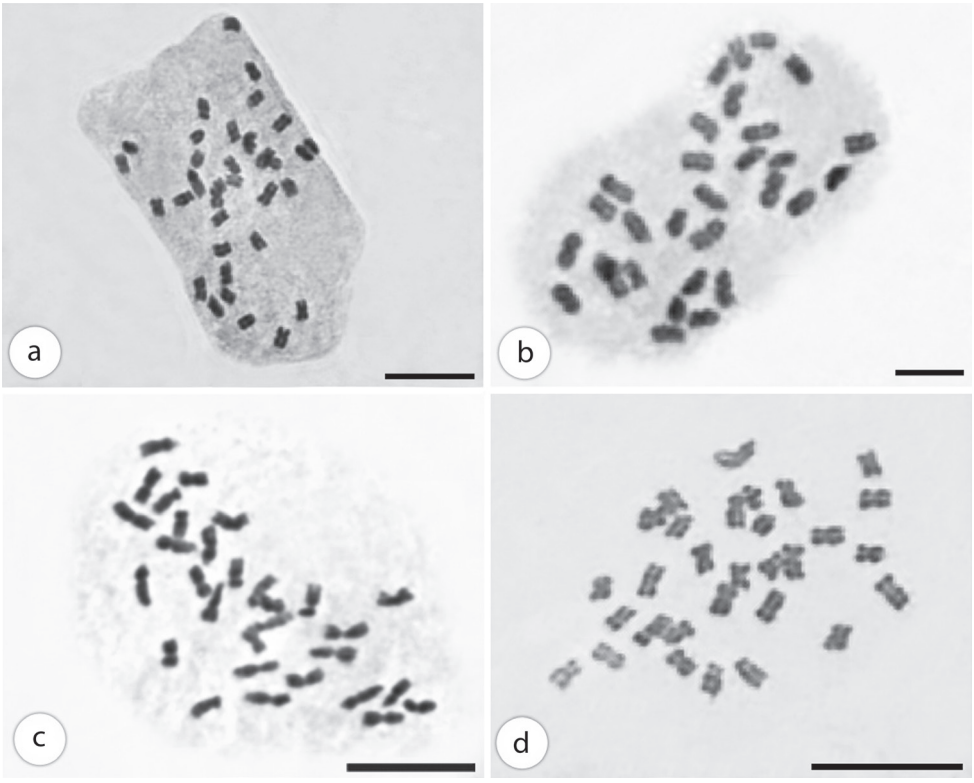

Figure 1. Mitotic metaphases. *P. amboinicus*, 2n=34 (**A**), *P. barbatus* (from Lavras-MG), 2n=30 (**B**), *P. grandis*, 2n=30 (**C**), *P. neochilus*, 2n=30 (**D**). Scale bars: 10 μm.

The position of the centromere was coincident among the four species only for the pairs 6 and 15, with these being classified as metacentric (Table 2).

Also among the plants of *P. barbatus* little karyotypic similarity could be established. Only the pairs 4, 5, 6 and 15 had chromosomes with coinciding classification. Moreover, these same pairs are also coincident in *P. grandis*, which presented greater karyotypic similarity with the plants of *P. barbatus* (Santa Maria), differing only in the pairs 3, 8 and 10 (Table 2).

The contrasts accomplished through the statistical test of least significant difference (LSD) among the plants revealed that *P. barbatus* (Campinas) differs statistically from *P. grandis* in relation to the pairs 2 and 12. The pair 2 presents relative length with mean values of 8.78 and 8.10, and the pair 12 shows 5.45 and 5.92 for *P. barbatus* and *P. grandis*, respectively. The pair 8 differed among the plants of *P. barbatus* originated from Lavras and Santa Maria, with respective averages of 6.68 and 6.93 (Tables 3–4). The remaining pairs did not present significant differences for this variable among the plants of the species.

Table 2. Data regarding arm relation and chromosome type in species of *Plectranthus* genus.

	P. a	*P. b*			*P. g*	*P. n*
		UFLA	IAC	UFSM		
1	1.43m	2.35sm	1.88sm	1.21m	1.34m	1.79sm
2	1.21m	2.15sm	1.18m	1.25m	1.34m	1.17m
3	1.37m	1.42m	1.98sm	1.38m	1.96sm	2.00sm
4	2.35sm	1.44m	1.29m	1.28m	1.20m	2.13sm
5	1.39m	2.07sm	1.88sm	1.79sm	2.02sm	1.13m
6	1.36m	1.32m	1.17m	1.37m	1.18m	1.19m
7	2.19sm	1.32m	1.30m	2.23sm	2.07sm	1.92sm
8	1.27m	2.22sm	1.79sm	1.30m	1.98sm	1.11m
9	1.38m	1.53m	1.97sm	2.22sm	2.27sm	1.81sm
10	1.99sm	2.19sm	1.40m	1.35m	2.15sm	1.25m
11	1.29m	2.25sm	1.38m	1.17m	1.37m	1.26m
12	1.36m	2.01sm	1.41m	2.06sm	2.20sm	1.39m
13	1.29m	1.20m	1.21m	2.27sm	2.04sm	1.43m
14	1.97sm	1.43m	2.11sm	1.47m	1.21m	1.84sm
15	1.17m	1.24m	1.20m	1.21m	1.35m	1.25m
16	1.18m					
17	1.24m					

**P. a* (*P. amboinicus*); *P. b* (*P. barbatus*); *P. g* (*P. grandis*); *P. n* (*P. neochilus*); m = metacentric; sm = submetacentric.

Table 3. Data regarding the total length (μm) and relative length (%) of each chromosome of *Plectranthus* spp.

Pair	*P. amboinicus*		*P. barbatus*						*P. grandis*		*P. neochilus*	
			UFLA		IAC		UFSM					
1	3.04	(8.89)	2.99	(8.56)	3.57	(9.42)	2.93	(9.09)	2.95	(8.37)	2.82	(8.55)
2	2.87	(8.38)	2.87	(8.22)	3.33	(8.78)	2.63	(8.15)	2.85	(8.10)	2.75	(8.33)
3	2.49	(7.26)	2.75	(7.87)	3.09	(8.15)	2.52	(7.82)	2.72	(7.72)	2.55	(7.75)
4	2.42	(7.06)	2.60	(7.44)	2.94	(7.75)	2.43	(7.55)	2.65	(7.52)	2.42	(7.34)
5	2.31	(6.73)	2.52	(7.20)	2.80	(7.38)	2.34	(7.26)	2.58	(7.32)	2.41	(7.31)
6	2.13	(6.23)	2.51	(7.17)	2.72	(7.17)	2.27	(7.03)	2.50	(7.09)	2.36	(7.15)
7	2.05	(5.98)	2.44	(6.97)	2.61	(6.89)	2.26	(7.00)	2.37	(6.72)	2.30	(6.97)
8	2.02	(5.90)	2.34	(6.68)	2.59	(6.83)	2.23	(6.93)	2.36	(6.69)	2.25	(6.82)
9	1.94	(5.68)	2.23	(6.37)	2.35	(6.20)	2.10	(6.50)	2.31	(6.57)	2.14	(6.48)
10	1.86	(5.42)	2.18	(6.23)	2.25	(5.95)	2.06	(6.39)	2.18	(6.19)	2.04	(6.19)
11	1.78	(5.20)	2.11	(6.02)	2.23	(5.87)	2.00	(6.21)	2.14	(6.09)	1.97	(5.97)
12	1.72	(5.02)	1.95	(5.58)	2.07	(5.45)	1.84	(5.71)	2.09	(5.92)	1.89	(5.72)
13	1.68	(4.90)	1.94	(5.56)	2.03	(5.35)	1.66	(5.15)	1.95	(5.52)	1.78	(5.39)
14	1.61	(4.70)	1.83	(5.23)	1.78	(4.71)	1.57	(4.88)	1.86	(5.29)	1.74	(5.26)
15	1.57	(4.59)	1.71	(4.89)	1.56	(4.10)	1.40	(4.34)	1.72	(4.89)	1.57	(4.76)
16	1.45	(4.22)										
17	1.32	(3.85)										

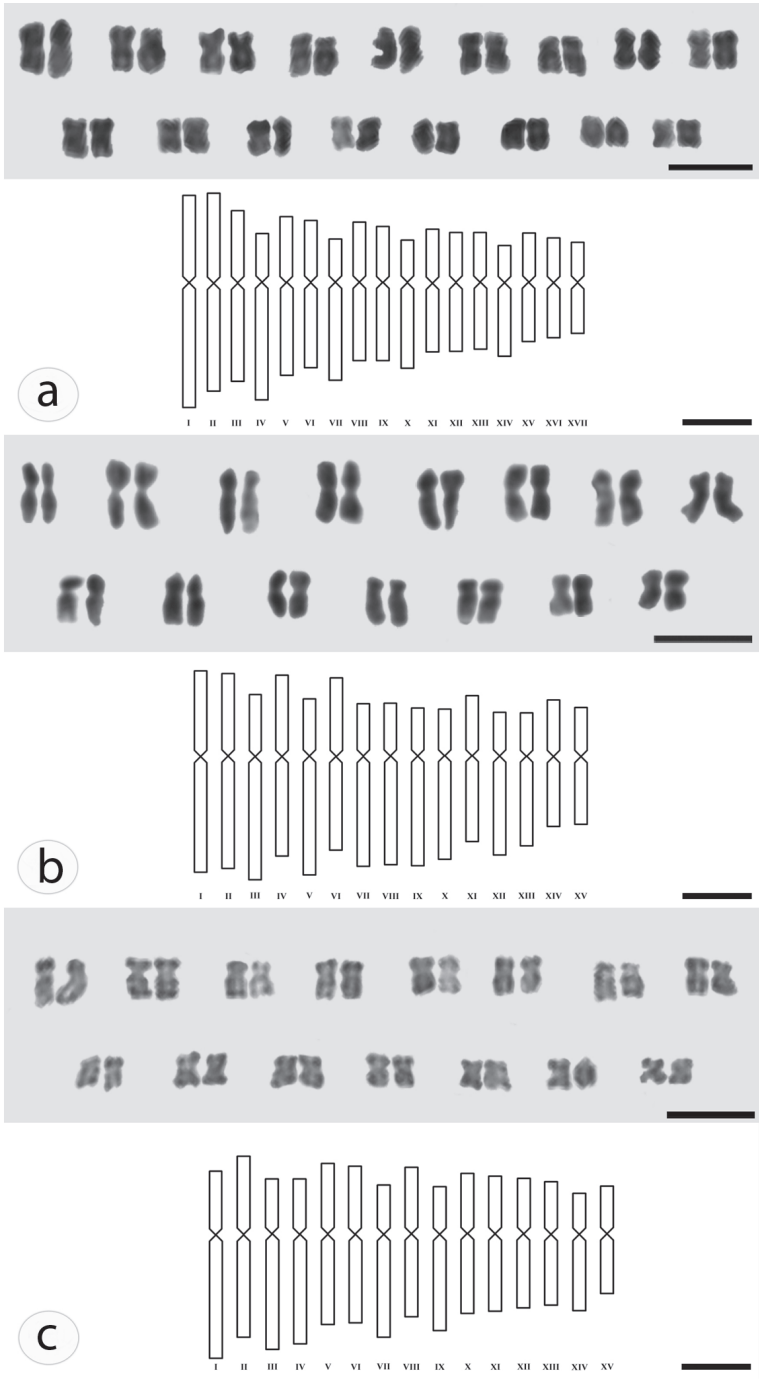

Figure 2. Karyograms and idiograms. *P. amboinicus* (**A**), *P. grandis* (**B**), *P. neochilus* (**C**). Scale bars: Karyograms (5 μm); idiograms (1 μm).

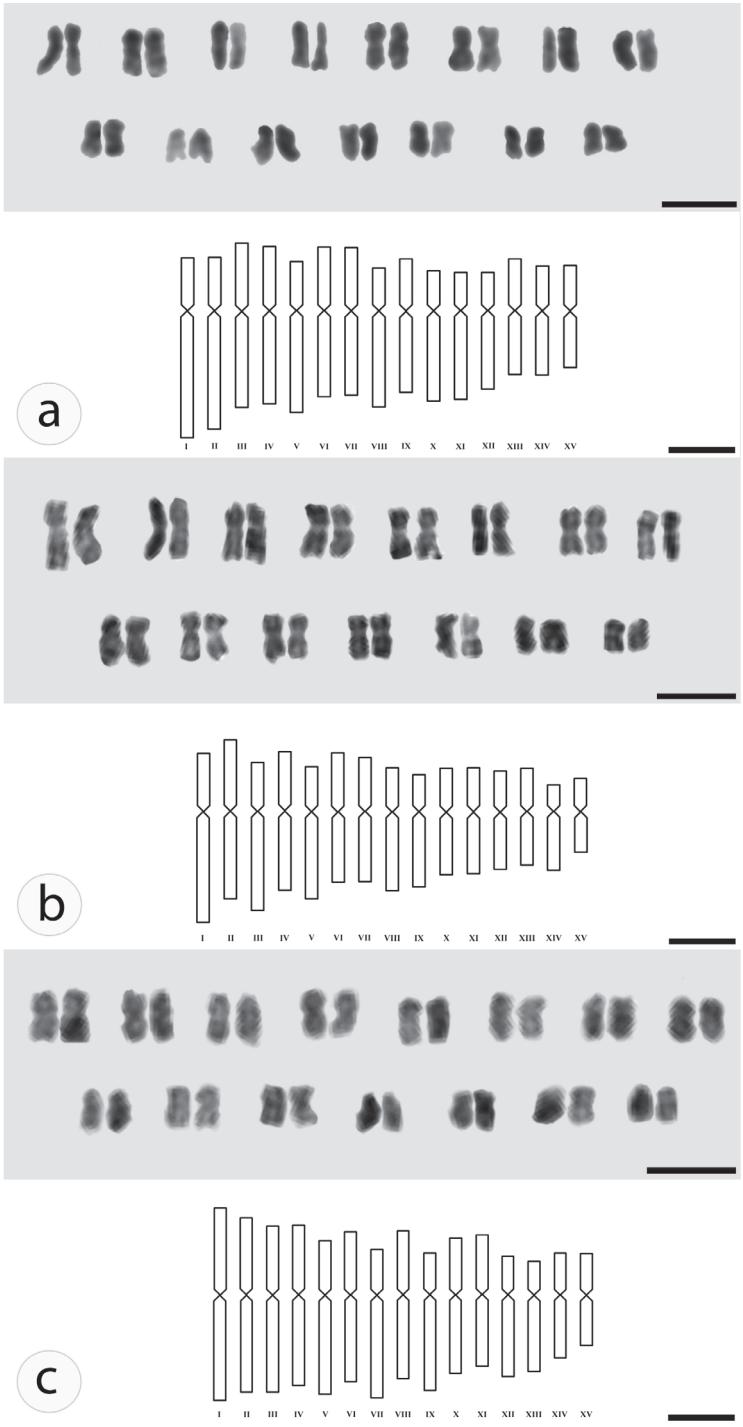

Figure 3. Karyograms and idiograms for *P. barbatus* from different localities. Lavras (**A**), Campinas (**B**), Santa Maria (**C**). Scale bars: Karyograms (5 μm); idiograms (1 μm).

Table 4. Comparison of relative lengths of the chromosome pairs 2, 8 and 12 of *Plectranthus* plants by LSD test.

	***P. b* (UFLA)**	***P. b* (IAC)**	***P. b* (UFSM)**	***P. g* (IAC)**
P. b (UFLA)	-	ABC	AbC	ABC
P. b (IAC)	ABC	-	ABC	aBc
P. b (UFSM)	AbC	ABC	-	ABC
P. g (IAB)	ABC	aBc	ABC	-

*Lowercase letters indicate statistically different mean values. Pair 2 (A); Pair 8 (B); Pair 12 (C). *P. b* (*P. barbatus*); *P. g* (*P. grandis*).

Table 5. Values of karyotypic asymmetry indices of *Plectranthus* species, according to criteria proposed by Zarco (1986) (A_1: intrachromosomal asymmetry, A_2: interchromosomal asymmetry) and proposed by Paszko (2006) (AI: asymmetry index).

	P. a	***P. b***			***P. g***	***P. n***
		UFLA	**IAC**	**UFSM**		
A_1	0.29	0.39	0.32	0.32	0.38	0.33
A_2	0.23	0.17	0.22	0.19	0.15	0.16
AI	3.09	2.60	2.93	2.82	2.48	2.06

**P. a* (*P. amboinicus*); *P. b* (*P. barbatus*); *P. g* (*P. grandis*); *P. n* (*P. neochilus*).

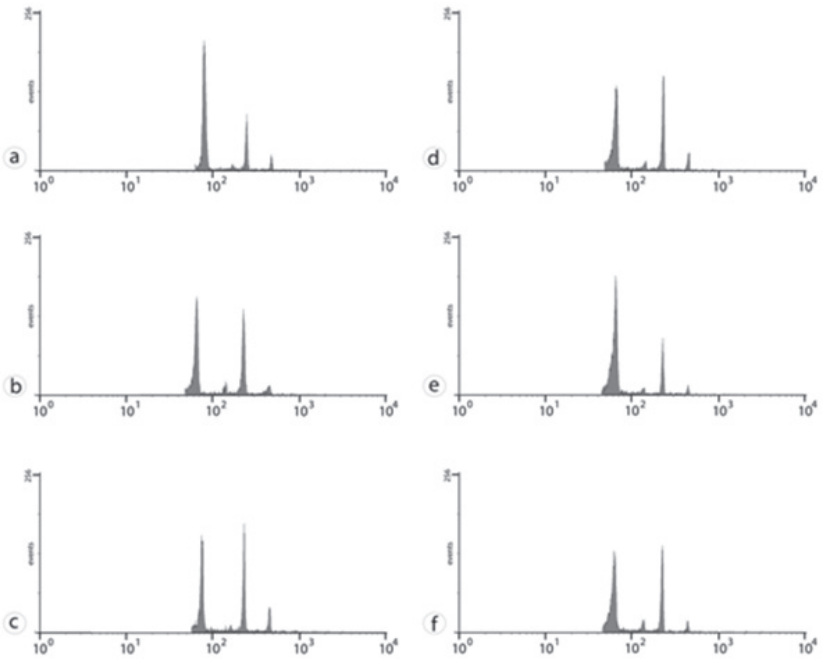

Figure 4. Flow cytometry histograms. *P. amboinicus* **(A)**, *P. grandis* **(B)**, *P. neochilus* **(C)**, *P. barbatus* **(D–F)** from UFLA **(D)**, IAC **(E)**, UFSM **(F)**. The first peak in each histogram refers to the G1 peak of each of the *Plectranthus* species, and the second G1 peak corresponds to the reference sample (*Pisum sativum*). The abscissa represents the DNA amount, and the ordinate the number of nuclei.

Table 6. Mean values of 2C DNA and coefficient of variation obtained by flow cytometry technique for *Plectranthus* plants.

Species/Plant	DNA (pg)[1]	CV (%)
P. amboinicus (UFLA)	5.98 a	0.79
P. amboinicus (IAC 465)	5.79 a	0.72
P. amboinicus (IAC 2193)	5.81 a	0.57
Mean	5.86 A	
P. barbatus (UFLA)	5.20 a	0.57
P. barbatus (IAC)	5.17 a	0.70
P. barbatus (UFSM)	5.69 b	0.57
Mean	5.35 B	
P. grandis	5.23 B	0.64
P. neochilus (UFLA)	5.99 a	0.56
P. neochilus (IAC)	5.94 a	0.54
P. neochilus (UFSM)	6.00 a	0.55
Mean	5.98 A	

[1]Averages followed by the same lowercase letters within each group of species, and averages followed by the same capital letters do not differ statistically by Tukey test at 5% probability.

According to Stebbins (1958), *P. amboinicus* and *P. barbatus* (Campinas and Santa Maria) have karyotypes included in the category 3b. Differently, the karyotypes of *P. neochilus*, *P. grandis* and *P. barbatus* (Lavras) were included in the category 3a.

The studied species of *Plectranthus* have close karyotypic asymmetry indices (Table 5).

P. amboinicus has the largest difference in relation to total size of the chromosomes, besides having the greatest asymmetry index as proposed by Paszko (2006). *P. neochilus* has one of the smallest values, both for intrachromosomal (A_1) and interchromosomal asymmetry (A_2), as proposed by Zarco (1986). It also has the smallest asymmetry index (AI) value as described by Paszko (2006) (Table 5).

In relation to DNA amount in the evaluated *Plectranthus* plants, two groupings were identified (Table 6): *P. amboinicus* (5.86 pg) and *P. neochilus* (5.98 pg) had the highest mean values (Fig. 4a, c), whereas *P. grandis* (5.23 pg) and *P. barbatus* (5.35 pg) had the lowest (Fig. 4b, d–f).

Discussion

Distinct chromosome numbers for *P. amboinicus* have already been described in the literature (2n=16, 24, 30, 32, 34, 48 and 56) in works that treated the species with different synonymies (Scheel 1931, Basavaraja and Krishnappa 1982, Saggoo and Bir 1983, Thoppil 1993).

Thoppil (1993), studying the synonymy *Coleus aromaticus* Bentham, 1831, found 2n=32 as most common chromosome number and that the relative lengths of the largest and smallest chromosomes were 8.19 and 4.37, respectively. The values of relative

length described here, for plants with 34 chromosomes, are slightly similar to those found by Thoppil (1993) (Table 2), suggesting the occurrence of rearrangements of breakage or fusion type in the chromosomes of intermediary size.

Karyotypic studies on *P. neochilus* are rare in the literature. De Wet (1958) and Riley and Hoff (1961), using the synonymy *Coleus pentheri* Gürke, 1905 described 32 chromosomes for plants originated from the east and south of Africa. These authors did not report details of chromosome morphology for this species. The occurrence of 30 chromosomes in *P. neochilus* is reported for the first time in the present work.

The occurrence of 30 chromosomes in *P. barbatus* that was observed for different accessions of this species in the present study corroborates the number reported earlier by different authors (Cherian and Kuriachan 1981, Sagoo and Bir 1983, Bahl and Tyagi 1988, Thoppil 1993). Other descriptions regarding variation in chromosome number (2n=28 to 34) have been related for *P. barbatus* (Reddy 1951, Saggoo and Bir 1983). Riley and Hoff (1961) were the first to find a specimen with 32 chromosomes. According to Thoppil (1993), specimens of this species from the south of India with 28 chromosomes, have autotetraploid genome with basic number x=7.

The statistical differences observed for the pairs 2, 8 and 12 in *P. barbatus* and *P. grandis* suggest the occurrence of chromosomal rearrangements, seeing that some of these pairs present variation both in relative length as well as in centromeric position. This way, the chromosomes of pairs 8 and 12 classified as submetacentric may have undergone alterations, namely deficiency in one of the chromosome arms, giving rise to the metacentric form, or duplications in one of the arms of these chromosomes, thus rendering them submetacentric. The differences seen in the pair 2 for *P. barbatus* (Campinas) and *P. grandis* did not express variations in centromere position, which suggests events of duplication/deficiency in both chromosome arms.

The remaining chromosome pairs of the evaluated *P. barbatus* and *P. grandis* plants did not present significant statistical differences regarding relative length. Nevertheless, the centromere position in some of the pairs of *P. barbatus* plants and of the *P. grandis* plant appeared altered. Taking the chromosome pair 1 as example, the plants from Lavras and Campinas had it classified as submetacentric, and that from Santa Maria, together with *P. grandis*, had the same pair classified as metacentric. These changes in classification of the chromosome pair as to centromere position may be justified by the occurrence of inversions, since the relative lengths are statistically similar. Also, other mechanisms may drastically modify the chromosome structure, among which centromeric repositioning, as reported by Rocchi et al. (2012). These authors propose that centromeric repositioning is an alteration that occurs in the chromosome structure without changes in the base sequence of the DNA. This event creates a new centromere (neocentromere), apparently through epigenetic factors, and substitutes the original one. This finding has deeply modified the interpretation of karyotypic evolution in various mammals.

Different pressures exerted by the different environments can be other reason for karyotypic variations mentioned for *P. barbatus*, since this hypothesis was considered previously by Shah (1989), who evaluated populations of this species from distinct

geographical origins and identified variation in the chromosome morphology among plants of this species.

Passinho et al. (1999) and Bandeira et al. (2010) also evaluated different populations of *Plectranthus* species by means of AFLP (Amplified Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA), respectively, and according to these authors there are intra and interpopulational genetic variation.

The occurrence of differentiated karyotypic formulas for plants of *P. barbatus* and the fact that *P. barbatus* presents nuclear DNA content statistically similar to that of *P. grandis*, are not able to indicate that *P. barbatus* and *P. grandis* have enough differences to be considered distinct species. Therefore, more experiments using molecular cytogenetic techniques are needed in order to understand the relationship between both species.

Regarding to asymmetry of karyotype, based on the methods of Stebbins (1958), *P. amboinicus* is included in a more asymmetric category in relation to the other studied species. According to Stebbins (1958), the karyotypic symmetry is characterized by the predominance of metacentric and submetacentric chromosomes of approximately same size. Nonetheless, this species presents the largest proportion between the smallest and largest chromosome, thus being interchromosomally more asymmetrical.

Based on asymmetry indexes proposed by Zarco (1986) and Paszko (2006), *P. amboinicus* also exhibited the most asymmetric karyotype, as it presented the highest AI value (Table 5). According to Paszko (2006), high values are indicative of more elevated levels of karyotypic heterogeneity. *P. neochilus* can be considered the species with lowest asymmetry in relation to the others, both from the intrachromosomal as well as interchromosomal point of view, due to presenting the lowest coefficients of variation for centromeric index and total chromosome length; these data consequently generate a value inferior to AI (Table 5). Both species are the most distant ones in terms of karyotypic asymmetry. These species have probably undergone structural rearrangements of karyotype, without great losses or gains of DNA sequences, as both have statistically similar amounts of nuclear DNA. Even though the dispersion diagram may indicate different degrees of asymmetry, the studied *Plectranthus* species are strictly related, which can be observed by the gradual variations in AI values (Table 5).

The variation among karyotypes of kindred species and among plants, associated with the differences in nuclear DNA content found in this work, supports the hypothesis that, karyotypically, *P. amboinicus* and *P. neochilus* are more stable species and the variation found among plants of *P. barbatus*, regarding chromosome morphology, express differences among populations.

Conclusions

The populations of *P. amboinicus* and *P. neochilus* present coinciding karyotypes among their respective plants.

P. barbatus is a species undergoing active process of karyotypic variation.

The karyotypic intraspecific variation in *P. barbatus* is an indication that *P. grandis* is one of the events of variation in the species, since the species exhibit the same morphological characteristics.

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RESEARCH ARTICLE

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Cytogenetic description of the earthworm *Drawida ghilarovi* Gates, 1969 (Oligochaeta, Moniligastridae) from the southern Russian Far East

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Abstract

Sixty-six specimens of the earthworm *Drawida ghilarovi* Gates, 1969 (Oligochaeta, Moniligastridae) from 15 localities of the southern Russian Far East were studied cytogenetically. We examined chromosome sets during mitosis and diakinesis as well as DNA content in the spermatogenous and somatic cell nuclei. The populations and morphs displayed no differences in karyotype and ploidy levels estimated in terms of both chromosome number and DNA mass index: n = 10, 2n = 20; c = 1.1 pg, 2c = 2.2 pg. We conclude that polyploidy as a species- or race-forming factor is not typical of these earthworms.

Keywords

Karyotype, chromosomal set, nuclear DNA content, earthworm, *Drawida*, Oligochaeta, Moniligastridae

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Introduction

The karyology of Oligochaeta is mainly studied in the earthworms of the family Lumbricidae. A distinctive feature of the evolution of their karyotypes is polyploidy, which is widespread in the family. The polyploid races and subspecies occur as frequently as the diploid ones (Bulatova et al. 1987, Perel 1987, Viktorov 1993, 1997, Kashmenskaya and Polyakov 2008, Garbar et al. 2009, Vlasenko et al. 2011). Di- and polyploid forms tend to be spatially isolated, with the latter occurring predominantly at the edges of the species' ranges (Grebelnyi 2008, Kotsyuba et al. 2010, Onyschuk and Garbar 2010). In addition, conspecific races of different ploidy levels may occupy different ecological niches, vary in size and coloration, and belong to different life forms (e.g., properly edaphic and soil-litter), with *Eisenia nordenskioldi* (Eisen, 1873) from Siberia and the Russian Far East being an example (Vsevolodova-Perel and Bulatova 2008, Vsevolodova-Perel and Lejrih 2014). At the same time, it was shown that polyploidy is not the cardinal mechanism of the microevolution of the lumbricid species and subspecies in the Caucasus (Bakhtadze et al. 2008).

There are no similar data on earthworms of the family Moniligastridae, in particular, *Drawida ghilarovi* Gates, 1969. The members of this family invaded the South-Eastern Asia after the collision of the Indian and Asian lithospheric plates in the Tertiary period, i.e., 66–1.6 m.y.a. (Easton 1981). In the provinces of the North-Eastern China bordering on Russia, six tropical species of the genus *Drawida* Michaelsen, 1900 are found, and 4 and 8 *Drawida* species are reported for the Korean Peninsula and Islands of Japan, respectively (Blakemore 2003, 2007). The southern part of the *Drawida* range covers India and, possibly, Ceylon (Gates 1969, Ganin et al. 2014). *Drawida ghilarovi*, listed in the Red Books of the Russian Federation and Khabarovsk Territory, occurs in the southern Russian Far East, the northern part of its range. It was described as a new species based on its grey color morph found in forest biotopes of the Kedrovaya Pad' and Ussuriysky Nature Reserves (Gates 1969).

Polyploidy is known to be accompanied by polymorphism. As the amount of available data increased, new color morphs of *D. ghilarovi* were described in the Russian literature, in particular, light-bluish (Gates 1969), aquamarine, bluish grey (Vsevolodova-Perel 1997), pitch black (Ganin 1997), greenish or bluish (The Red Book of the Russian Federation 2001), bluish-black with a metal tint, brownish, and bluish-grey (Ganin et al. 2014).

It was found out that at the northern limit the tropical moniligastridae distribution the Red Book species *Drawida ghilarovi* Gates, 1969 exists in two distinct life forms: "soil-litter" (=epigeic) inhabitants of the floodplain meadow-wetland biotopes and "aneciques" of the forest biotopes (Ganin 2013). Moreover, forest drawidas are represented by two morphs, stable in color and size, living together in different soil horizons. Larger brownish worms with a dark pigmented part close to the belt inhabit the fermentative layer A_o; and a gray morph of worms smaller in size inhabits the 0-10 cm soil layer. Forest gray drawidas can survive in peat and wetland soils, whereas black meadow-wetland worms die in forest soils. Besides, the sympatry in the wetland and

forests inhabitants is not observed, which ensures their reproductive isolation. Phenology of the two forms of worms is also different. Forest drawidas have obligate winter diapause whereas meadow-wetland species can be active all year round and do without freezing. The range of the black morph is limited to the basin of pra-Amur River in Late Neogene. Probably, the floodplain of the river had habitats typical of *D. ghilarovi* at that time (Ganin et al. 2014).

We are not aware of any data on the cytogenetics of *Drawida* or Moniligastridae species in general. Some information was provided in our previous report (Ganin et al. 2014). The goal of the present study is to describe the karyotype and determine the ploidy levels in geographically remote *D. ghilarovi* populations from various biotopes of the northern, western, southern, and eastern parts of the species' Far Eastern range. In particular, we intended to reveal potential polyploid races or subspecies, a proposal anticipated by the presence of similar forms in lumbricids.

Materials and methods

Specimens of *D. ghilarovi* were collected in fifteen localities of the southern Russian Far East (Fig. 1, Table 1). Worms from northern part of the range were collected in the vicinity of the Slavyanka village and Anyuisky National Park (Nanaysky District, Khabarovsk Territory); and in the western part, in the Bastak Nature Reserve (Jewish Autonomous Province); in the central part of the range, in floodplain meadows of the Bolshekhekhtsirskii Nature Reserve and adjacent areas, and cedar forest in Shivki Mountain (Bikinsky District). In the Primorsky Territory, worms were sampled in submontane and montane coniferous and mixed coniferous-broad-leaved biotopes of the Ussuriysky, Kedrovaya Pad', Sikhote-Alin', and Lasovsky Nature Reserves, in the vicinity of Vostok Biological Station (shore of the Sea of Japan), Lazovsky Ridge (about 1000 m above sea level, Partizansky District), and in floodplain meadow biotopes of Razdol'naya River (Nadezhdinsky District) and Ilistaya River (Lake Khanka Nature Reserve, Spassky District). Based on the life form, sampled worms were subdivided into two groups: marsh-meadow and forest. Colored morphs were taken into account too. Worms were kept under laboratory conditions in accordance with soil-zoology requirements. Three sexually mature worms were taken from each of the above groups. In total 66 specimens were studied. Most of these worms were collected in summer 2010–2013 and kept under laboratory conditions.

In accordance with conventional cytogenetic method, 0.04% colchicine solution was introduced into the body cavity for 18–20 h. For chromosome analysis, air-dried preparations of spermatogenous cells were made from suspended content of seminal vesicles incubated in 0.56% KCl solution and fixed with 3:1 mixture of ethanol and glacial acetic acid at 4 °C (Bulatova et al. 1987). In addition, squash preparations were made from fixed portions of seminal vesicles using an original method of tissue squashing through cellophane (Anisimov 1992). Smears of somatic cells were prepared from coelomic fluid and fixed as above. Some preparations were stained with 5%

Table 1. Geographical locality, life form and colored morphs of examined *D. ghilarovi*.

Locality No.	Locality and biotope	Geographical coordinates	Life form and colored morph
Pra-Amur and Amur Rivers basin from north to south			
1†	Khabarovsk Territory, Nanaysky District, Slavyanka village, marsh	49°27'N, 136°46'E	epigeic, black
2†	Khabarovsk Territory, Nanaysky District, Anyuisky National Park, marsh	49°20'N, 137°03'E	epigeic, black
3†	Jewish Autonomous Province, Bastak Nature Reserve, marsh	48°59'N, 135°03'E	epigeic, black
4.1†	Khabarovsk Territory, Lazo District, Bolshekhekhtsirskii Nature Reserve, floodplain of Chirki River, marsh	48°09'N, 135°08'E	epigeic, black
4.2†	The same place, marsh	48°09'N, 135°08'E	epigeic, black-reddish
5†	Khabarovsk Territory, Lazo District, Bolshekhekhtsirskii Nature Reserve, floodplain of Odyr River, marsh	48°06'N, 134°52'E	epigeic, black
6	Primorsky Territory, Spassky District, Lake Khanka Nature Reserve, meadow	44°38'N, 132°49'E	epigeic, black
7†	Primorsky Territory, Nadezhdinsky District, floodplain of Razdol'naya River, meadow	43°33'N, 131°54'E	epigeic, black
West macro-slope of the southern Sikhote-Alin			
8.1	Khabarovsk Territory, Bikinsky District, Shivki Mountain, forest	47°00'N, 134°22'E	aneciques, grey
8.2	The same place, forest	47°00'N, 134°22'E	aneciques, brownish
9.1†	Primorsky Territory, Ussuriysky Nature Reserve, forest	43°33'N, 132°21'E	aneciques, greenish-grey
9.2	The same place, forest	43°33'N, 132°21'E	aneciques, yellow-brown
10	Primorsky Territory, Mountain-taiga Biological Station, forest	43°41'N, 132°09'E	aneciques, yellow-brown
Black Mountains, Chanbaishan Plateau			
11†	Primorsky Territory, Khasansky District, Kedrovaya Pad' Nature Reserve, forest	42°26'N, 130°38'E	aneciques, bluish-grey
East macro-slope of the southern Sikhote-Alin			
12.1†	Primorsky Territory, Vostok Biological Station, forest	42°54'N, 132°44'E	aneciques, brownish, long
12.2†	The same place, forest	42°54'N, 132°44'E	aneciques, brownish, short
13.1†	Primorsky Territory, Lazovsky Ridge, forest	43°30'N, 133°35'E	aneciques, brownish, long
13.2†	The same place, forest	43°30'N, 133°35'E	aneciques, brownish, short
14.1	Primorsky Territory, Lasovsky Nature Reserve, forest	43°00'N, 133°44'E	aneciques, grey
14.2	The same place, forest	43°00'N, 133°44'E	aneciques, brownish
15.1	Primorsky Territory, Sikhote-Alin Nature Reserve, forest	45°14'N, 136°30'E	aneciques, grey
15.2	The same place, forest	45°14'N, 136°30'E	aneciques, yellow-brown

† - partly studied localities (Ganin et al. 2014), other localities are presented for the first time.

Giemsa solution, and the other, with cytochemical Feulgen nuclear reaction, which stains exclusively DNA (Bancroft and Cook 1999). Feulgen reaction allows one to use cytophotometry to measure relative DNA content in nuclei and separate chromo-

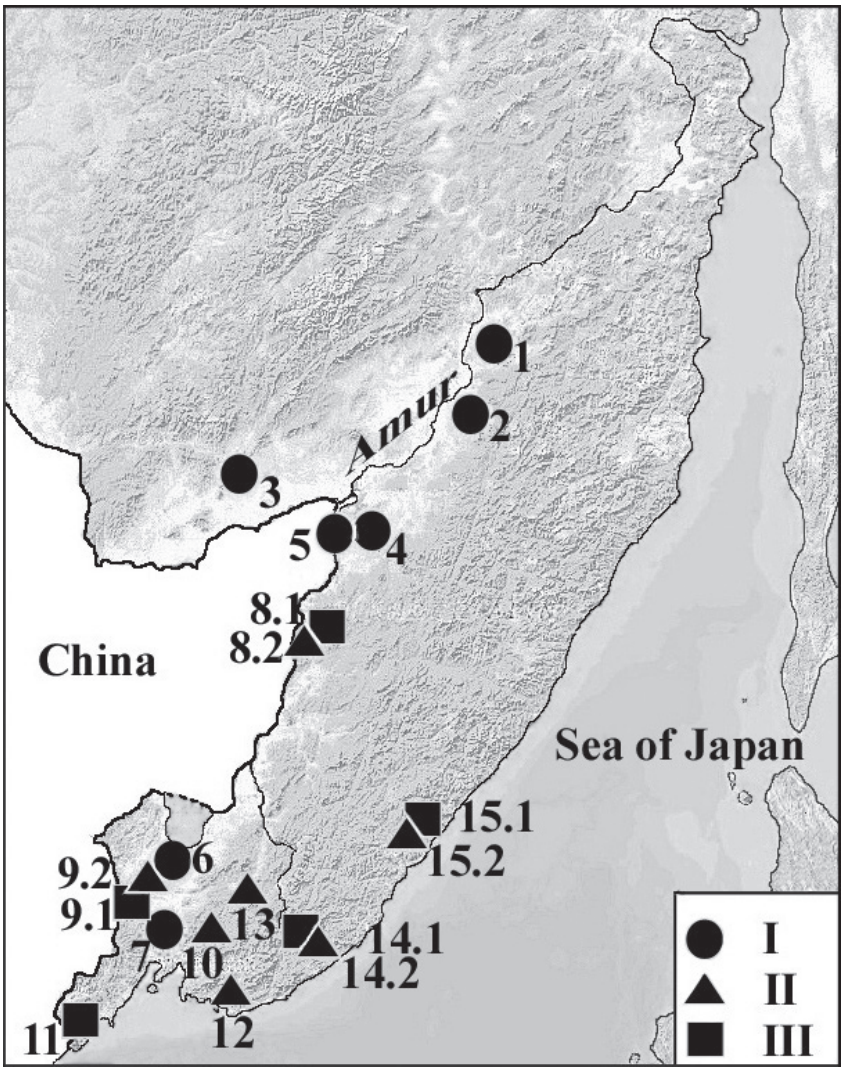

Figure 1. Map showing collection sites (1–15, see Table 1) of different morphs (I-III) of the tropical worm *Drawida ghilarovi* in the southern Russian Far East. I – black, black-reddish; II – brownish, yellow-brownish; III – aquamarine, bluish-grey, grey, greenish-grey, brownish-blue morphs.

somes and determine cell ploidy levels. Samples of 100–150 spermatogenous cells and 30–40 coelomocytes were obtained from each worm. The cells were photographed using an AxioImager A1 and Axioscop 40 microscopes equipped with a digital camera (Carl Zeiss). The chromosome analysis and computer cytophotometry of nuclei were performed using Adobe Photoshop CS3. The relative DNA mass was obtained as follows: m(DNA) = NA*(BI – NCI), where m(DNA) is conventional DNA mass; NA, nuclear area expressed as number of pixels; BI, background intensity level; and NCI, nuclear chromatin intensity level. To estimate the absolute mass of diploid DNA (pg),

cultured rat cells with known genome mass (Gregory 2005) were fixed, stained, and subject to cytophotometry under the same conditions as worm cells. For karyotypes characteristics, the length of the chromosomes was measured in micrometers at the images using AxioVision 4.8.2. Chromosomes were classified on the centromere index using the criteria of Levan et al. (1964). Centromere index is the length of the short arm divided by the total chromosome length and multiplying by 100 percentage. Data were statistically treated and histograms were built using Microsoft Excel.

Results and discussion

Preparations made from seminal vesicles contained spermatogenous cells of different maturity depending on sampling period (May–October). In the first half of the summer, spermatogonia and lepto-, zygo-, and pachytene spermatocytes-I predominated, while in the second half, diplotene, diakinetic spermatocytes, cells undergoing meiosis, spermatids, and mature sperm cells mostly occurred. Worms collected in autumn (September–October) or spring (May) were sexually inactive. Samples obtained from them contained degenerating spermatogenous cells of different maturity and rare spermatogonia foci. In summer samples, spermatogenous cells at all maturity stages were found.

Karyotypes were determined based on the joint analysis of chromosome sets in mitotic metaphase plates of dividing spermatogonia, in spermatocytes-I at diakinesis and metaphase I of the first meiotic division. The content of nuclear DNA was measured in coelomocytes and spermatogenous cells at all maturity stages, from spermatogonia to spermatids including chromosome plates. It was also measured in spermatozoa. However, due to high optical density of chromatin, values of DNA content in spermatozoa were lower than in spermatids. They were therefore excluded from further analysis.

Photometric estimation of nuclear DNA content and chromosome analysis did not reveal genotypic differences among worms sampled from different regions or biotopes or having differences in coloration.

Nuclear DNA content

Nuclear DNA content in spermatogenous cells was trimodally distributed, as expected, with twofold increment between neighboring nuclear classes. In most cases, a sparse fourth class was also observed (Fig. 2). Accounting for morphological parameters of spermatogenous cells (size and shape of nuclei and chromatin structure), the class exhibiting the lowest DNA content was attributed to spermatids and spermatozoa; it is therefore a haploid class (c). The next class, a diploid one (2c), was formed by spermatogonia, rare spermatocytes II, and somatic cells of seminal vesicles. The tetraploid class (4c) comprised premeiotic spermatocytes-I (including easily identifiable pachytene, diplotene, and diakinetic stages) and mitotic and G_2-phase spermatogonia. The

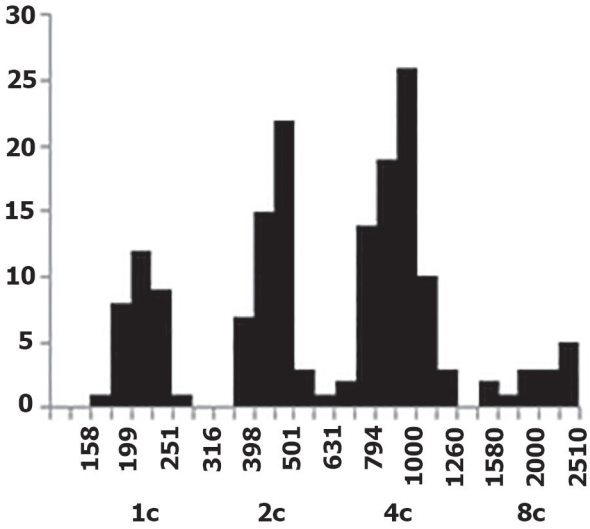

Figure 2. Distribution of nuclear DNA content in spermatogenous and somatic cells from seminal vesicles of *D. ghilarovi* sampled in the Kedrovaya Pad' Nature Reserve. Abscissa, DNA content (conventional units and haploid "c" units). Ordinate, number of nuclei.

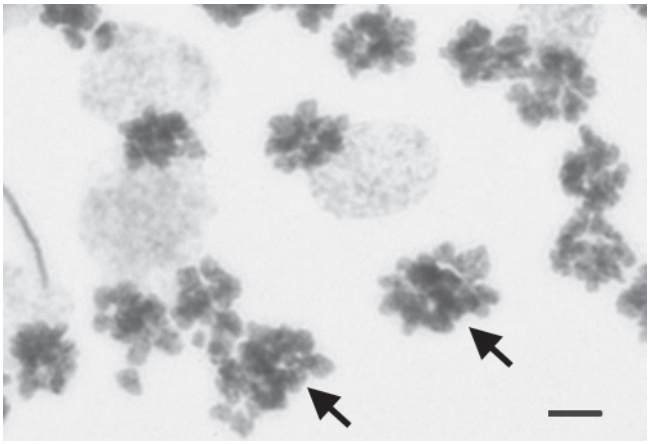

Figure 3. Cluster of synchronously dividing spermatogonia from a seminal vesicle of *D. ghilarovi* sampled in the Lasovsky Nature Reserve. Arrows point at two tetraploid (4n8c) mitoses among ordinary diploid ones (2n4c). Scale bar: 5 μm.

small octoploid class (8c), atypical of sexual cell population, was formed by apparently meiotic prophase nuclei twice as large as normal tetraploid nuclei.

In accordance with these results, tetraploid (4n8c) mitotic figures occurred in clusters of normal diploid mitoses (Fig. 3). Obviously, a small portion (<0.5–1%) of spermatogonia undergo polyploidizing mitosis in the last cell cycle to form a subpopulation of anomalous spermatocytes with octoploid DNA content. In several cases,

diakinetic nuclei with an increased number of bivalent chromosomes were encountered. Diploid spermatids and spermatozoa resulting from such polyploid meiosis only occasionally occurred. Anyway, their number did not correspond to that of divided octoploid spermatocytes.

The estimates of DNA content in coelomic fluid cells confirm the above results, in particular, the presence of octoploid spermatocytes. Coelomocytes used as a coarse reference of diploid DNA content normally had the expected DNA amount (2c), coinciding with the second peak of the distribution of DNA content in sexual cells (appr. 450 conventional units). Occasionally, the distribution of DNA content in coelomocytes displayed hypodiploid asymmetry, possibly, due to mass degradation (apoptosis) of these cells.

Polyploidization of a portion of spermatogenous cells may be considered analogous to somatic polyploidy (endopolyploidy, localized polyploidy) widespread in plants and animals. In somatic polyploidization, a portion of a cell population (occasionally, the whole population) switches to incomplete mitotic cycles including abortive mitosis, endomitosis, or DNA endoreplication in polytene chromosomes (Edgar and Orr-Weaver 2001, Anisimov 2005, Lee et al. 2009, Davoli and Lange 2011). In *Drawida* spermatogenous cells, the polyploidy is facultative and has no apparent adaptive or population-genetic value.

Using cytophotometry of spermatogenous cells and coelomocytes, the averaged diploid DNA content (in conventional units) was estimated for each of *D. ghilarovi* populations (life forms and colored morphs) (Table 2). As is seen, different populations displayed no substantial differences in 2c DNA content of the standard (diploid) chromosome set. The estimates varied from 433±6 to 479±8 conventional units. Some between-population differences in 2c DNA content were statistically significant. For example, the values 442±9 (locality No. 1) and 463±5 (No. 3) differ with $p<0.05$; 462±7 (No. 9.1) and 436±5 (No. 9.2) display $p<0.01$; while limits 433±6 (No. 8.1)

Table 2. Mean diploid DNA content in *D. ghilarovi* locality as determined by cytophotometry. SE – standard error.

Locality No. (see table 1)	Mean 2c DNA content ± SE (in conventional units)	Locality No. (see table 1)	Mean 2c DNA content ± SE (in conventional units)
1†	442±9	9.2	436±5
2†	458±9	10	479±8
3†	463±5	11†	477±5
4.1†	444±8	12.1†	445±8
4.2†	465±9	12.2†	442±6
5†	467±8	13.1†	448±8
6	474±6	13.2†	460±8
7†	467±9	14.1	468±7
8.1	433±6	14.2	467±7
8.2	442±6	15.1	466±8
9.1†	462±7	15.2	470±5

† - partly studied localities (Ganin et al. 2014), other localities are presented for the first time.

and 479±8 (No. 10) have $p<0.001$. Noteworthy, similar differences were occasionally observed between samples from the same population. Probably, they are the result of methodical or seasonal variations during collection and/or keeping of worms. Anyway, the present results revealed no signs of organism-level polyploidy in the *D. ghilarovi* populations studied, which would cause genome-scale differences as early as in zygote.

The size of *D. ghilarovi* genome expressed as absolute DNA mass (pg) was estimated as follows. The photometric amount of 2c DNA averaged for 22 samples was 458 conventional units. Hence, c = 229 conventional units. The photometric amount of rat 2c DNA determined from cultured cell preparations using the same staining protocol was 1284 conventional units, which gives 642 conventional units per haploid amount (c). The absolute haploid DNA mass of the rat genome is 3.1 pg (see reference base in Gregory 2005). The proportion (229 × 3.1 : 642) gives the haploid (c) mass of *D. ghilarovi* DNA to be 1.1 pg.

Karyotype characteristics

Chromosome analysis revealed that worms belonging to different populations and color morphs, with dividing spermatogonia having 20 chromosomes in the diploid set (2n = 20) (Fig. 4a). The pairing of homologous chromosomes is easily discerned in karyogram (Fig. 4b). As is seen, the *D. ghilarovi* karyotype comprises 10 chromosome pairs, whose mean length, centromeric index and morphology are presented in the Table 3.

At early diakinesis almost all bivalents had a ring-like morphology, except two bivalents which were rod-shaped (Fig. 5a). The analysis of spermatocytes-I at the stages of diakinesis to metaphase-I containing the haploid number of bivalent chromosomes also showed that all *D. ghilarovi* specimens had the same chromosome set (n = 10) (Fig. 5). In accordance to this, 10 single chromocenters could be observed in early spermatids.

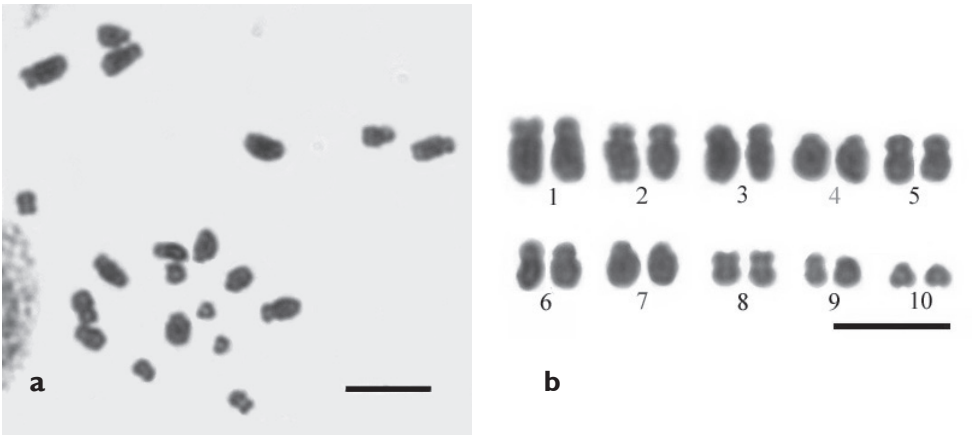

Figure 4. Mitotic metaphase (**a**) and karyogram (**b**) of *D. ghilarovi* from the Sikhote-Alin Nature Reserve. 2n = 20. Scale bars: 5 μm.

Table 3. Mean length (ML), its standard deviation (SD) and centromere index (CI) of the chromosome pairs in six metaphase plates of *D. ghilarovi*. SE – standard error; m – metacentric, sm – submetacentric, st – subtelocentric chromosomes.

Chromosome pair	ML ± SE (μm)	SD of ML	CI ± SE	Centromere position
1	3.03±0.05	0.17	28.97 ±1.18	sm
2	2.71±0.07	0.25	27.65±1.14	sm
3	2.46±0.05	0.16	29.91±1.47	sm
4	2.33±0.06	0.21	24.10±0.37	st
5	2.20±0.05	0.17	44.93±0.87	m
6	2.06±0.06	0.19	30.16±1.62	sm
7	1.94±0.05	0.15	22.92±0.38	st
8	1.75±0.04	0.13	38.91±0.82	m
9	1.53±0.04	0.13	39.80±0.97	m
10	1.23±0.05	0.16	23.12±0.62	st

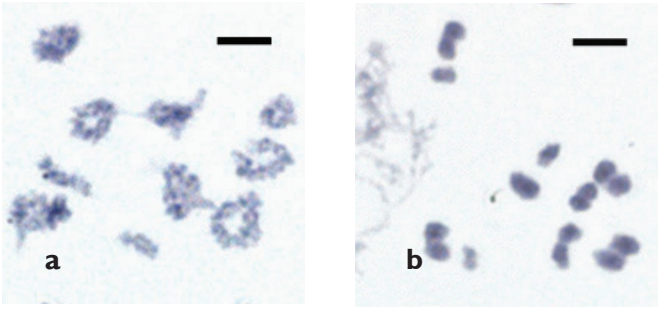

Figure 5. Early diakinesis (**a**) and meiotic metaphase-I (**b**) in *D. ghilarovi* from the Kedrovaya Pad' Nature Reserve. n = 10. Scale bars: 5 μm.

The differences between chromosomes in relative DNA content were determined by cytophotometry of separate bivalents in several diakinetic plates. Their ranked series is presented in Table 4. It begins with bivalent 1, the largest one (15.8% of the total DNA content) and ends with bivalent 10 only comprising 3.6% of the total DNA content. In further research, these data together with morphometric results may be used as an additional parameter when comparing *Drawida* karyotypes from new ranges.

As was mentioned above, there are no available data on the cytogenetics of Moniligastridae, in particular, *Drawida*. However, karyotypes of Lumbricidae worms are relatively well studied. Approximately half of the members of this group are polyploid. In most lumbricids, the basic haploid set includes 18 chromosomes, and diploid, 36 chromosomes (Viktorov 1993, Vsevolodova-Perel and Bulatova 2008, Bakhtadze et al. 2008, Kashmenskaya and Polyakov 2008). In polyploid members of the family, the number of chromosomes is usually a multiple of 18. However, some diploid species of the family have haploid sets of 11, 15, 17, or 19 chromosomes (Bakhtadze et al. 2008, Onyschuk and Garbar 2010). For example, in different populations of *Eisenia*

Table 4. DNA cytophotometry data of genome mass (%) distribution in chromosomes of individual diakinetic spermatocytes of *D. ghilarovi*. M – mean, SE – standard error.

Locality No. / specimen No.	Chromosome No.									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1/1	16.01	14.07	13.12	10.83	10.75	10.00	9.87	6.63	5.27	3.46
3/1	15.70	14.27	12.95	10.50	10.17	10.12	9.51	6.54	6.54	3.71
6/1	15.84	13.80	11.95	11.17	11.25	9.89	9.88	6.74	6.06	3.41
6/2	15.68	13.47	12.43	10.93	10.15	9.92	9.61	7.96	6.16	3.69
7/1	15.30	13.48	12.85	11.07	10.88	9.93	9.40	6.90	6.38	3.80
11/1	16.36	14.05	12.76	11.62	11.43	10.29	9.35	5.74	4.80	3.60
11/2	15.40	12.94	12.93	11.58	11.38	10.25	9.97	6.21	5.85	3.48
M ± SE	15.76 ±0.14	13.73 ±0.17	12.71 ±0.15	11.10 ±0.15	10.86 ±0.20	10.06 ±0.06	9.65 ±0.09	6.67 ±0.26	5.87 ±0.24	3.59 ±0.06

foetida, the haploid and diploid sets comprise 11 and 22 chromosomes, respectively (Vitturi et al. 1991, Viktorov 1993, Bakhtadze et al. 2008). Thus, *D. ghilarovi* has the smallest chromosome set among all earthworms studied so far (n = 10 and 2n = 20). In the family Lumbricidae, the chromosome size varies usually within 2–10 μm, while the largest chromosome in *D. ghilarovi*, scarcely exceeds 3 μm (cf. Fig. 4 and Table 3), and the size of incompletely compacted chromosomes in that, up to 5 μm (Ganin et al. 2014). At the same time, the genome mass in *D. ghilarovi* presently estimated to be about 2.2 pg (2c) is substantially greater than in other lumbricids. Thus, in *Octodrilus complanatus*, 2c = 1.72 pg (at 2n = 36), and in *E. foetida*, 2c = 1.4 pg (at 2n = 22) (Vitturi et al. 2000). Obviously, comparative cytogenetic characterization of Oligochaeta requires further research using various morphological and cytochemical parameters.

Conclusion

To summarize the above, all examined *D. ghilarovi* populations from the southern Russian Far East had the same karyotype and ploidy level in terms of both chromosome number and DNA mass, exactly, n = 10, 2n = 20; c = 1.1 pg, 2c = 2.2 pg. In other words, polyploidization as a species- or race-forming factor is not typical of this group.

Acknowledgement

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Chromosome analysis of *Endochironomus albipennis* Meigen, 1830 and morphologically similar *Endochironomus* sp. (Diptera, Chironomidae) from water bodies of the Volga region, Russia

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Abstract

Based upon the detailed chromosome map of polytene chromosomes of the eurybiont species *Endochironomus albipennis* Meigen, 1830, the localization of the centromere regions using a C-banding technique is defined. Chromosomal polymorphism in populations from two water bodies in the Volga region has been studied, 17 sequences are described. Polytene chromosomes of *Endochironomus* sp. (2n=6), having larvae morphologically similar to those of *E. albipennis* Meigen, 1830 (2n=6) are described for the first time.

Keywords

Diptera, Chironomidae, *Endochironomus albipennis*, *Endochironomus* sp., karyotype, polytene chromosomes, chromosomal polymorphism, Volga River

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Introduction

Larvae of *Endochironomus albipennis* Meigen, 1830 inhabit water bodies of different types. They are typical epibiotic organisms inhabiting submerged objects in the littoral zone, sometimes occurring also inside strongly decomposed plant residues (Kalugina 1963, Belyanina 1981). In Russia, this species is widely spread in the South and Center of the European part, in Siberia and in Kamchatka (Kalugina 1963, Belyanina 1981, Petrova and Michailova 1989).

The first data about the chromosome number of *E. albipennis* (2n=6) were reported by Konstantinov and Belyanina-Nesterova (1971). Later, a description of the karyotype and chromosomal polymorphism in a population from the Volga River was done by Belyanina (1981). This author indicated the chromosomes as: chromosome I (arms AB); chromosome II (arms CD); chromosome III (arms EF). Another description of chromosome arms including marking the chromosome regions was made by Michailova and Gercheva (1982) and Michailova (1987, 1989) for the Bulgarian and Swiss populations.

Kiknadze et al. (1991) mapped *E. albipennis* chromosomes using the photomap of Michailova (1987). Nevertheless designation of arms in chromosome III in their article does not conform to this system, i.e. numeration of parts (from 1 to 12) begins from the arm defined as F, whereas the same arm in photomap of Michailova (1987) is defined as arm GE. Arm GE in the photomap of Kiknadze et al. (1991) conforms to arm F in photomap of Michailova (1987). Chromosomal polymorphism of *E. albipennis* is still poorly studied, but several types of inversions have been described by Belyanina (1981) and Petrova and Michailova (1989).

There is neither a unified system of chromosome mapping nor a catalogue of chromosome sequences for *E. albipennis*. The few available photomaps are partially incomparable with each other. Therefore it is impossible to establish the limits of chromosome rearrangements in the populations of this species.

The main objectives of the present work were to study the chromosome polymorphism in two populations of *E. albipennis* from the Volga region and to present the list of chromosome sequences of the species. In addition, our aim was to provide the first description of polytene chromosomes of *Endochironomus* sp., larvae of which are similar in morphology to those of *E. albipennis*.

Material and methods

The investigations were carried out in three stations in the Volga region (near Saratov). Sixty eight larvae of *E. albipennis* were collected in Sazanka Lake, Engels (51°29'52"N, 46°4'11"E) and in a pond near Novo-Aleksandrovka village (48°21'00"N, 31°29'00"E). Thirteen larvae of *Endochironomus* sp. were collected 11.08.2010 in Saratovka River (51°31'9"N, 46°15'57"E) inside decomposing rhizomes of *Nuphar luteum* (Linnaeus, 1753).

The species were identifying using larval morphology (Pankratova 1983, Pinder and Reiss 1983). The preparations of the polythene chromosomes were made from squashes of salivary glands cells stained with the ethanol-orcein method (Demin and Shobanov 1990). For detection of heterochromatin and centromere regions in chromosomes, a method of C-banding described by Belyanina and Sigareva (1978) was used.

Designation of the polythene chromosome arms was made according to Michailova (1987). In the chromosome map of *E. albipennis* (Figs 1a, 2a, b, 3a) we have saved the marking of large regions (marked by large numerals at the pictures) conforming to the mapping system developed by Michailova and Gercheva (1982) and Michailova (1987, 1989). We developed here a more detailed mapping including the separation of the small regions (marked by small numerals under the chromosome) of chromosomes (Table 1, Figs 1–3).

Designation of the band patterns conforms to the order of their description: *alb*A1, *alb*A2 etc. Genotypic combinations of banding sequences in every arm were designated as A1.1, A1.2, A2.2, etc., respectively. For analyzing chromosomal polymorphism we calculated the frequencies for every combination of chromosome sequences in each chromosome arm and also the mean number of heterozygous inversions per individual.

Table 1. Chromosome arms and banding sequences in the polytene chromosomes of *Endochironomus albipennis*.

Chromosome arms (Belyanina 1981)	Chromosome arms (Michailova 1987, 1989)	Banding sequences (Michailova 1987)	Banding sequences (this study)
C	A	1–6a	*alb*A1 (1–16)
		-	*alb*A2 (inversion of section 4–14)
		-	*alb*A3 (inversion of section 4–15)
D	D	6b-15	*alb*D1 (17–33)
		inversion of section 10–13	*alb*D2 (inversion of section 24–31)
		-	*alb*D3 (inversion of section 22–31)
A	B	1–5	*alb*B1 (1–17)
		-	*alb*B2 (inversion of section 9–12?)
		-	*alb*B3 (inversion of section 5–8)
B	C	6–12	*alb*C1 (19–35)
		-	*alb*C2 (inversion of section 18–32)
		-	*alb*C3 (inversion of section 24–34)
E	GE	1–6	*alb*GE1 (1–16)
		-	*alb*GE2 (inversion of section 3–10)
		-	*alb*GE3 (some inversion steps)
F	F	7–12	*alb*F1 (17–32)
		inversion of section 8–9	*alb*F2 (inversion of section 21–25)

Analysis of slides was performed under the microscope MBI-11У4.2. For photomicrography a digital photographic camera Panasonic LS80 LUMIX was used. In the description of the larval morphology the terminology by Saether (1980) was used.

Results

Endochironomus albipennis. 2n=6. (Figs 1–4)

Karyotype. Centromeres are not distinct morphologically. Based on the C-banding patterns, chromosomes I (AD) and II (BC) are metacentric, whereas chromosome III (GEF) is acrocentric (Fig. 3). Chromosome arms designated previously by different authors are offered in Table 1. Frequencies (%) of chromosome inversions are presented in Table 2.

Chromosome I (AD).The centromere region was detected using C-banding technique (Fig. 4b) as a thin indistinct C-band on the boundary of sections 16 and 17.

Arm A (Fig. 1a) has the following band sequence: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 (Table 1, Fig. 1a). Balbiani Ring (BR_1) is located in section 15. Section 5 contains a weakly active puff; on the boundary of sections 10 and 11 there is a constriction. Sequence *alb*A2 apparently was formed on sequence *alb*A1 as a result of inversion of sections 4-14. Sequence *alb*A2 was present both in homo- and heterozygous states (Fig. 1b). Homozygous inversion A2.2 was found for the first time; heterozygous inversion A1.2 was described previously as C/C1 by Belyanina (1981) and was observed with high frequency in larvae from the Volga River (Table 2). The chromosome sequence *alb*A3 found here for the first time arose apparently as a result of inversion of sections 4-15 (Fig. 1c) and occurred only in a heterozygous state – A1.3 (Table 2).

Arm D (Fig. 1a) has the band sequence: 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33. In section 17, BR_2 is situated (Fig. 1a). The Nucleolus Organizer Region (NOR) is located in section 22 and shows a variable degree of activity. Sequence *alb*D2 was apparently formed on sequence *alb*D1 as a result of inversion of sections 24-31 and was present in the heterozygous state – D1.2 (Fig. 1b, c). The new sequence *alb*D3 was apparently formed on the sequence *alb*D1 as a result of inversion of sections 22-31. The homozygous inversion D2.2 and heterozygous inversion D1.3 were found for the first time, heterozygous inversion D1.2 was described previously as D/D1 by Belyanina (1981) and was also observed in larvae from Volga (Table 2).

Chromosome II (BC).The centromere region is detected using C-banding (Fig. 4 a) as an indistinct C-disc on the boundary between sections 18 and 19.

Arm B (Fig. 2a) has the band sequence: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18. The NOR is situated in section 5; no other active regions are present in this arm. For the first time we revealed the chromosome sequence *alb*B3, that had apparently arisen on sequence *alb*B1 as a result of inversion of sections 5-8 and was present both in the homozygous (Fig. 2b, c) and heterozygous states. The heterozygous inversion

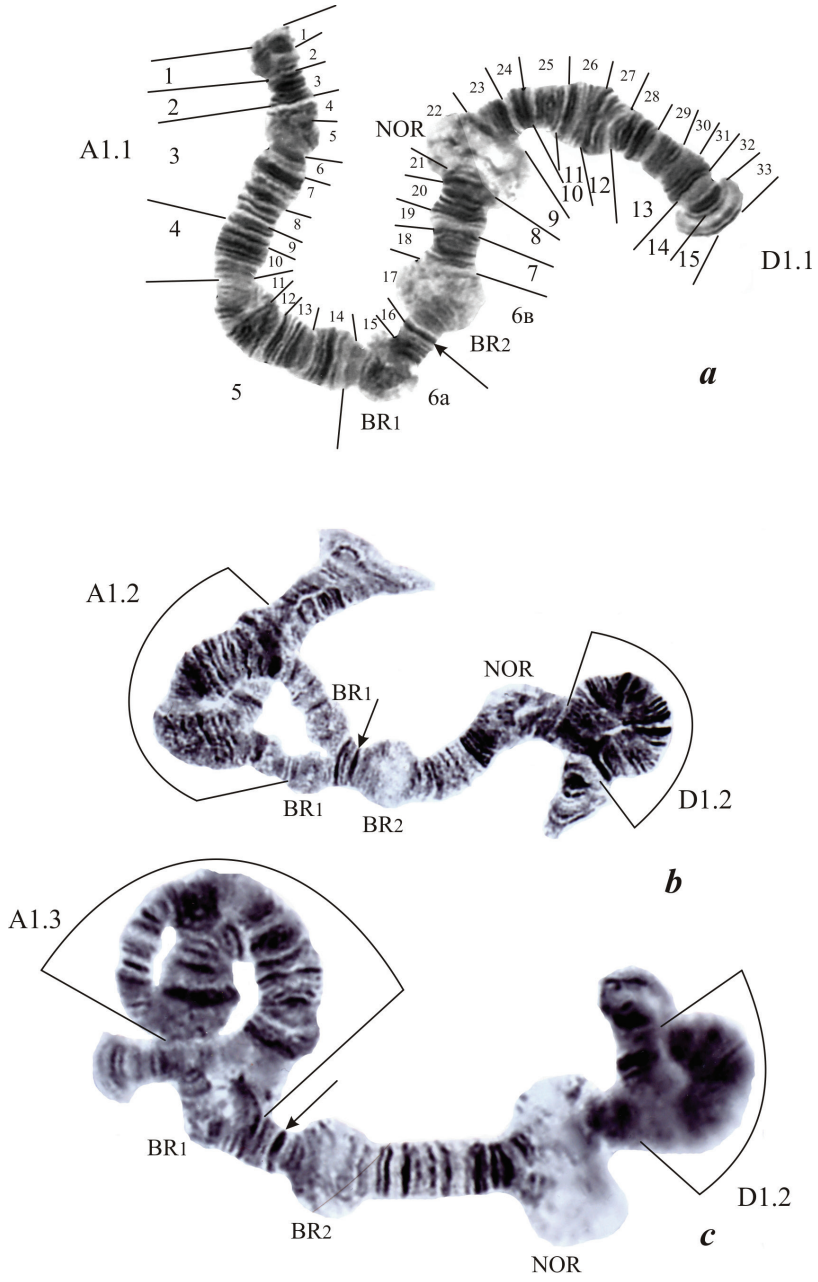

Figure 1. Chromosome I (AD) in the karyotype of *E. albipennis*: **a** homozygous for chromosomal sequences in the arms A (A1.1) and D (D1.1) **b** chromosome I (AD) with two heterozygous inversions – in the arm A (A1.2) and D (D1.2) **c** chromosome I with two heterozygous inversions – in the arm A (A1.3) and D (D1.2). Chromosome arms after Michailova (1987). The large regions of chromosome are presented according to Michailova (1987), small regions of chromosome done in this study were marked over the chromosome. The regions with inversions are marked by the brackets, Nucleolus Organizer (NOR), Balbiani ring (BR), puff (p), arrows indicates the centromere of the chromosome I (AD).

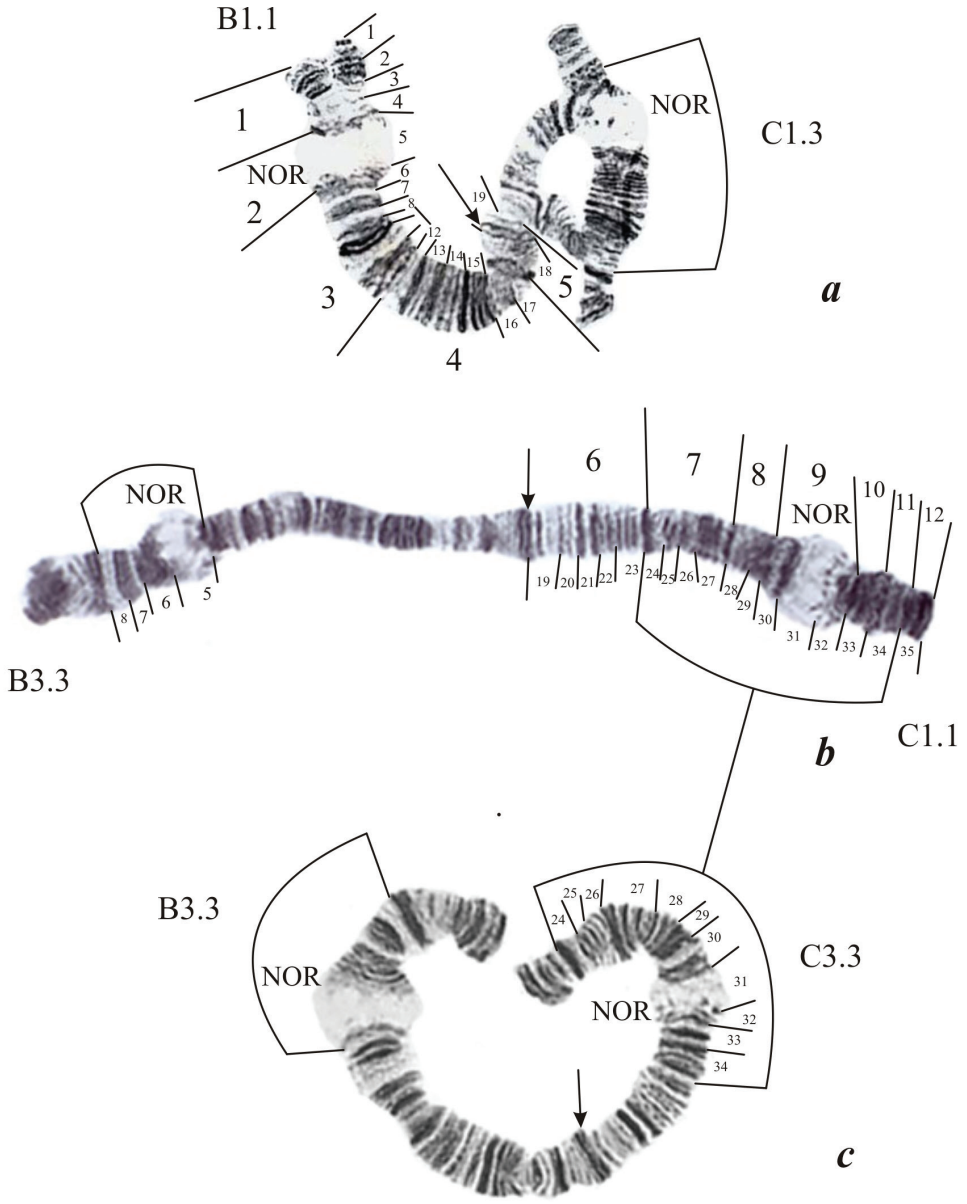

Figure 2. Chromosome II (BC) in the karyotype of *E. albipennis*: **a** homozygous for chromosomal sequences B1 in the arm B (B1.1) and heterozygous for the sequence C3 (C1.3) **b** chromosome II (BC), for B3 (B3.3) and C1 (C1.1) **c** chromosome II (BC) with two homozygous inversions (B3.3 and C3.3). The designations are the same as in Fig. 1.

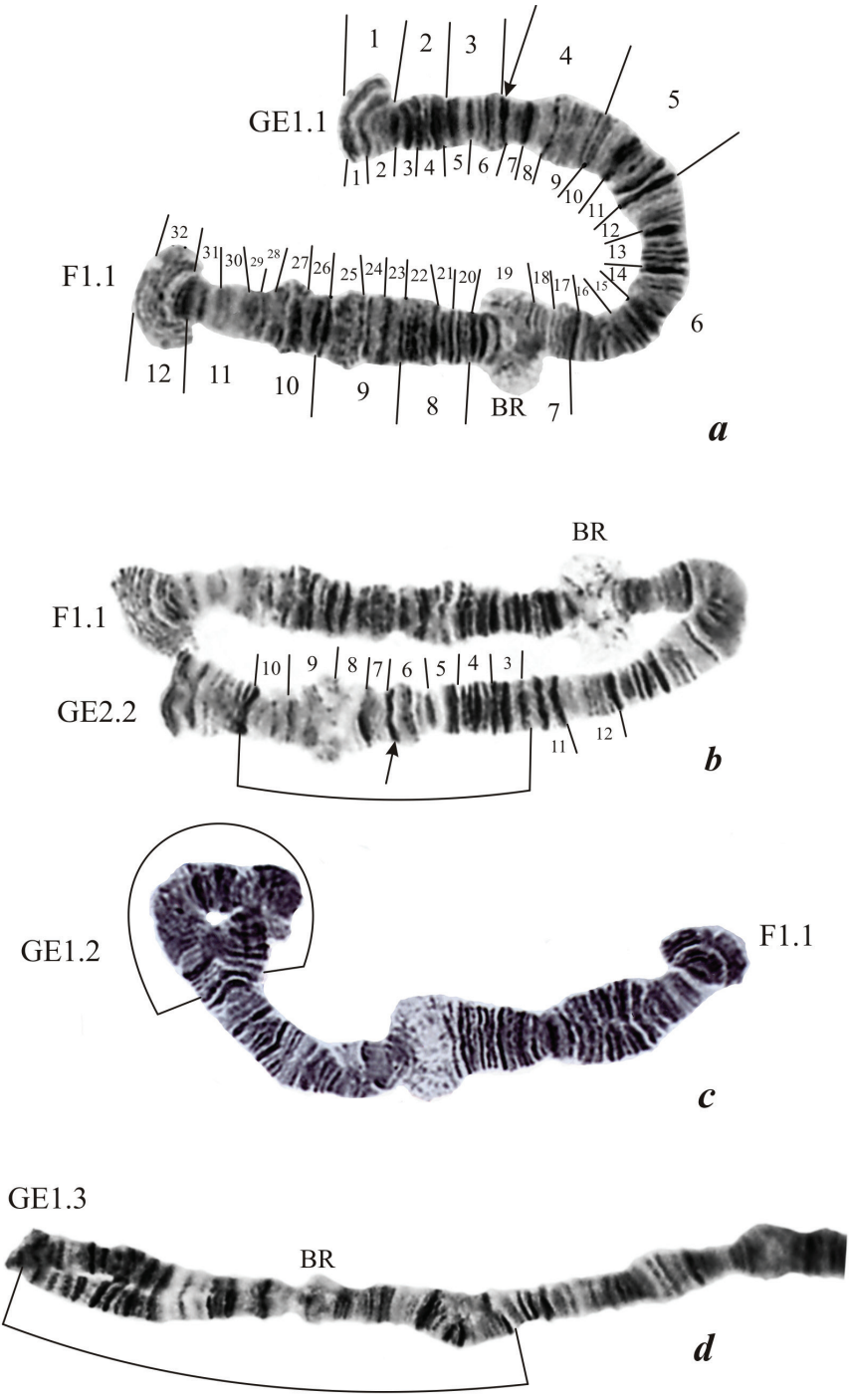

Figure 3. Chromosome III (GEF) in the karyotype of *E. albipennis*: **a** homozygous for chromosomal sequences in the arms GE (GE1.1) and in the arm F (F1.1) **b** homozygous inversion GE2.2 **c** heterozygous inversion GE1.2 **d** heterozygous inversion GE1.3. The designations are the same as in Fig. 1.

Table 2. Frequencies (%) of chromosome inversions in the polytene chromosomes of *Endochironomus albipennis*.

Genotypic combinations	Frequencies (%)		
	Sazanka Lake, Engels, 12.10.2008, 51 individuals (Present data)	**Pond near Novo-Aleksandrovka village, 10.05.2009, 17 individuals (Present data)**	**Volga River, Saratov, 1971–1972, 1976–1979; 126 individuals (Belyanina 1981)**
A1.1	22.7	52.9	39.7
A1.2	46.0	23.5	60.3 (C/C1)
A2.2	3.9	-	-
A1.3	27.4	23.5	-
D1.1	60.7	88.2	71.4
D1.2	23.5	5.8	28.6 (D/D1)
D2.2	1.9	5.8	-
D1.3	11.7	-	-
B1.1	37.2	29.4	85.7
B1.2	-	-	14.3 (A/A1)
B3.3	43.1	58.8	-
B1.3	17.6	11.7	-
C1.1	43.1	29.4	25.4
C1.2	-	-	74.6 (B/B1)
C1.3	23.5	17.6	-
C3.3	27.4	47.0	-
EG1.1	25.4	58.8	-
EG1.2	43.1	23.5	-
EG2.2	9.8	-	-
EG1.3	19.6	17.6	-
F1.1	98.1	100	-
F1.2	1.9	-	-
Number of heterozygous inversions per individual	1.6	1.3	3.2

B1.2 was described previously as A/A1 by Belyanina (1981) and was not observed in our study (Table 2).

Arm C (Fig. 2b) has the band sequence: 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35. The NOR is situated in section 31. Chromosome sequence *alb*C3 was formed as a result of inversion in sections 24-34 and was present in both states, heterozygous – C1.3 (Fig. 2a) or homozygous – C3.3 (Fig. 2c). The inversions C1.3 and C3.3 were found for the first time; the heterozygous inversion C1.2 was previously described as B/B1 by Belyanina (1981) and was observed only in larvae from the Volga (Table 2).

Chromosome III (GEF). Previously it was suggested that this chromosome is the result of tandem fusion of the short chromosome IV with arm E of chromosome EF

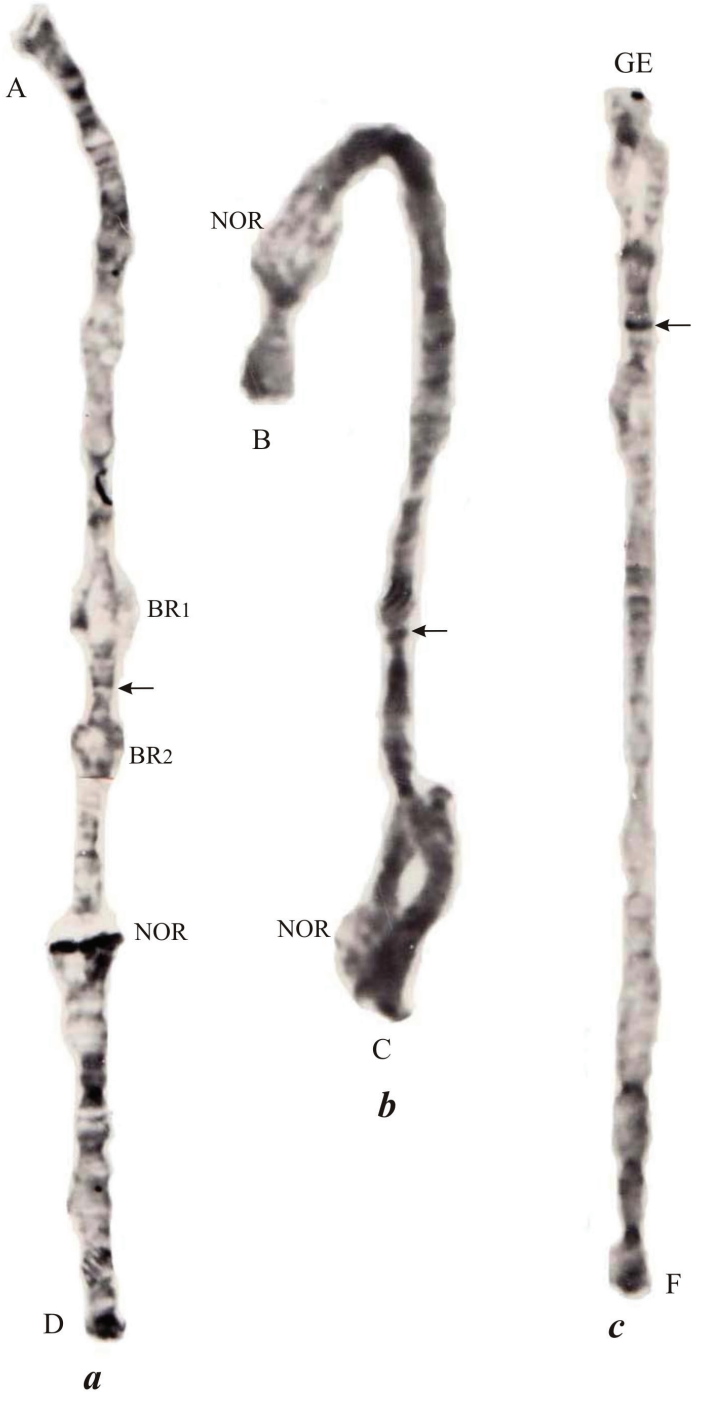

Figure 4. Localization of the centromere regions in the polythene chromosomes of *E. albipennis* by C band staining: **a** chromosome I (AD) **b** chromosome II (BC) **c** chromosome III (GEF). The designations are the same as in Fig. 1.

but the division into arms «GE» and F was made without using C-staining (Michailova 1987). C-banding in this chromosome has clearly detected a C-disc (Fig. 4) on the boundary of sections 6 and 7. This C-positive disc is possibly the active centromere suggesting thus the chromosome III is heterobrachial with short arm G and long arm EF.

Arm GE (Fig. 3a) has the band sequence: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16. The active regions in this arm were absent. We have discovered here three chromosome sequences, among them *alb*GE1, accepted as a standard, and two sequences defined as *alb*GE2 and *alb*GE3 respectively. Sequence *alb*GE2 was formed as a result of inversion of sections 3-10 and found in both states, heterozygous GE1.2 (Fig. 3c) and homozygous GE2.2 (Fig. 3b). Sequence *alb*GE3 was found only in a heterozygous state (Fig. 3d); this is a complicated inversion formed through several inversion steps. The heterozygous inversions GE1.2 and GE1.3 were found in two reservoirs, whereas homozygous GE2.2 in the Sazanka Lake only (Table 2).

Arm F (Fig. 3a) has the band sequence: 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32. *BR* is situated in section 19. We have found one inversion sequence defined as *alb*F2. Sequence *alb*F2 was formed on sequence *alb*F1 as a result of inversion of sections 21-25 and was present only in the heterozygous state. Frequency of this inversion was very low, 1.9%. It was only found in the population from the Sazanka Lake.

Analysis of chromosome polymorphism was performed in comparison with the data of Belyanina (1981) and Michailova (1987) (Table 2). A total of 17 chromosome sequences were recorded, which were found in the studied populations in homozygous and heterozygous states (Table 2).The level of *E. albipennis*`s chromosomal polymorphism in populations from different water bodies was essentially lower (number of heterozygous inversions per individual was 1.6 in Sazanka Lake, and 1.3 in pond near Novo-Aleksandrovka village), than in the Volga River near Saratov – 3.2 (Belyanina 1981).

Endochironomus sp. 2n=6. (Figs 5, 6).

Larva. Body is yellow, maximal length - 10 mm. The head capsule is light yellow. Submentum of *Endochironomus* sp. (Fig. 5b) with a small pigment spot, as opposed to submentum of *E. albipennis* (Fig. 5a), which does not have a spot. Both species are similar in structure mental teeth (Fig. 5c, d), but differ significantly in structure of ventromental plates (VmP): VmP of *E. albipennis* (Fig. 5e) extend in width, the ratio of width to the length (VmPR) is 4.1–4.5 (4.2), VmP of *Endochironomus* sp. (Fig. 5f) less elongated in width, VmPR is 2.3–3.6 (3.0). Anterior edge of the ventromental plate of *E. albipennis* with a row of small, not protruding teeth, anterior edge of the VmP of *Endochironomus* sp. with a well-visible row of teeth. Seta subdentalis (SSd) of *E. albipennis* is lanceolate and straight (Fig. 5g), but SSd of *Endochironomus* sp. (Fig. 5h) is lanceolate and slightly curved.

Karyotype. Centromeres are not distinct morphologically. Chromosome arms were designated in accordance with the photomap of *E. albipennis*: I (AD), II (BC), III (GEF), I<II=III.

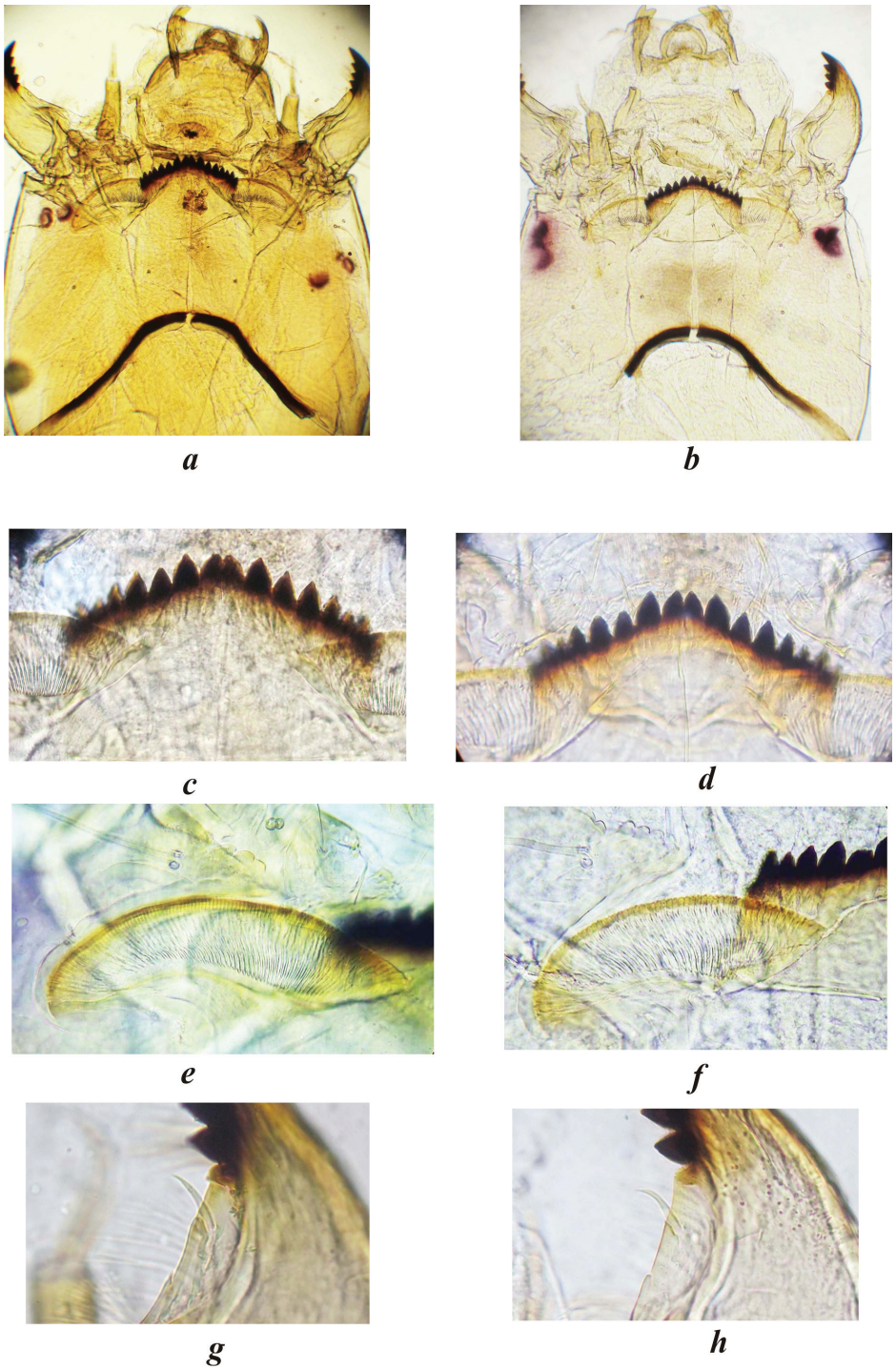

Figure 5. Larvae of *E. albipennis* (**a, c, e, g**) and *Endochironomus* sp. with 2n=6 (**b, d, f, h**): **a**, **b** head capsule (ventral view) **c**, **d** mentum **e**, **f** VmP (ventromental plates) **g**, **h** SSd (seta subdentalis).

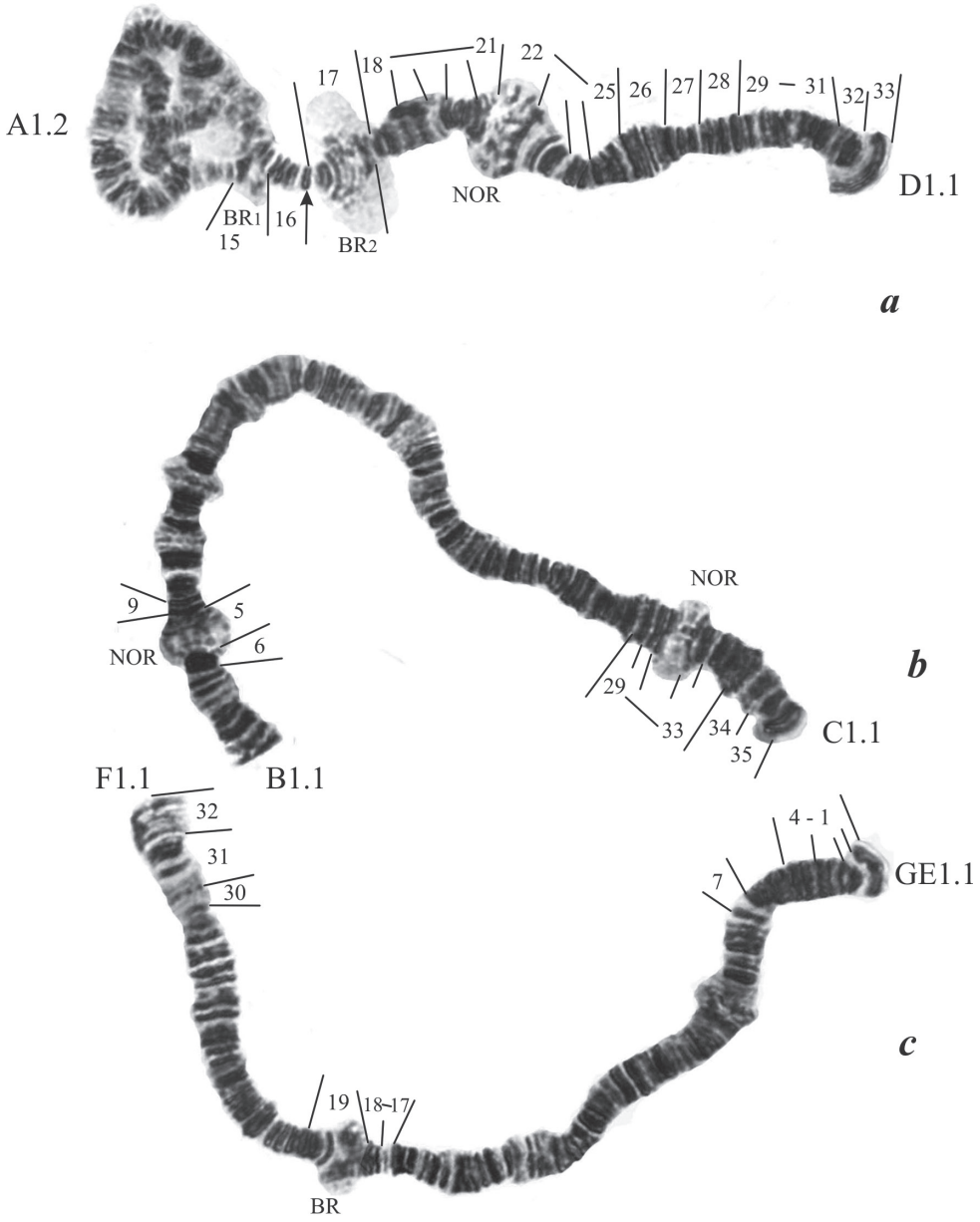

Figure 6. Karyotype of *Endochironomus* sp. (2n=6): **a** chromosome I (AD) **b** chromosome II (BC) **c** chromosome III (GEF). The designations are the same as in Fig. 1.

Arm A (Fig. 6a) has the region including sections 15-16 which is homeologous with arm A of *E. albipennis.* There is an active BR_1 in section 15.

Arm D (Fig. 6a) has the band sequence: 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33. In section 17, BR_2 is localized, in section 22 one NOR is situated.

Arm B (Fig. 6b) has the small region including sections 5-6 which is homeologous with arm B of *E. albipennis*. In section 5, a NOR is situated.

Arm C (Fig. 6b) has the region including sections 29-35 which is homeologous with arm C of *E. albipennis*. In section 31, a NOR is situated.

Arm GE (Fig. 6c) has the only the region including sections 1-4 which is homeologous to arm GE of *E. albipennis*.

Arm F (Fig. 6c) has the sites including sections 17-19 and 30-32 which are homeologous with arm F of *E. albipennis*. In section 19, BR is situated.

Discussion

Among all *Endochironomus* species detailed cytophotomaps of polytene chromosomes have been earlier compiled only for *E. tendens* Fabricius, 1775 (Durnova 2009), so a comparative analysis of polytene chromosomes of *E. tendens*, *E. albipennis* and *Endochironomus* sp. is currently hampered. Karyotypes of *E. tendens* and *E. albipennis* differ strongly both in disc patterns and in distinctness of centromere regions: in *E. tendens* centromere regions appear as thick heterochromatin blocks, whereas in *E. albipennis* they are morphologically not distinct. With the differential staining of chromosomes of *E. albipennis* using C-technique described by Sigareva (1985), centromeric heterochromatin was only defined clearly and permanently as a thin C-disc in the chromosome III (Fig. 4). Centromere regions of the chromosomes I and II were stained indistinctly, which is apparently connected with the very low amount of paracentromeric heterochromatin in these chromosomes.

The evolution of *E. tendens* apparently proceeded as a narrow specialization because larvae of this species are the typical miners in the tissues of littoral macrophytes (Kalugina 1963, Durnova 2009). Larvae of *E. albipennis* are eurybiontic and inhabit different biotopes being epibiotic organisms of different submerged littoral substrata in the water bodies. Molecular data (Durnova et al. 2014) have shown that by the nucleotide sequences of the mitochondrial gene *COI E. tendens* displays greater similarity to *Synendotendipes kaluginae* Durnova, 2010 than to *E. albipennis*, which indicates a high degree of divergence between *E. tendens* and *E. albipennis* not only at the chromosome level, but at the molecular level.

Larvae of *Endochironomus* sp. (2n=6) are morphologically similar to those of *E. albipennis* (Durnova et al. 2011). The degree of homeology in chromosome I (AD) between *E. albipennis* and *Endochironomus* sp. is relatively high; arms D are identical in banding patterns. These species differ in many sections of the chromosomes II (BC) and III (GEF), and only in few regions some common banding patterns can be seen. The number of discs in the central part of the chromosome II (BC) of *Endochironomus*

sp., in which no homeology is observed, is much higher than in *E. albipennis*. Probably during a process of differentiation of these species, duplication of chromosome material took place. The degree of homeology between two species in chromosome I (GEF) is also low, length of arms F and GE of *Endochironomus* sp. exceeds considerably length of *alb*F and *alb*GE, which is probably related to the duplication of the chromosome material.

Thus, *Endochironomus* sp. distinctly differs from *E. albipennis* by the polytene chromosome band patterns, which undoubtedly argues for its separate species status. The chromosome differentiation of these two species was evidently accompanied not only by inversions, but also by duplications of chromosome material (in chromosome I and chromosome II), as indicated by larger number of discs in chromosomes of *Endochironomus* sp. as compared to *E. albipennis*.

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Chromosomal distribution of microsatellite repeats in Amazon cichlids genome (Pisces, Cichlidae)

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Abstract

Fish of the family Cichlidae are recognized as an excellent model for evolutionary studies because of their morphological and behavioral adaptations to a wide diversity of explored ecological niches. In addition, the family has a dynamic genome with variable structure, composition and karyotype organization. Microsatellites represent the most dynamic genomic component and a better understanding of their organization may help clarify the role of repetitive DNA elements in the mechanisms of chromosomal evolution. Thus, in this study, microsatellite sequences were mapped in the chromosomes of *Cichla monoculus* Agassiz, 1831, *Pterophyllum scalare* Schultze, 1823, and *Symphysodon discus* Heckel, 1840. Four microsatellites demonstrated positive results in the genome of *C. monoculus* and *S. discus*, and five demonstrated positive results in the genome of *P. scalare*. In most cases, the microsatellite was dispersed in the chromosome with conspicuous markings in the centromeric or telomeric regions, which suggests that sequences contribute to chromosome structure and may have played a role in the evolution of this fish family. The comparative genome mapping data presented here provide novel information on the structure and organization of the repetitive DNA region of the cichlid genome and contribute to a better understanding of this fish family's genome.

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Keywords
Karyotype evolution, fluorescence *in situ* hybridization, repetitive DNA, genome organization

Introduction

The fish family Cichlidae exhibits high species richness with approximately 3,000 species distributed in Central and South America, Africa, and South India (Kullander 1998, Kocher 2004). The evolution of this family is characterized by repeated adaptive radiation and sympatric speciation (Schliewen et al. 1994, Seehausen 2006). Moreover, these fish are considered to be an excellent model for evolutionary studies because of their morphological and behavioral adaptations to a wide diversity of explored ecological niches (Lowe-McConnell 1991). Approximately 400 species have been identified in a wide range of habitats in the Neotropical region. The Amazon exhibits the highest diversity of Cichlidae, with more than 300 identified species (Kullander 2003).

In Neotropical cichlids, the diversity of morphological adaptations does not result from variations in the diploid number because most species have 48 chromosomes (Feldberg et al. 2003). However, the species of this family exhibit a dynamic genome with variations in structure and karyotype composition and organization, as demonstrated by the DNA sequencing and the physical chromosome mapping of several repetitive DNA sequences, such as telomere sequences; retrotransposons isolated from *Xiphophorus maculatus* Günther, 1866 (*Rex*1, *Rex*3, *Rex*6); retrotransposon isolated from *Astronotus ocellatus* Agassiz, 1831 similar to *Rex3* (Ao*Rex*3); long interspersed elements isolated from *Astronotus ocellatus* (AoLINE); retrotransposon isolated from *Cichla kelberi* Kullander & Ferreira, 2006 (RCk); transposon isolated from *Caenorhabditis elegans* Maupas, 1900 (*Tc*1); the 18S and 5S ribosomal gene sequences; and U1 spliceosomal small nuclear RNA (U1 snRNAs) (Vicari et al. 2006, Gross et al. 2009, Mazzuchelli and Martins 2009, Teixeira et al. 2009, Gross et al. 2010, Poletto et al. 2010, Cabral-de-Mello et al. 2012, Valente et al. 2011, Schneider et al. 2013a, Schneider et al. 2013b). Moreover, in this fish family, repetitive DNAs, such as transposable elements, co-localize or are associated with ribosomal DNAs, which suggests their roles in the duplication and dispersion of repetitive rDNA sequences (Gross et al. 2010, Schneider et al. 2013a, Schneider et al. 2013b, Nakajima et al. 2012).

Repetitive DNA sequences display a high degree of polymorphism because of the variation in the number of repetitive units, which results from a specific evolutionary dynamics. Among these elements, microsatellites (or short tandem repeats) are the most polymorphic and are formed of short sequences of one to six nucleotides repeated in tandem throughout the DNA (Tautz and Renz 1984). Because of their supposed neutral evolution, these molecular markers have been widely used in population genetics, to identify taxonomic limits, and in hybridization and forensic studies (Goldstein and Pollock 1997, Filcek et al. 2005, Racey et al. 2007, McCusker et al. 2008). However, recent research has demonstrated that certain microsatellites are

functional and may affect gene regulation (Kashi and King 2006, Gemayel et al. 2010, Sonay et al. 2015); evolution of orphan genes (Palmieri et al. 2014, Schlötterer 2015); involved in chromosomal rearrangements (Kamali et al. 2011) and be involved with increased or diminishes likelihood of disease related alleles size (Ananda et al. 2014; Padeken et al. 2015).

The chromosomal mapping of microsatellite sequences has been little examined. This approach is primarily used to study the evolution of different sex-determining chromosome systems (Li et al. 2010, Pokorná et al. 2011, Terencio et al. 2013). Considering that microsatellites are the most dynamic genomic component, a better understanding of their chromosomal organization is important for improving knowledge regarding the role of repetitive DNA elements in the mechanisms of chromosomal evolution and heterochromatin composition.

Cichla monoculus Agassiz, 1831 has a karyotype with 2n = 48 subtelo/acrocentric (st/a) chromosomes, described as basal for cichlids, and little heterochromatin. Although *Pterophyllum scalare* Schultze, 1823 also has 2n = 48 chromosomes, this species differs in karyotype formula with meta/submetacentric (m/sm) chromosomes due to chromosomal inversions and accumulation of heterochromatin in the pericentromeric regions. The highest diploid number described for this group is found in species of the genus *Symphysodon* Heckel, 1840, which has 2n = 60 chromosomes, as well as large heterochromatic blocks (Schneider et al. 2013a). Thus, this study provides a physical mapping of microsatellite sequences on the chromosomes of three Neotropical cichlid fish species (*C. monoculus*, *P. scalare*, and *S. discus*), that occupy different phylogenetic positions, and contributes to a better understanding of the chromosomal organization and evolution of this fish family.

Methods

Specimens of *C. monoculus* (four males and four females), *P. scalare* (three males and three females) were collected in Catalão Lake, confluence of the Negro/Solimões Rivers (3°09'47.44"S / 59°54'51.39"W) and *S. discus* (two males and two females) in Negro River (0°56'06.43"S / 62°56'22.61"W). The specimens were caught in the wild with sampling permission (ICMBio SISBIO 10609-1/2007). All of the individuals were euthanatized with Eugenol (clove oil).

Mitotic chromosomes were obtained from kidney cells using an air-drying protocol (Bertollo et al. 1978).

Eight microsatellites (Amado et al. 2008; Passos et al. 2010) were mapped using fluorescence *in situ* hybridization (FISH) during the mitotic metaphase of *C. monoculus*, *P. scalare*, and *S. discus* (Table 1). The repetitive sequence probes were labeled with digoxigenin-11-dUTP or biotin-16-dUTP (Dig-Nick Translation Mix and BioNick Translation Mix; Roche) using nick translation reactions following the manufacturer's instructions. Anti-digoxigenin rhodamine (Roche) and streptavidin/Alexa Fluor 488 (Life Technologies) were used to detect the signal. FISH was performed on mitotic

Table 1. Repetitive sequences hibridized to cichlid chromosomes. (+) positive hybridization signals detected; (-) absence of hibridization signals.

Repeat motif	*C. monoculus*	*P. scalare*	*S. discus*
$(CA)_{16}$	+	+	+
$(AC)_{7}$	+	-	-
$(GT)_{13}$	+	+	-
$(GA)_{12}$	-	+	-
$(GAATA)_{8}$	+	+	+
$(GAGAA)_{12}$	-	+	-
$(GT)_{9}CA(GT)_{7}CG(GT)_{19}$	-	-	+
$(CT)_{14}GT(CT)_{5}(CG)_{2}(CT)_{9}$	-	-	+

chromosome spreads (Pinkel et al. 1986). The FISH was performed with high stringency (2.5 ng/μl of DNA, 50% deionized formamide, 10% dextran sulfate and 2xSSC at 37 °C for 18 h). The chromosomes were counterstained with DAPI (2 μg/ml) in the Vectashield mounting medium (Vector).

Results

Four microsatellites, among which three were dinucleotides and one was a pentanucleotide, exhibited positive reactions in the genome of *C. monoculus* (Table 1). Hybridizations with the pentanucleotide microsatellite $(GAATA)_{8}$ displayed dispersed signals in all of the chromosomes. Moreover, conspicuous markings were observed in several chromosome pairs. However, one chromosome pair did not exhibit any homology with the probe (Fig. 1a). The microsatellite $(CA)_{16}$ was distributed in all of the chromosomes of *C. monoculus*, except for one pair. In most chromosomes, the microsatellite displayed a dispersed distribution, and in several cases, the markings were conspicuous (Fig. 1b). A dispersed pattern was observed after hybridization with the microsatellite $(GT)_{13}$, whereas a strong signal occurred in the telomeric, interstitial or centromeric regions of the chromosomes (Fig. 1c). Conversely, hybridizations with the microsatellite $(AC)_{7}$ resulted in only two positive chromosome pairs, one with markings in the telomeric region of the short arm and in the interstitial region of the long arm and the other with markings in both telomeric regions (Fig. 1d).

Five microsatellites were mapped in the genome of *P. scalare*: three dinucleotides and two pentanucleotides (Table 1). The microsatellites $(GAATA)_{8}$, $(CA)_{16}$ and $(GT)_{13}$, which were dispersed in *C. monoculus*, exhibited clustered signals in *P. scalare*. The microsatellite $(CA)_{16}$ was mapped on centromeric blocks of five chromosome pairs (Fig. 1f), other signals were located in telomeric, centromeric and interstitial regions of most chromosomes (Fig. 1e and 1g). Additionally, the microsatellites $(GA)_{12}$ and $(GAGAA)_{12}$ exhibited similar patterns of conspicuous markings in the centromeres. However, in the case of $(GAGAA)_{12}$, one chromosome pair did not display any markings (Fig. 1h, i).

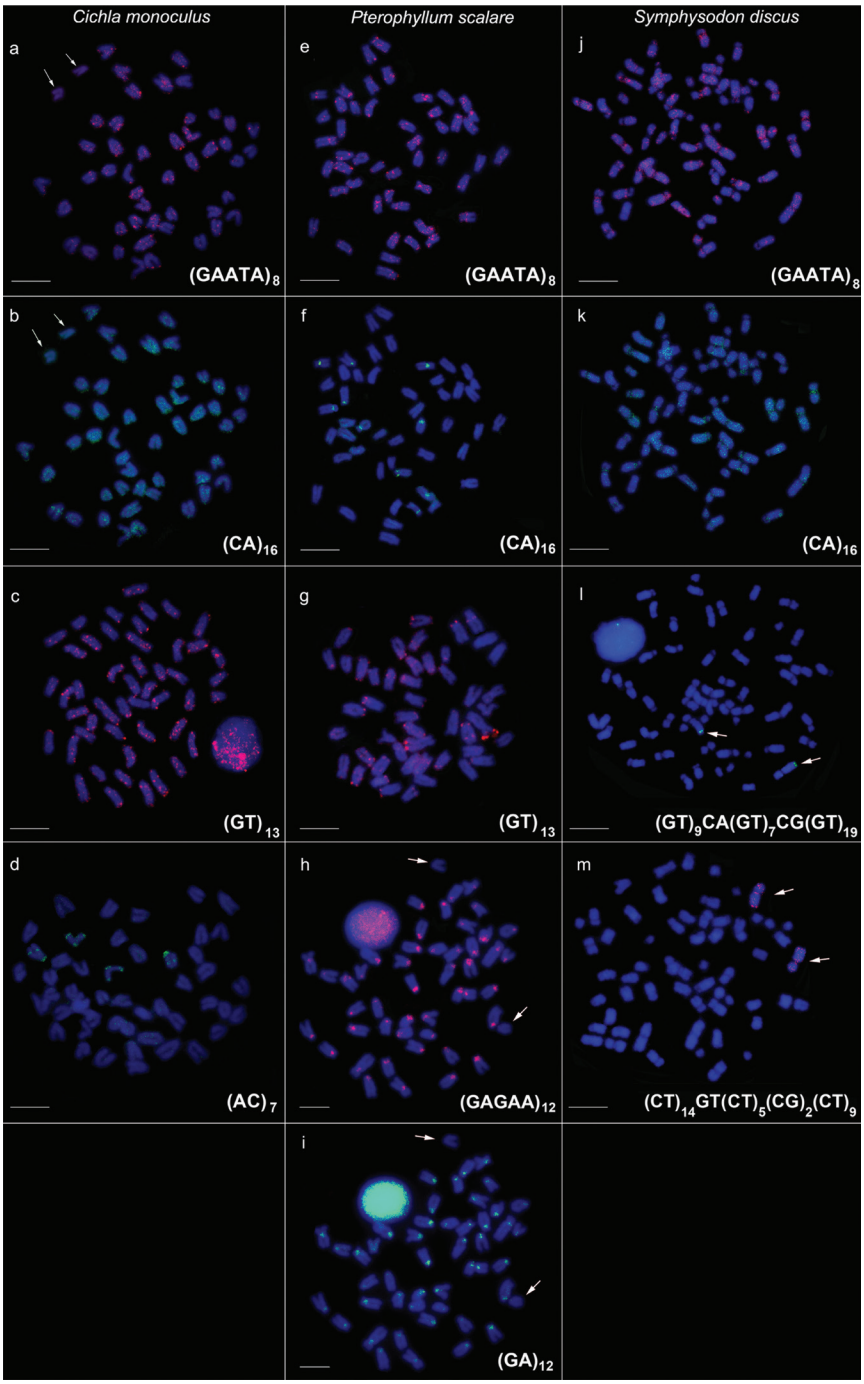

Figure 1. Metaphase chromosomes of *C. monoculus*, *P. scalare* and *S. discus* hybridized with microsatellite sequences (**a–m**). Arrow shows no signs of hybridizations in **a, b, h, i** and chromosomes positive for microsatellite in **l, m**. The probes detection was performed with streptavidin Alexa Fluor 488 (green) or anti-digoxigenin rhodamine (red). Chromosomes were counterstained with DAPI. Scale bar: 10 μm.

Four microsatellites were mapped in *S. discus*. In this species, the microsatellites $(GAATA)_8$ and $(CA)_{16}$ exhibited patterns similar to that of *C. monoculus* with dispersed signals and conspicuous markings in the centromeric region (Fig. 1j, 1k). Only one chromosome pair displayed positive results for microsatellites $(GT)_9CA(GT)_7CG(GT)_{19}$ and $(CT)_{14}GT(CT)_5(CG)_2(CT)_9$. The first was located in the telomeric region of the long arm, and the latter was located in the centromeric and telomeric regions (Fig. 1l, m).

Discussion

The repetitive regions of the genome typically do not undergo the selective pressure that affects non-repetitive sequences, and most microsatellite sequences evolve neutrally and supposedly do not affect an individual phenotype (Schlötterer 2000). However, recent studies have identified functional microsatellites that affect the physical aspect of an individual (Kashi and King 2006, Gemayel et al. 2010, Padeken et al. 2015). These putative functional microsatellites are primarily located in or near gene regions, and there is variation in the number of times that the motif is repeated, which is related to the ability of the microsatellites to modify gene expression or change protein sequences (Wren et al. 2000, Li et al. 2004, Vinces et al. 2009, Gemayel et al. 2010). In addition to this functional aspect, repetitive DNA variants that include microsatellites may serve as efficient agents for adaptive evolution (King and Kashi 2009).

In all classes of repetitive DNA, there appears to be a general trend of increased matrix length throughout evolutionary time. Moreover, highly repetitive sequences tend to accumulate in regions of low recombination, such as centromeres and telomeres, whereas repetitive regions in euchromatin are much more susceptible to crossing-over (Pathak and Ali 2012).

Overall, the chromosome hybridization of microsatellites demonstrated contrasting patterns of abundance and localization of these sequences in the chromosomes of *C. monoculus*, *P. scalare* and *S. discus*, which indicates that the repetitive sequences have accumulated differently among the genomes. Although the three species exhibited a wide distribution of microsatellites $(GAATA)_8$ and $(CA)_{16}$ in their genome, clustering of these markers was observed in *P. scalare*, which represents a derived species in the phylogeny of Cichlinae (Smith et al. 2008). Clustering of repetitive sequences in derived species was also observed for transposable elements of this fish family (Gross et al. 2009; Valente et al. 2011; Schneider et al. 2013b). The association between microsatellites and the abundance of retrotransposable elements has been suggested as a mechanism that may increase the number of microsatellites (Nadir et al. 1996). However, in several organisms, a relationship between the high density of transposable elements and a high rate of microsatellites is not observed (Schlötterer 2000, 2015).

Position of the microsatellite sequences mapped in this study was similar to that observed for retroelements in the same species, with signals scattered throughout chromosomes and others clustered in terminal and centromeric regions (Schneider et al. 2013b). This outcome suggests that these sequences may be involved in the structural

formation of the centromere and the telomere. Moreover, the microsatellites present in the centromeric and subtelomeric regions differ among cichlid species, which reinforces the importance of these sequences in the evolution of the different groups.

The centromere is an essential structure with several functional roles in the segregation of replicated chromosomes to daughter cells. These roles include genetic/epigenetic marking and the assembly of the protein complex of the kinetochore during the cell cycle, providing checkpoints to control mitosis, the release of sister-chromatid cohesion, chromosome migration to the cellular poles and cytokinesis (Santaguida and Musacchio 2009, Allshire and Karpen 2008, Perpelescu and Fukagawa 2011). Centromeres comprise long stretches of tandem repeats of satellite and microsatellite DNA, which are essential for binding with protein complexes (Verdaasdonk and Bloom 2011, Kalitsis and Choo 2012). Centromeric DNA sequences typically present high evolutionary rates and variation among species or chromosomes of the same species is common (Plohl et al. 2008). Thus, the centromeric region of *P. scalare* is rich in $(GAGAA)_{12}$ microsatellite sequences, but one chromosome pair does not display any hybridization signals. As well, in *C. monoculus* and *S. discus*, the centromere region is not rich in $(GA)_{12}$ and $(GAGAA)_{12}$.

Another chromosome region with a high evolutionary rate is the subterminal region. Typically, this region is composed of different classes of repetitive DNA that may help stabilize the terminal portion of the chromosomes because of the possibility of amplifying these sequences even in the absence of telomerase (Jain et al. 2010, Torres et al. 2011). In *S. discus*, the microsatellites $(GT)_9CA(GT)_7CG(GT)_{19}$ and $(CT)_{14}GT(CT)_5(CG)_2(CT)_9$ are present in the subterminal region of two chromosome pairs, whereas these markers were not observed in the chromosomes of *C. monoculus* and *P. scalare*. The same result was obtained for the microsatellite $(AC)_7$, which was observed only in *C. monoculus*. The microsatellites $(GAATA)_8$ and $(GT)_{13}$ were observed in the subterminal regions of *C. monoculus* and *P. scalare* but not in *S. discus*. In *P. scalare*, a conspicuous marking of the microsatellite $(GT)_{13}$ was observed in the terminal region of the largest chromosome pair, where the 18S ribosomal gene is located (Schneider et al. 2013a), indicating synteny between these two classes of repetitive DNA.

Still, heterochromatin of the cichlids analyzed here was located in the centromeric or pericentromeric regions in most of the chromosomes (Schneider et al. 2013). These regions show positive signals of hybridization for different microsatellites analyzed, as well as other repetitive elements (Schneider et al. 2013). The most common cellular mechanism that prevents activation and expansion of repetitive elements is the formation of heterochromatin over their sequences and three epigenetics pathways interconnected ensure the silencing of their elements: methylation of H3K9, DNA methylation and the germ-line specific PIWI pathway (Padeken et al. 2015).

The regulation of the repetitive sequences is not yet clear and depends largely on new technologies to clarify their function (Padeken et al. 2015), but the comparative mapping data presented provide novel information on the structure and organization of the repetitive region of the genome of cichlids and suggest that microsatellites contribute to chromosome structure and may have played a role in the evolution of this fish family.

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SHORT COMMUNICATIONS

COMPARATIVE Cytogenetics
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First description of the karyotype of a eucharitid wasp (Hymenoptera, Chalcidoidea, Eucharitidae)

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Abstract

The haploid karyotype of *Kapala* sp. (Eucharitidae), a parasite of the Neotropical ant *Dinoponera lucida* Emery, 1901 (Hymenoptera, Formicidae), is reported for the first time. It consists of four metacentric chromosomes. Chromosomes in the family Eucharitidae were unknown so far; therefore, our results confirm that multiple parallel chromosomal fusions have taken place in several lineages within the superfamily Chalcidoidea.

Keywords

Kapala, parasitoid wasp, Eucharitidae, cytogenetics

Introduction

Eucharitidae are a comparatively small family of hymenopteran parasitoid wasps with 423 described species (Noyes 2014). They belong to the superfamily Chalcidoidea, an economically important and extremely diverse group with more than 22,000 described species out of an estimated number of about 500,000 species living today (Gokhman

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2013, Noyes 2014). According to Murray et al. (2013), the exclusive association between parasitic wasps within this family and ants started approximately 72 Mya ago. Eucharitids are one of the few wasp groups that were able to break the ants' communication codes used in kin recognition among colony members via behavioral, morphological, and chemical adaptations (Murray et al. 2013).

Despite the large number of described species, less than 1% of chalcid wasps have been karyotyped so far (Gokhman 2009). Two groups of families used to be recognized within Chalcidoidea based on karyotypes: one group with species showing high chromosome numbers (n=9–11) and a second group with species showing low chromosome numbers (n=3–7) (Gokhman 2005, Gokhman and Gumovsky 2009). Recent studies, however, have revealed an even more complex pattern (Gokhman 2013). For instance, species with lower chromosome numbers (n = 5–7) were found in the families Eurytomidae and Encyrtidae, which initially were placed into the "high- numbered" group (Gokhman 2013). Karyotype evolution in this group has been a matter of discussion for the past decade since chromosome rearrangements are likely to have played an important role in the diversification of parasitoid wasps. However, it is difficult to analyze karyotype changes within a phylogenetic framework since despite recent progress (Munro et al. 2011, Heraty et al. 2013) phylogenetic relationships within Chalcidoidea are still largely unknown. It is noteworthy that depending on the outgroup used, one can arrive at different conclusions based on the same cytogenetic data, as in the case of Eupelmidae (Fusu 2008, Gokhman and Gumovsky 2009).

Wasps of the genus *Kapala* Cameron, 1884 (Hymenoptera: Eucharitidae) are specialized ant parasitoids that are associated with several poneromorph ant genera such as *Ectatomma* F. Smith, 1858, *Gnamptogenys* Roger, 1863, *Typhlomyrmex* Mayr, 1862 (Ectatomminae), *Hypoponera* Santschi, 1938, *Neoponera* Emery, 1901, *Odontomachus* Latreille, 1804, *Pachycondyla* F. Smith, 1858, *Pseudoponera* Emery, 1900 and *Dinoponera* Roger, 1861 (Ponerinae) (Pérez-Lachaud et al. 2006, Buys et al. 2010, Lachaud et al. 2012, Murray et al. 2013, Lachaud and Pérez-Lachaud 2015). So far, only 17 out of more than 60 estimated species have been described within this genus, which is widespread and most commonly collected in the Neotropical region (Heraty 2002, Pérez-Lachaud et al. 2006, Lachaud et al. 2012). Brazil harbors a high diversity of *Kapala* wasps, however, only eight species have been reported to date from this country (Noyes 2014).

In this paper, we present the first description of the karyotype in a eucharitid wasp (*Kapala* sp.) and discuss the importance of these results for the understanding of karyotype evolution in parasitic wasps.

Material and methods

Two specimens of *Kapala* sp. (presumably a new species according to J. Heraty, 2014, in litt.) were found inside the cocoons of *Dinoponera lucida* Emery, 1901 (Formicidae, Ponerinae), a young adult male (Fig. 1) and a female pupa. The adult specimen was

Figure 1. Adult male specimen of *Kapala* sp.

deposited at the University of California Riverside Entomology Research Museum, USA. The host colony of *D. lucida* was collected in the fields of the Barrolândia station of CEPLAC in Belmonte, state of Bahia, Brazil (47°73'02"S, 82°21'24"W). The pupa was dissected and the cerebral ganglia were used for obtaining mitotic metaphases according to Imai et al. (1988). Metaphases were stained using Giemsa stain (1:30) and analyzed with an Olympus BX60 microscope equipped with a digital camera. The adult specimen and pupa were studied and photographed with an Olympus SZX7 stereomicroscope. The photographs of the collected insect specimens and metaphases were taken using the Image Pro Plus® version 4.1 analysis software (Media Cybernetics). Karyotypes were digitally mounted and the chromosomes were grouped according to Levan et al. (1964).

Results and discussion

This is the first description of the karyotype of a eucharitid wasp. A total of four metaphases were obtained, all of them showing 2n=8 chromosomes. The karyotype of this species (Fig. 2) showed four pairs of metacentric chromosomes with the first pair longer than the remaining three. This karyotype pattern is similar to others described in parasitic wasps with a low chromosome number (n=3–5) (Gokhman and Gumovsky 2009). Considering the known phylogenetic relationships, Eucharitidae together with Perilampidae and Pteromalidae belong to a derived family group of parasitic wasps that probably has undergone independent reductions in chromosome number during karyotype evolution (Gokhman and Gumovsky 2009, Murray et al. 2013). These assump-

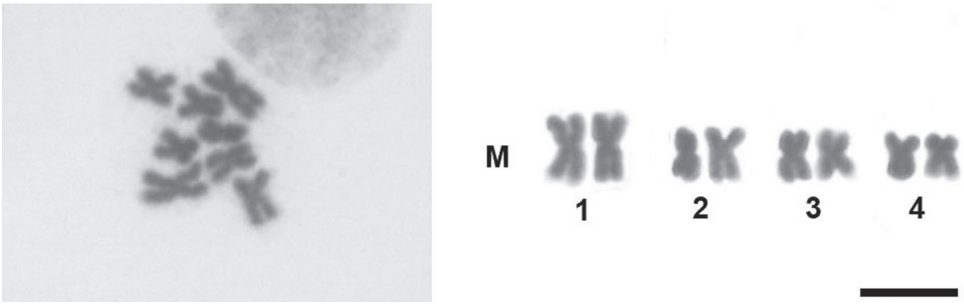

Figure 2. Metaphase plate and diploid karyotype of *Kapala* sp. Bar=10 μm.

tions are based on the observations that the haploid chromosome number n=11 could be an ancestral character state for the superfamily Chalcidoidea (Gokhman 2013). Species with a low chromosome number are assumed to have undergone chromosome fusions, which would lead to the reduction in chromosome number and consequently to an increase in chromosome size.

The chromosome number of n=4 in *Kapala* sp. (Eucharitidae) is quite similar to those reported in other Chalcidoidea families such as Perilampidae (n=3) and Pteromalidae (n=4–7). *Perilampus ruschkai* Hellén (Perilampidae), n=3 (Gokhman 2005), was clustered in the same clade with Eucharitidae and some Pteromalidae subfamilies in a phylogenetic tree of Chalcidoidea (Gokhman and Gumovsky 2009). According to the latter paper, in the clade comprising Eucharitidae, Perilampidae, and Pteromalidae, a reduction in chromosome number from n=9–11 to n=3–7 during karyotype evolution occurred independently from other clades such as Trichogrammatidae + Eulophidae and Chalcididae + Leucospidae. As discussed in Gokhman and Gumovsky (2009) and Munro et al. (2011), Perilampidae (even if sometimes recovered as a paraphyletic clade) is the sister group of Eucharitidae. According to Gokhman (2011), although studies on the evolution of karyotypes in parasitic wasps are still scarce, it is known that the chromosome rearrangements involved in events of karyotype evolution are mainly fusions (centric and tandem), pericentric inversions, and rare cases of fission followed by the growth of constitutive heterochromatin. This author also points out that similar karyotypes have been described in groups somewhat distinct taxonomically, a phenomenon called karyotypic orthoselection by White (1973).

The karyotype of *Kapala* sp. analyzed herein falls into the seventh karyotype pattern group defined by Gokhman (2011), in which n values vary from 3-7, with a metacentric: acrocentric ratio ≥ 1 and the first chromosome pair less than 1.5 times longer than the remaining three pairs. According to Gokhman (2011), this karyotype pattern represents the final stage of pairwise chromosomal fusions for parasitoid wasps. This first description of the karyotype of a *Kapala* species corroborates a previous hypothesis that this group of wasps would have a low-numbered karyotype (Gokhman and Gumovsky 2009) and therefore confirms that multiple parallel chromosomal fusions have taken place in several lineages within the superfamily Chalcidoidea (Gokhman

2013). Further analyses including larger samples and other cytogenetic techniques will provide more information for the better understanding of the role of chromosomal rearrangements in the evolution of parasitoid wasps.

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RESEARCH ARTICLE

COMPARATIVE Cytogenetics
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Comparative cytogenetic study of three *Macrolophus* species (Heteroptera, Miridae)

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Abstract

Macrolophus pygmaeus (Rambur, 1839) (Insecta, Heteroptera, Miridae) is a predator of key vegetable crop pests applied as a biocontrol agent in the Mediterranean region. *M. pygmaeus* and *M. melanotoma* (A. Costa, 1853) are cryptic species with great morphological similarity which results in their misidentification and negative consequences for the conservation of their populations on greenhouse and outdoor crops. In order to find out specific markers for their separation we studied the karyotype, male meiosis and heterochromatin composition of these species and additionally of a third species (as a reference one), *M. costalis* Fieber, 1858. We demonstrate here that all the three species share achiasmate male meiosis and sex chromosome pre-reduction. On the other hand, the species differ in karyotype, with 2n=28 (26+XY) in *M. pygmaeus*, 2n=27 (24+X_1X_2Y) in *M. costalis*, and 2n=34 (32+XY) in *M. melanotoma*, and heterochromatin distribution and composition. In addition, the species differ in sperm morphology: sperm cells of *M. costalis* are significantly longer with longer head and tail than those of *M. melanotoma* and *M. pygmaeus,* whereas sperm cells of *M. melanotoma* have a longer tail than those of *M. pygmaeus.* All these characters can be used as markers to identify the species, in particular the cryptic species *M. melanotoma* and *M. pygmaeus*.

Keywords

Macrolophus, Miridae, Heteroptera, karyotype, sex chromosomes, achiasmate meiosis, sex chromosome pre-reduction, sperm morphology

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Introduction

The Miridae are the largest family of true bugs (Heteroptera, Cimicomorpha) with approximately 10000 species described (Schuh 1995). Cytogenetical data are presently available for about 200 species (Ueshima 1979, Nokkala and Nokkala 1986, Grozeva 2003, Grozeva et al. 2006, 2007, Grozeva and Simov 2008a, b, 2009, Kuznetsova et al. 2011). The mirid bugs share some cytogenetic characteristics with all the Heteroptera: they possess holokinetic (or holocentric) chromosomes and most of them are characterized by an inverted sequence of reductional and equational division of the sex chromosomes (post-reduction) in male meiosis (Ueshima 1979). On the other hand, they have some unique chromosomal characteristics. Chiasmata are absent in male meiosis, the achiasmate meiosis being of a collochore type (Nokkala and Nokkala 1986, Kuznetsova et al. 2011). In the three hitherto studied *Macrolophus* Fieber, 1858 species, *M. costalis* Fieber, 1858, *M. pygmaeus* (Rambur, 1839) and *M. geranii* Josifov, 1961, both autosomes and sex chromosomes divide pre-reductionally during the achiasmate male meiosis (Grozeva et al. 2006, 2007).

The species from the present study, *M. costalis, M. melanotoma* (A. Costa, 1853), and *M. pygmaeus*, occur on a variety of plant species in the Mediterranean region. *M. pygmaeus* is an efficient predator of several key vegetable crop pests in Europe produced commercially and used widely as a biocontrol agent (Alomar et al. 2002, 2006, van Lenteren 2003, Messelink et al. 2014). *M. pygmaeus* and *M. melanotoma* are cryptic species with great morphological similarity which results in their misidentification and negative consequences for the conservation of their populations on greenhouse and outdoor crops. In order to find specific markers for their separation we here studied the karyotype, male meiosis and heterochromatin composition of these species and additionally of a third species, *M. costalis*. The species have recently been separated based on differences of their genetic profiles, cuticular hydrocarbon composition and on the fact that interspecies crosses do not produce viable progeny (Martinez-Cascales et al. 2006, Gemeno et al. 2012, Castañé et al. 2013). *M. costalis* can be easily distinguished morphologically from *M. pygmaeus* or *M. melanotoma* by the black dot on the scutellum, but it was included in our study as a reference species. In earlier cytogenetic studies (Grozeva et al. 2006, 2007), karyotype of two of the three species here examined was reported. Such characters, as highly asymmetric karyotype (2n=24+$X_1X_2X_3Y$) with two extra-large autosome pairs and interstitial distribution of C-heterochromatin in them (Grozeva et al. 2006), provide excellent cytogenetic markers to distinguish *M. costalis* from other *Macrolophus* species. The karyotype of *M. pygmaeus* (2n=26+XY) is asymmetric, as in *M. costalis*, but with different number of autosomes and a simple XY sex chromosome system (Grozeva et al. 2007). The species share sex chromosome pre-reduction, but can easily be differentiated by their karyotype and pattern of C-heterochromatin distribution.

Sperm morphology is significant in fertilization (Evans and Simmons 2008). Franco et al. (2011) have recently shown that the species in which sperm competition occurs also displayed the longest sperm length.

With the aim of distinguishing between the cryptic *Macrolophus* species, both karyotype and male meiosis were studied for the first time in *M. melanotoma* and reinvestigated in *M. pygmaeus* and (as a reference species) in *M. costalis* using standard chromosome staining and fluorochromes DAPI and CMA_3. In addition, morphology of sperm cells was examined in each of the three species.

Material and methods

Insects

Males and females of *M. costalis*, *M. pygmaeus* and *M. melanotoma* were collected in Catalonia, NE of Spain, in the vicinity of Mataró (Barcelona) (41.556 North, 2.475 East) from *Cistus albidus* Linnaeus, 1753, commercial tomato fields and *Dittrichia viscosa* (Linnaeus) Greuter, respectively. Colonies from collected individuals were set-up under controlled conditions (25 ± 1°C, 70 ± 10% RH and L16:D8 photoperiod) on tobacco plants (*Nicotiana tabacum* Linnaeus, 1753) with *Ephestia kuehniella* Zeller, 1879 (Lepidoptera, Pyralidae) eggs as a prey (Agusti and Gabarra 2009a, b). All *Macrolophus* specimens were preliminarily identified following Josifov (1992). However, due to morphological similarity between *M. melanotoma* and *M. pygmaeus*, their identification was additionally tested by conventional PCR using methodology and specific primers described in Castañe et al. (2013).

Karyotype

The abdomen of 20 *M. pygmaeus*, 23 *M. melanotoma* and 13 *M. costalis* males were placed in 3:1 fixative (96% ethanol-glacial acetic mixture) and the thorax in 70% ethanol for later species identification by DNA analysis (Castañe et al. 2013). Dissected gonads were squashed in a small drop of 45% acetic acid. The cover slips were removed by the dry ice technique. Slides were dehydrated in fresh fixative (3:1) and air dried. Part of the preparations was stained using Schiff-Giemsa method of Grozeva and Nokkala (1996) to check the number of chromosomes and their behaviour in meiosis. For other slides, DNA- binding fluorochromes, GC-specific chromomycin A_3 (CMA_3) and AT-specific 4'-6'-diamino-2-phenylindole (DAPI) were applied following Schweizer (1976) and Donlon and Mafenis (1983), with minor modifications as described in Kuznetsova et al. (2001).

Chromosomes were analyzed using light/fluorescent microscopy (Axio Scope A1 – Carl Zeiss Microscope) at 100× magnification and documented with a ProgRes MFcool – Jenoptik AG digital camera. All cytogenetic preparations and remains of the specimens are stored at the Institute of Biodiversity and Ecosystem Research, BAS in Sofia.

Sperm morphology

In every species, sperm cells from 10 males (other than those used for the karyotype analysis) were measured following Franco et al. (2011). On a slide, one drop of Beadle saline solution (128.3 mM NaCl, 4.7 mM KCl, 23 mM $CaCl_2$) was added to the male abdomen. The seminal vesicle was extracted and opened in 20 µl of saline solution to allow the sperm out. The sperm were diluted carefully with the aid of a fine needle, and then one drop was transferred and smeared across a microscope slide, allowed to dry and rinsed. Sperm cells were analyzed using a Leica DM 4000 light microscope. Twelve sperm cells per individual were measured (head and tail length) at 400x under dark field using the QWin 6.0 (Leica Microsystems, Germany) software package. In order to reduce measurement variation, each component was measured five times for each sperm cell. Data were analyzed by a one-way ANOVA and means separation by Tukey multiple range test.

Results

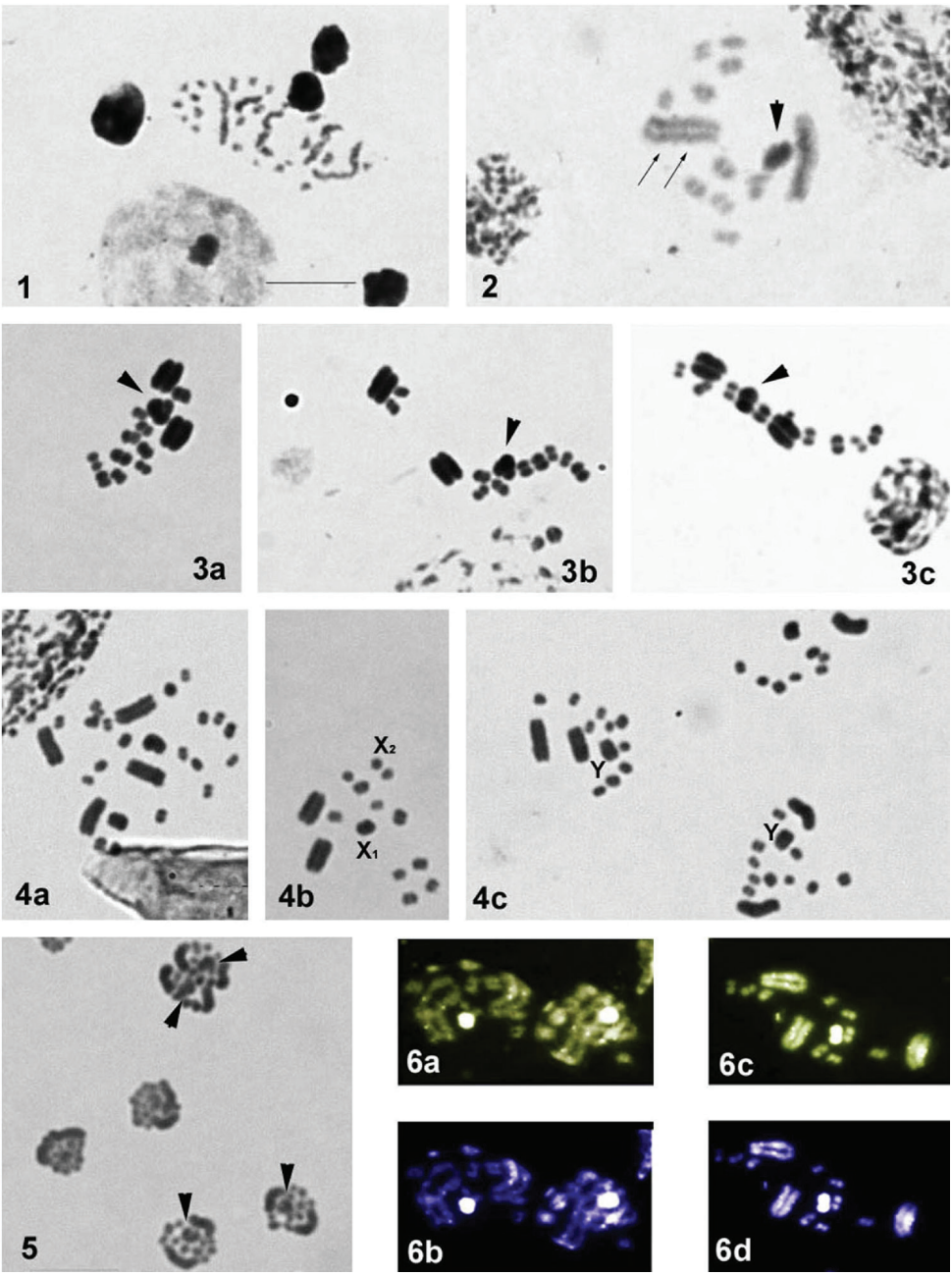
Karyotype

Macrolophus costalis, 2n=27 (24+X_1X_2Y)

Published data: 2n=28 (24+$X_1X_2X_3Y$) (Grozeva et al. 2006)

Spermatogonial metaphases consisted of 5 large (incl. Y) and 22 similar in size chromosomes (incl. two X) (Fig. 1). In meiosis, condensation stage was most abundant and showed 12 autosomal bivalents plus a positively heteropycnotic sex chromosome body (Fig. 2). Size differences between the bivalents were observed. The complement included two extremely large bivalents, four to five times the size of the other 10 similar size bivalents. Bivalents consisted of parallel-aligned homologous chromosomes without chiasmata, i.e. the male meiosis was achiasmate. After the Schiff-Giemsa staining, it was easy to see that conspicuous interstitial heterochromatic bands in both large bivalents divide them into the three almost equal parts. At metaphase I (MI), the sex chromosomes were seen either as a trivalent (Fig. 3a, b) or as a bivalent (Fig. 3c). They clearly segregated at anaphase I (Fig. 4a) resulting in two types of metaphase II, with 12 autosomes plus two X chromosomes (Fig. 4b) and with 12 autosomes plus the Y (Fig. 4c). Male meiosis was hence pre-reductional both for autosomes and sex chromosomes. After second division, every cell produced daughter cells possessing either 2X or Y chromosome respectively (Fig. 5). Thus, the chromosome formula of male *M. costalis* was determined as 2n=27 (24+X_1X_2Y) in contrast to 2n=28 (24+$X_1X_2X_3Y$) earlier reported in Grozeva et al. (2006).

After staining with fluorochromes, bright DAPI- and CMA_3- positive bands were observed in the same locations on the larger autosomal bivalents and sex chromosomes (Fig. 6a–d).



Figures 1–6. Male meiosis in *Macrolophus costalis*. (**1–5** conventional staining **6** fluorochrome staining: **6a, c** CMA_3 **6b, d** DAPI) **1** spermatogonial metaphase **2** condensation stage **3a–c** metaphase I **4a–c** metaphase II **5** telophase **6a, b** condensation stage **6c, d** metaphase I. Sex-chromosomes are indicated by arrowheads. Heterochromatin blocks are indicated by arrows. Bar = 10 μm.

Macrolophus pygmaeus, 2n=28 (26+XY)

Published data: 2n=28 (26+XY) (Grozeva et al. 2007)

Spermatogonial metaphases consisted of 5 large (incl. X) and 23 similar size chromosomes (incl. Y) (Fig. 7). In meiosis, condensation stage showed 13 autosomal bivalents and a positively heteropycnotic sex chromosome body (Fig. 8). The complement included two extremely large bivalents (~five times the size of the others), and eleven bivalents similar in size. The bivalents consisted of parallel-aligned homologous chromosomes; chiasmata were absent and the male meiosis was achiasmate. MI was nonradial (i.e., the autosomes did not form a ring), and the sex chromosomes formed a pseudobivalent (Fig. 9). The sex chromosomes segregated at AI resulting in two MII cells each with 14 chromosomes (13 + X or Y) (Fig. 10). Male meiosis was hence pre-reductional both for autosomes and sex chromosomes. Thus, the chromosome formula of *M. pygmaeus* was confirmed as 2n=28 (26+XY) in line with that earlier reported in Grozeva et al. (2007).

After staining with fluorochromes, bright DAPI- and CMA_3 -positive bands were observed on both sex chromosomes (Fig. 11a–d). In addition, a weak DAPI- positive / CMA_3 -negative signal was registered in a telomere of one of the larger bivalents (Fig. 11a, b).

Macrolophus melanotoma 2n=34 (32+XY)

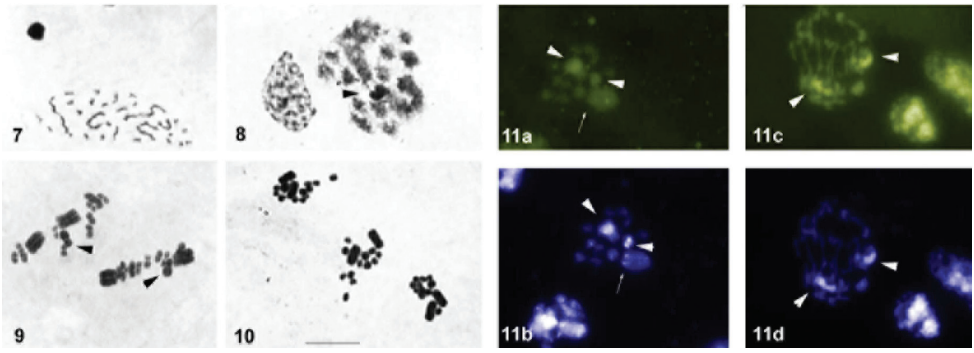
Published data: absent

At spermatogonial metaphase, there were 34 chromosomes (Fig. 12) gradually decreasing in size and the sex chromosomes were difficult to distinguish. At meiotic condensation stage, autosomal bivalents consisted of parallel lying homologs, and the sex chromosomes appeared as a heteropycnotic body (Fig. 13). At MI, there were 16 autosomal bivalents and X and Y chromosomes (Fig. 14a–c). Note that both sex chromosomes were occasionally placed in the center of a ring formed by autosomal bivalents (Fig. 14b). The autosomal bivalents constituted a decreasing size series and the X was more than twice the size of the Y. As a result of pre-reductional division of sex chromosomes at AI (Fig. 15), two types of MII (Fig. 16) raised, both with 17 chromosomes while with X or Y chromosome respectively.

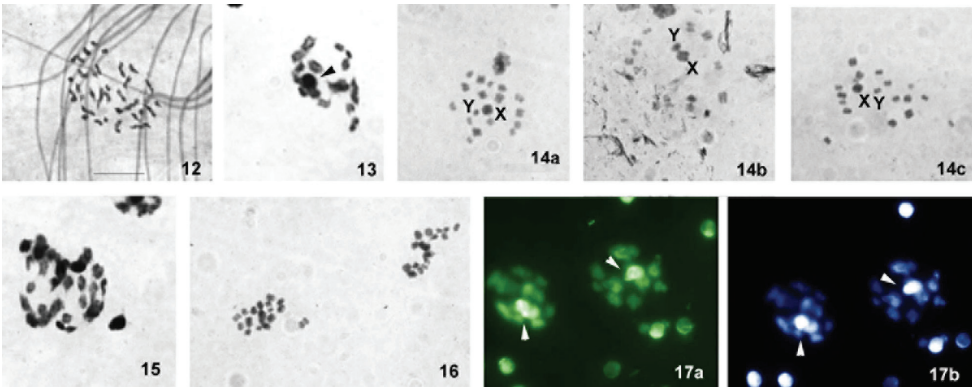
After staining with fluorochromes, bright DAPI- and CMA_3 bands were observed on the sex chromosomes (Fig. 17).

Sperm morphology

Sperm cells of the species studied were of similar shape, with a long and filiform head (Fig. 18). However, species are different in the total length (mean ± SE) of the sperm cells ($F_{2,27}$=15.53; P<0.0001), in the length of head ($F_{2,27}$=38.89; P<0.0001) and tail ($F_{2,27}$=25.43; P<0.01) (Table 1).



Figures 7–11. Male meiosis in *Macrolophus pygmaeus*. (**7–10** conventional staining **11** fluorochrome staining: **11a, c** CMA_3 **11b, d** DAPI) **7** spermatogonial metaphase **8** condensation stage **9** metaphase I **10** metaphase II **11a, b** metaphase I **11c, d** anaphase. Sex-chromosomes are indicated by arrowheads. CMA_3/DAPI signals are indicated by arrows. Bar = 10 μm.



Figures 12–17. Male meiosis in *Macrolophus melanotoma*. (**12–16** conventional staining **17** fluorochrome staining: **17a** CMA_3 **17b** DAPI) **12** spermatogonial metaphase **13** condensation stage **14a–c** metaphase I **15** anaphase I **16** metaphase II **17** condensation stage. Sex-chromosomes are indicated by arrowheads. Bar = 10 μm.

Table 1. Sperm cells lenght (means ± standard error) of *M. costalis*, *M. melanotoma* and *M. pygmaeus.* Within each row, means followed by the same letter do not differ significantly (P≤0.005).

Sperm length (μm)	*M. costalis*	*M. melanotoma*	*M. pygmaeus*
Total	236±0.9 **a**	220.4±0.9 **b**	215±1.4 **c**
Head	61.3±0.4 **a**	51.3±0.4 **b**	50.6±0.4 **b**
Tail	174.7±0.8 **a**	169.1±1 **b**	164.6±1.2 **c**

Discussion

The genus *Macrolophus* belongs to the tribe Dicyphini of the subfamily Bryocorinae (Heteroptera, Miridae). In Bryocorinae, besides the modal for the Miridae chromosome number 2n=34 (32+XY), some higher (2n=36+XY and 2n=46+XY/X_1X_2Y) and

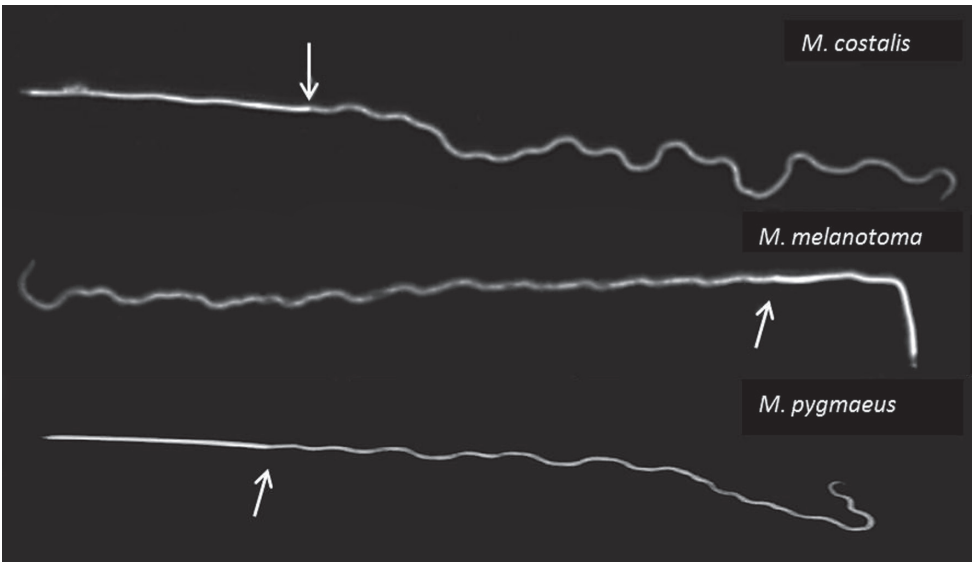


Figure 18. Sperm morphology of *Macrolophus* species. The arrow shows the end of sperm head.

lower (2n=16-26+XY) chromosome numbers have also been described (Ueshima 1979, Grozeva 2003, Grozeva et al. 2006, 2007, 2008a). The chromosome formula of the reference species *M. costalis* was reported earlier as 2n=28 ($24+X_1X_2X_3Y$) for a Bulgarian population collected from the tobacco plants, which was the first report of three X chromosomes in the family (Grozeva et al. 2006). In contrast, the chromosome formula of the NE Spanish population here studied appeared to be different, with two instead of three X chromosomes. As the specimens come from different geographic regions we could speculate that they represent chromosomal races within a species. At taxonomic level, they may probably be considered as subspecies, but to clarify this hypothesis a chromosomal analysis of individuals (males and females) from natural populations of this species over the whole distribution range will be necessary. *M. pygmaeus* showed 2n=28 (26+XY) both in Bulgaria (Grozeva et al. 2007) and Spain (present study). The third species, *M. melanotoma*, studied here for the first time, appeared to differ from *M. costalis* and *M. pygmaeus* in karyotype. Besides difference in the chromosome number, *M. melanotoma* lacks two large autosome pairs characteristic of the two other species allowing for the cryptic *M. melanotoma* and *M. pygmaeus* to be reliably differentiated.

Differences in molecular organisation of chromatin revealed after fluorochrome staining, suggest an additional chromosome marker to differentiate *Macrolophus* species. In *M. costalis,* bright DAPI/CMA_3 bands were observed in the same locations on the large autosomal bivalents and sex chromosomes whereas in two remaining species bright fluorescent bands were observed only on the sex chromosomes. In turn, *M. pygmaeus* differed from *M. melanotoma* in that it showed some additional weak DAPI-positive signals in a telomeric region of a larger bivalent.

Franco et al. (2011) have recently reported data on sperm morphology in *M. pygmaeus* and another dicyphine species *Nesidiocoris tenuis* Reuter, 1895. *M. pygmaeus* males were shown to have significantly smaller sperm cells (213.18 μm), with longer (50.94 μm) and wider heads and shorter tails (162.94 μm) than *N. tenuis*. In our study, the data on *M. pygmaeus* sperm cell size were confirmed. On the other hand, we found that *M. costalis* males have significantly longer sperm cells, with a longer head and tail compared to *M. melanotoma* and *M. pygmaeus*. In turn, *M. melanotoma* males have significantly longer sperm tails compared to *M. pygmaeus* (Table 1).

Conclusion

As mentioned in Introduction, the cryptic species *M. pygmaeus* and *M. melanotoma* can be differentiated from each other based on the cuticular hydrocarbon profiles and specific molecular primers (Gemeno et al. 2012, Castañé et al. 2013). In our study, we provide some alternative characters, such as karyotype (number and size of chromosomes, sex chromosome system, and amount and distribution of heterochromatin) and sperm cells' morphology, allowing for reliable identification of *M. pygmaeus*, *M. melanotoma* and *M. costalis*.

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RESEARCH ARTICLE

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Cytogenetic analyses of five amazon lizard species of the subfamilies Teiinae and Tupinambinae and review of karyotyped diversity the family Teiidae

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Abstract

Lizards of the family Teiidae (infraorder Scincomorpha) were formerly known as Macroteiidae. There are 13 species of such lizards in the Amazon, in the genera *Ameiva* (Meyer, 1795), *Cnemidophorus* (Wagler, 1830), *Crocodilurus* (Spix, 1825), *Dracaena* (Daudin, 1801), *Kentropyx* (Spix, 1825) and *Tupinambis* (Daudin, 1802). Cytogenetic studies of this group are restricted to karyotype macrostructure. Here we give a compilation of cytogenetic data of the family Teiidae, including classic and molecular cytogenetic analysis of *Ameiva ameiva* (Linnaeus, 1758), *Cnemidophorus* sp.1, *Kentropyx calcarata* (Spix, 1825), *Kentropyx pelviceps* (Cope, 1868) and *Tupinambis teguixin* (Linnaeus, 1758) collected in the state of Amazonas, Brazil. *Ameiva ameiva*, *K. calcarata* and *K. pelviceps* have 2n=50 chromosomes classified by a gradual series of acrocentric chromosomes. *Cnemidophorus* sp.1 has 2n=48 chromosomes with 2 biarmed chromosomes, 24 uniarmed chromosomes and 22 microchromosomes. *Tupinambis teguixin* has 2n=36 chromosomes, including 12 macrochromosomes and 24 microchromosomes. Constitutive heterochromatin was distributed in the centromeric and terminal regions in most chromosomes. The nucleolus organizer region was simple, varying in its position among the species, as evidenced both by $AgNO_3$ impregnation and by hybridization with 18S rDNA probes. The data reveal a karyotype variation with respect to the diploid number, fundamental number and karyotype formula, which reinforces the importance of increasing chromosomal analyses in the Teiidae.

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Keywords
Macroteiidae, Chromosome, Heterochromatin, Differential staining, rDNA-FISH

Background

The family Teiidae is composed of lizards formerly known as macroteiids that are restricted to the New World (Giugliano et al. 2007, Harvey et al. 2012). Harvey et al. (2012) recently divided Teiidae in three subfamilies: (1) Teiinae, including the genera *Ameiva* (Meyer, 1795), *Ameivula* (Spix, 1825), *Aurivela* (Bell, 1843), *Aspidoscelis* (Fitzinger, 1843), *Contomastix* (Dumésil and Bibron, 1839), *Cnemidophorus* (Wagler 1830), *Dicrodon* (Dumésil and Bibron, 1839), *Holcosus* (Cope, 1862), *Kentropyx* (Spix, 1825), *Medopheos* (Bocourt, 1874) and *Teius* (Merrem, 1820); (2) Tupinambinae, including the genera *Crocodilurus* (Spix, 1825), *Dracaena* (Daudin, 1801), *Salvator* (Dumésil & Bibron, 1839) and *Tupinambis* (Daudin, 1802); and (3) Callopistinae, which contains the single genus *Callopistes* (Gravenhorst, 1837) (Harvey et al. 2012). However, the phylogenetic hypothesis of Teiidae based on molecular data (Reeder et al. 2002, Giugliano et al. 2007) differs substantially from the hypothesis proposed by Harvey et al. (2012).

Most chromosome data for teiid lizards refer only to the determination of diploid numbers and karyotype formulae (Fritts 1969, Gorman 1970, Lowe et al. 1970, Robinson 1973, Cole et al. 1979, de Smet et al. 1981, Navarro et al. 1981, Ward and Cole 1986, Cole et al. 1995, Markezich et al. 1997, Rocha et al. 1997, Walker et al. 1997, Manriquen-Moran et al. 2000, Veronese et al. 2003). Some species of this family have, however, been analyzed in detail with respect to their chromosomal structure and organization, as revealed by differential staining techniques, such as the detection of heterochromatin and nucleolar organizer regions (NORs), as well as chromosomal physical mapping of DNA sequences (Bickham et al. 1976, Bull 1978, Peccinini-Seale and Almeida 1986, Porter et al. 1991, Rocha et al. 1997, Veronese et al. 2003, Peccinini-Seale et al. 2004, Santos et al. 2007, Santos et al. 2008).

The family Teiidae can be divided into two chromosomal groups: the *Dracaena* group (currently the subfamily Tupinambinae), which has a karyotype with 34–38 chromosomes and a clear distinction of macrochromosomes (M) from microchromosomes (mi), and the *Ameiva* group (currently the subfamily Teiinae), which has a diploid number ranging from 46–56 chromosomes, with no distinction between macrochromosomes and microchromosomes (Gorman 1970).

We did a cytogenetic study of five species in the family Teiidae (*Ameiva ameiva* (Linnaeus, 1758), *Cnemidophorus* sp.1, *Kentropyx calcarata* (Spix, 1825), *Kentropyx pelviceps* (Cope, 1868) and *Tupinambis teguixin* (Linnaeus, 1758)) using classical as well as molecular cytogenetic markers (conventional staining, heterochromatin patterns, NOR locations and chromosomal physical mapping of 18S rDNA sequences). Karyotype organization in the family is discussed.

Methods

Thirty-three specimens belonging to the subfamilies Teiinae and Tupinambinae were collected in the state of Amazonas, Brazil, in the following localities: the riverside forests of the Jatapu river, the city of São Sebastião do Uatumã (0°50'E 01°55'S; 58°50'E 60°10'W), the Darahá and Ayuanã rivers, both in the city of Santa Isabel do Rio Negro (0°24'24"N; 65°1'1"W), the city of Manaus (3°07'13.03"S; 60°01'440"W) and the Purus riverside in the city of Tapauá (5°42'115"S; 63°13'684"W). All of the collections were conducted with permission from the Brazilian Environmental Protection Agency (ICMBio/SISBIO 41825-1). The collection sites are located in public lands (Table 1, Figure 1). The animals were euthanized soon after capture in the field with a lethal dose of the anesthetic sodium thiopental to avoid being deprived of food or water. This research was approved by the Ethics Committee for Animal Experimentation of the Fundação Universidade do Amazonas / Universidade Federal do Amazonas (UFAM) (number 041/2013). No endangered or protected species were used in this research study. The animals underwent cytogenetic procedures and were then fixed with 10% formaldehyde (injected in the coelom and digestive tract), preserved in 70% alcohol. Voucher specimens were deposited in the Herpetological Collection of the Instituto Nacional de Pesquisas da Amazônia (INPA H31712, 33213, 34791, 34841, 35018).

Cellular suspensions were obtained from the bone marrow was removed soon after the euthanasia of animals in the field using an *in vitro* colchicine treatment (Ford and Hamerton 1956). Constitutive heterochromatin (CH) was detected using barium hydroxide (Sumner 1972) and the NORs were detected using silver nitrate staining (Howell and Black 1980).

Genomic DNA was extracted from muscle tissue using a phenol-chloroform protocol (Sambrook and Russell 2001) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). 18S rDNA was amplified by polymerase chain reaction (PCR) using primers 18Sf (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18Sr (5'-CCG AGGACC TCA CTA AAC CA-3') (Gross et al. 2010). PCR reactions were performed on a final volume of 15 µL, containing genomic DNA (200 ng), 10× buffer with 1.5 mM of $MgCL_2$, Taq DNA polymerase (5 U/µL), dNTPs (1 mM), forward and reverse primers (5 mM) and Milli-Q water. The amplification cycles followed these steps: 1 min at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 56 °C, 1 min 30 s at 72 °C and 5 min at 72 °C.

The PCR product of the 18S rDNA was labeled with digoxigenin-11-dUTP (Dig-Nick Translation mix; Roche), by nick translation according to the manufacturer's instructions. The antibody anti-digoxigenin rhodamine (Roche) was used for probing the signal. Homologue (DNA probes from the same species) and heterologue (probes of one species hybridized to the chromosome of another) hybridizations were made under stringency conditions of 77% (2.5 ng/µL of 18S rDNA, 50% formamide, 10% dextran sulfate, and 2× SSC at 37 °C for 18 h) (Pinkel et al. 1986). The chromosomes were counterstained with DAPI (2 mg/ml) in VectaShield mounting medium (Vector). The chromosomes were analyzed using an Olympus BX51 epifluorescence mi-

Table 1. Species of the Teiinae and Tupinambinae subfamilies: collection sites, number and the analyzed animals and voucher specimens (lots) are listed. AM: Amazonas.

Subfamily	Species	Collection sites	Number and sex the analyzed animals	Voucher specimens (lots)
	Ameiva ameiva	São Sebastião do Uatumã, AM Santa Isabel do Rio Negro, AM Tapauá, AM	11 (four males; three females; four without sex identification)	INPA H33213
	Cnemidophorus sp.1	Manaus, AM	13 (five males; eight females)	INPA H35018
Teiinae	*Kentropyx calcarata*	São Sebastião do Uatumã, AM	4 (three males; one females)	INPA H31712
	Kentropyx pelviceps	Tapauá, AM	3 (three females)	INPA H34841
Tupinambinae	*Tupinambis teguixin*	São Sebastião do Uatumã, AM Tapauá, AM	3 (two females; one without sex identification)	INPA H34791

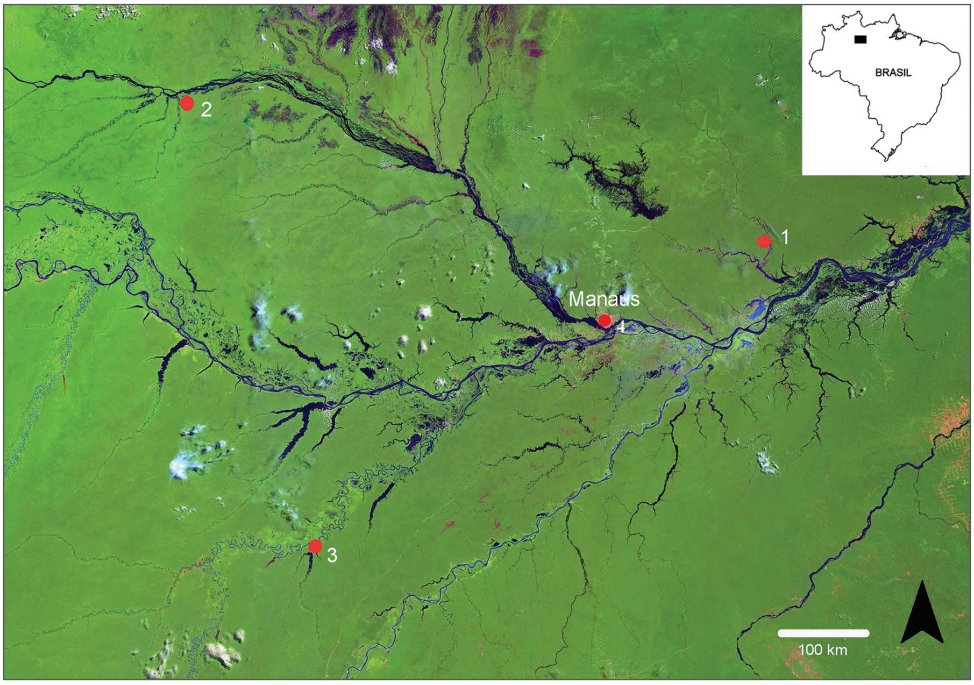

Figure 1. Satellite image of the Amazon basin showing the three different geographical areas; 1 = São Sebastião do Uatumã; 2 = Santa Isabel do Rio Negro; 3 = Tapauá; 4 = Manaus.

croscope and the images were captured with a digital camera (Olympus DP71) using Image-Pro MC 6.3 software. Mitotic metaphases were processed in Adobe Photoshop CS4 software and were measured using program ImageJ software. Chromosomes were organized by decreasing size, and chromosome morphology was determined based on

the arm ratio for metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) chromosomes (Levan et al. 1964). The karyotype formula was determined according to chromosomes that show a gradual series of acrocentric chromosomes, number of biarmed chromosomes, number of uniarmed chromosomes and number of macrochromosomes (M), and microchromosomes (mi) (Lowe and Wright 1966, Peccinini-Seale 1981). Macrochromosomes and microchromosomes are chromosomes that can be differentiated according to size; macrochromosomes are large and have one or two chromosome arms; microchromosomes are small (0.5–1.5 μm), puntiform and do not have any specific chromosome morphology.

Results

The diploid number for all specimens of *Ameiva ameiva*, *Kentropyx calcarata* and *Kentropyx pelviceps* was 50 chromosomes, and the karyotypic formula was classified by a gradual series of acrocentric chromosomes (Fig. 2a, i and m). *Cnemidophorus* sp.1 had 48 chromosomes with 2 biarmed chromosomes, 24 uniarmed chromosomes and 22 microchromosomes (Fig. 2e). *Tupinambis teguixin* had 36 chromosomes with 12 macrochromosomes (M) and 24 microchromosomes (mi). Pairs 1, 3, 4 and 5 of the macrochromosomes were metacentric and pairs 2 and 6 were submetacentric chromosomes (Fig. 3a). A secondary constriction was observed in the distal region of the long arms of pair 1 in *Cnemidophorus* sp.1, *Kentropyx calcarata* and *Kentropyx pelviceps* and in pair 2 in *Tupinambis teguixin* (Figs 2e, i, m and 3a). No differentiated sex chromosomes were observed in the analysed species.

Constitutive heterochromatin was observed in the centromeric and terminal regions in most chromosomes of *Ameiva ameiva*, *Cnemidophorus* sp.1, *Kentropyx calcarata* and *Kentropyx pelviceps* (Figs 2b, f, j, n). In *Tupinambis teguixin*, heterochromatic blocks were located in the centromeric region of all the macrochromosomes. However, tenuous blocks were observed in the terminal regions in macrochromosomes and microchromosomes (Fig. 3b).

The NORs were located in the terminal region of the long arms of pair 7 in *Ameiva ameiva* (Fig. 2c). In *Cnemidophorus* sp.1, *Kentropyx calcarata* and *Kentropyx pelviceps*, NORs were seen in the distal region of the long arms of pair 1 and in pair 2 in *Tupinambis teguixin*, coincident with the secondary constriction present in the karyotypes of these species (Figs 2g, k, o and 3c, respectively). Fluorescent *in situ* hybridization (FISH) with an 18S rDNA probe revealed a chromosome pair bearing this site, coincident with the NOR sites in all of the five analyzed species (Figs 2d, h, l, p and 3d).

Discussion

Since the 1970s, cytogenetic analysis of the family Teiidae has shown that individuals could be categorized into two groups: the *Ameiva* group, with diploid number vary-

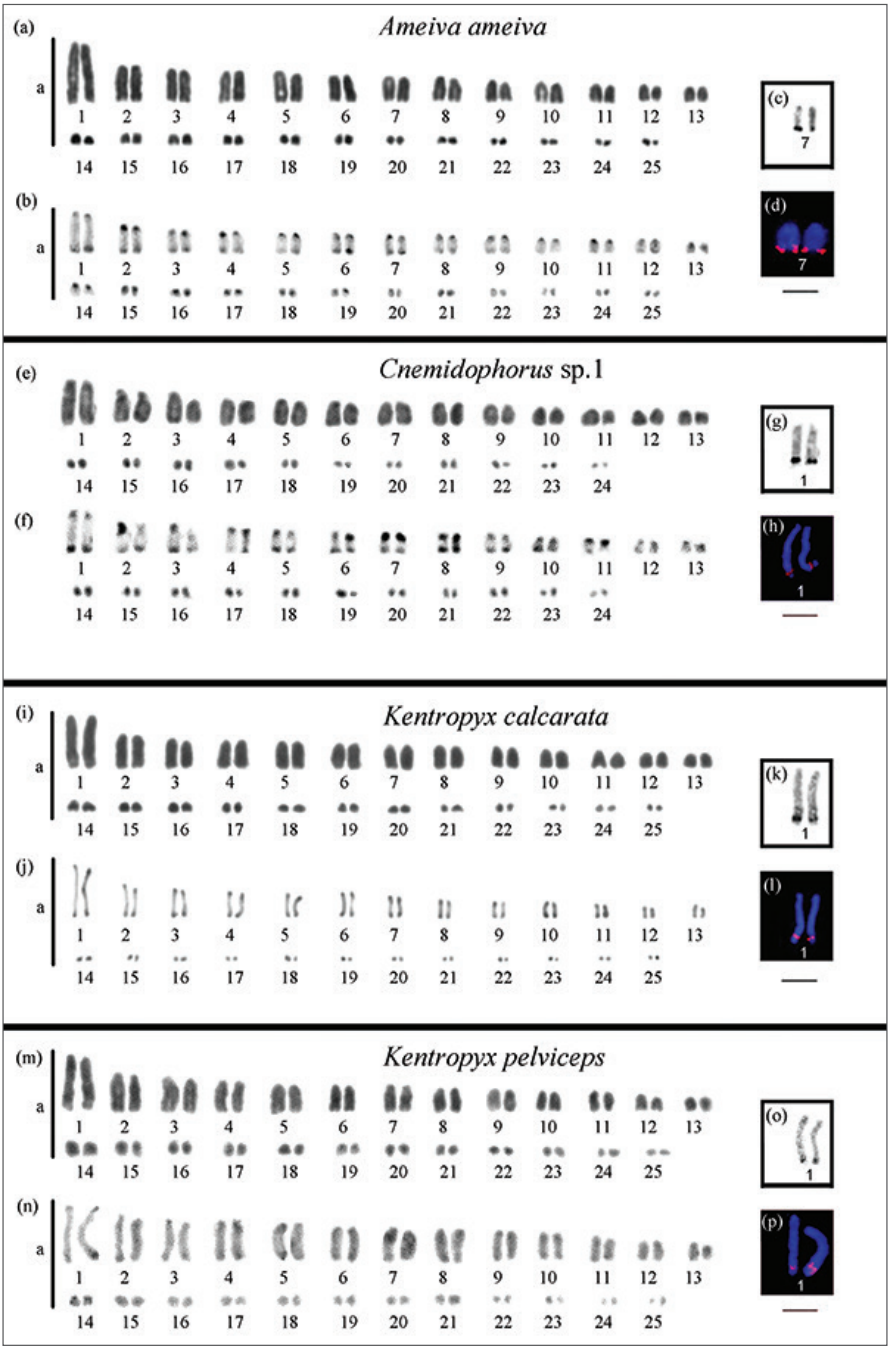

Figure 2. Karyotypes of species belonging to Teiinae: **a, e, i, m** in conventional Giemsa staining **b, f, j, n** Regions of heterochromatin evidenced by C-band technique **c, g, k, o** highlight the nucleolar pair impregnated with $AgNO_3$ **d**, **h**, **l**, **p** highlighted in the chromosome pair bearing the site of 18S rDNA (red) and chromosomes were counterstained with DAPI. m = Macrochromossome, mi = microchromossome. Scale bar = 10 μm.

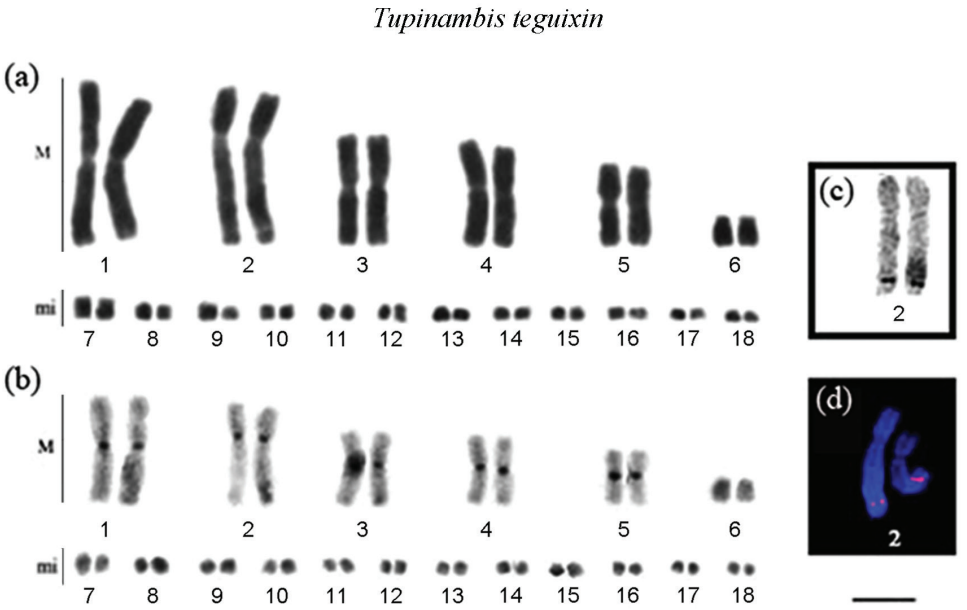

Figure 3. Karyotype of *Tupinambis teguixin*: **a** in conventional Giemsa staining **b** Regions of heterochromatin evidenced by C-band technique **c** highlight the nucleolar pair impregnated with $AgNO_3$ **d** highlight the chromosome pair bearing the site of 18S rDNA (red) and chromosomes were counterstained with DAPI. m = Macrochromossome, mi = microchromossome. Scale = 10 μm.

ing from 30–56 chromosomes, with no distinction between macrochromosomes and microchromosomes, and the *Dracaena* group, with a karyotype varying from 34–38 chromosomes, with a clear distinction between macrochromosomes and microchromosomes (Gorman 1970). By the end of the 1980s, several osteological and morphological studies corroborated the chromosomal data, thus supporting these two groups, which were subsequently considered subfamilies (Estes et al. 1988): Teiinae (*Ameiva* group) and Tupinambinae (*Dracaena* group).

Most karyotype data comes from species of the subfamily Teiinae, with descriptions of diploid numbers for 63 species. The karyotypes reveal a diploid number varying from 2n=30 in *Ameiva auberi* (Cocteau, 1838) to 2n=54 in *Teius oculatus* (D'orbigny & Bibron, 1837) and *Teius teyou* (Daudin, 1802), besides the presence of sex chromosomes of XX/XY in *Aspidocelis tigris tigris* (Baird & Girard, 1852) and *Ameivula littoralis* (Rocha, Bamberg Araújo, Vrcibradic, 2000). Some *Aspidoscelis* species show triploid numbers such as *Aspidoscelis tessalatus* (Say, 1823) with 69 chromosomes. Interspecific hybridization has been observed in some species of the genus *Aspidoscelis*, which were previously placed within the genus *Cnemidophorus* (Lowe et al. 1970, Walker et al. 1997, Lutes et al. 2010, Manriquez-Morán et al. 2000). Although the *Ameiva* group proposed by Gorman (1970) corresponds to the subfamily Teiinae, some species have a distinction between macrochromosomes and microchromosomes,

while most chromosomes are acrocentric. This finding is contrary to what was proposed by Gorman (1970) as a cytogenetic feature of the *Ameiva* group (Table 2).

Ameiva ameiva and *Kentropyx calcarata*, which belong to Teiinae, have the same diploid number (2n=50 chromosomes). This result corroborates the available data for these species from different localities (Gorman 1970, Beçak et al. 1972, Peccinini-Seale and Almeida 1986, Schmid and Guttenbach 1988, Sites et al. 1990, Veronese et al. 2003, Santos et al. 2007). However, in present study *Ameiva ameiva* and *Kentropyx calcarata* present a gradual series of acrocentric chromosomes characterized by absence of distinction between macrochromosomes and microchromosomes, similar to the results described by Cole et al. (1995) and Santos et al. (2007). The same finding is observed for *Kentropyx pelviceps*, whose cytogenetic characteristics are revealed for the first time in the present study.

Furthermore, karyotypic formulae composed of biarmed chromosomes, uniarmed chromosomes and microchromosomes has been described for *Ameiva ameiva* and *Kentropyx calcarata* and in the other species genera of the subfamily Teiinae (Lowe and Wright 1966, Gorman 1970, Beçak et al. 1972, Peccinini-Seale and Almeida 1986, Schmid and Guttenbach 1988, Sites et al. 1990, Veronese et al. 2003). These data show that some differences may result from different classification parameters adopted by several authors in their chromosomal analyses.

Currently, the genus *Cnemidophorus* is divided into four morphological groups: (1) *Cnemidophorus lemniscatus* including the species *Cnemidophorus arenivagus* (Markezich, Cole & Dessauer, 1997), *Cnemidophorus arubensis* (Lidth de Jeude, 1887), *Cnemidophorus cryptus* (Cole & Dessauer, 1993), *Cnemidophorus flavissimus* (Ugueto, Harvey & Rivas, 2010), *Cnemidophorus gramivagus* (Mccrystal & Dixon, 1987), *Cnemidophorus lemniscatus espeuti* (Boulenger, 1885), *Cnemidophorus lemniscatus gaigei* (Ruthven, 1924), *Cnemidophorus lemniscatus lemniscatus* (Linnaeus, 1758), *Cnemidophorus lemniscatus splendidus* (Markezich, Cole & Dessauer, 1997), *Cnemidophorus pseudolemniscatus* (Cole & Dessauer, 1993), *Cnemidophorus senectus* (Ugueto, Harvey & Rivas, 2010) and *Cnemidophorus* sp. B.; (2) *Cnemidophorus nigricolor* including the species *Cnemidophorus leucopsammus* (Ugueto & Harvey, 2010), *Cnemidophorus nigricolor* (Peters, 1873), *Cnemidophorus rostralis* (Ugueto & Harvey, 2010) and *Cnemidophorus* sp. A; (3) *Cnemidophorus murinus* including the species *Cnemidophorus murinus* (Laurenti, 1768) and *Cnemidophorus ruthveni* (Burt, 1935) and (4) *Cnemidophorus vanzoi* including the species *Cnemidophorus vanzoi* (Baskin & Williams, 1966) (Harvey et al. 2012). It is noteworthy that several new species of this genus have been described, showing that the taxonomy of this genus has not yet been elucidated, which emphasizes the need for morphological and molecular studies in this genus. Cytogenetically, some species of *Cnemidophorus* have 50 chromosomes, composed of biarmed chromosomes, uniarmed chromosomes and microchromosomes (Table 2, Peccinini-Seale and Almeida 1986). However, the karyotype of *Cnemidophorus* sp.1 from Manaus, in Amazonas state, differs from those described for other species of the genus. This species has 2n = 48 chromosomes with the absence of a pair of microchromosomes (Table 2, present study). Non-robertsonian chromosomal

rearrangements may be associated with chromosomal evolution of this genus, which favored changes in diploid number (reduction in diploid number). Another population in Amazonas state (county Manacapuru) identified as belonging to *Cnemidophorus lemniscatus* group has the expected diploid number of 50 chromosomes with the presence of biarmed chromosomes and uniarmed microchromosomes (0:26:24) (Sites et al. 1990). Our results show that the specimens we sampled from Manaus are karyotypically distinct from specimens we sampled from Manacapuru so *Cnemidophorus* sp.1 (*Cnemidophorus lemniscatus* group) could represent a new species.

Seven species from the subfamily Tupinambinae, have had their karyotypes analyzed, with diploid numbers varying from 2n=34–38 chromosomes, with the presence of both macrochromosomes and microchromosomes (Santos et al. 2008, present study). No sex chromosome system has been documented in the subfamily (Gorman 1970). *Tupinambis teguixin* has 2n=36 chromosomes (12M+24m) (Table 2) the same number and karyotype formula was found by other authors (Gorman 1970, de Smet et al. 1981, Santos et al. 2008). Beçak et al. (1972) described a diploid number of 38 chromosomes (12M+26m) for *T. teguixin*, with an additional pair of microchromosomes.

In the family Teiidae, heterochromatic blocks are located in the centromeric and terminal regions of almost all chromosomes. In some chromosomes, heterochromatic blocks are present in the pericentromeric, interstitial and terminal regions (Table 3). In the five species of the family Teiidae analyzed in this study, we observed a significant number of heterochromatic blocks in the centromere and terminal regions in the most of the chromosomes, which is consistent with similar patterns described in the literature.

The heterochromatin patterns for *Cnemidophorus* sp.1, *Kentropyx calcarata*, *Kentropyx pelviceps* and *Tupinambis teguixin* are described for the first time in this study. The heterochromatin distributional pattern is similar among the analyzed species, suggesting a common pattern for species in the family Teiidae. Three species in the subfamily Tupinambinae (*Crocodilurus amazonicus* (Spix, 1825), *Salvator merianae* (Duméril & Bibron, 1839) and *Tupinambis quadrilineatus* (Manzani & Abe, 1997), however, show species-specific heterochromatin patterns, with heterochromatic blocks in the centromeric, pericentromeric, interstitial and proximal regions of most chromosomes (Santos et al. 2008). The existence of such a distinctive pattern can likely be attributed to the addition of heterochromatin or the heterochromatization process during the evolution of these species. Heterochromatic regions are rich in repetitive DNA sequences usually located in the centromeric or terminal regions of chromosomes. This has often been considered important species-specific or population markers (Carvalho et al. 2012, Schneider et al. 2013). Even though heterochromatin may be located on the same chromosome region in different species, this does not mean it has the same genetic composition, which may differ in the amount of repetitive DNA sequences in the chromosomes (Chaiprasertsri et al. 2013).

Although the five species in the family Teiidae analyzed in the present study present a conserved karyotype macrostructure, some chromosomal characteristics differentiate the karyotype of these species. In *Cnemidophorus* sp.1, *Kentropyx calcarata*,

Table 2. Basic cytogenetic data compiled from the literature for the Teiidae family. Diploid number (2n), karyotypic formula (KF), fundamental number (FN). Three descriptions of karyotypic formulas: (a) number of biarmed chromosomes, number of uniarmed chromosomes and number of microchromosomes; (b) chromosomes that show a gradual series of acrocentric chromosomes; (c) macrochromosome chromosomes (M) and microchromosomes (mi). For data not included in the literature, "-" is indicated.

Subfamily	Genus	Species (*sensu* [2])	Species (initial description)	2n	Type of KF and description	FN	Reference
Callopistinae	*Callopistes*	*Callopistes flavipunctatus*	*Callopistes flavipunctatus*	2n=38	c (12M+26m)	50	2
		Callopistes maculatus	*Callopistes maculatus*	2n=38	c (12M+26m)	26, 50	2, 8
Teiinae	*Ameiva*	*Ameiva ameiva*	*Ameiva ameiva*	2n=50	a (0: 26: 24) b (gradual series of acrocentric chromosomes)	50	2, 18
Tupinambinae		*Ameiva auberi*	*Ameiva auberi*	2n=30	a (8: 10:12)	38	11
		Ameiva chrysolaema	*Ameiva chrysolaema*	2n=50	a (0: 22: 28), (6: 20: 24)	50, 56	2
		Ameiva dorsalis	*Ameiva dorsalis*	2n=50	a (4: 22: 24)	54	2
		Ameiva exsul	*Ameiva exsul*	2n=50	a (0: 26: 24)	50	2
		Ameiva maynardi	*Ameiva maynardi*	2n=50	a (4: 22: 24)	54	2
	Ameivula	*Ameivula nativo*	*Cnemidophorus nativo*	2n=50	a (5: 19: 24)	53	14
		Ameivula litorralis	*Cnemidophorus littoralis*	2n=46(XX/XY)	a (5: 19: 22)	51	9
		Ameivula ocellifera	*Cnemidophorus ocellifera*	2n=50	b(gradual series of acrocentric chromosomes)	-	18
	Aspidoscelis	*Aspidoscelis angusticeps*	*Cnemidophorus angusticeps*	2n=44, 46	a (6: 20: 18), a (2: 24: 20)	50, 48	3, 16
		Aspidoscelis burti	*Cnemidophorus burti*	2n=46	a (2: 24: 20)	48	3
		Aspidoscelis calidipes	*Cnemidophorus calidipes*	2n=46	a (2: 24: 20)	48	3
		Aspidoscelis ceralbensis	*Cnemidophorus ceralbensis*	2n=52	-	-	4
		Aspidoscelis communis	*Cnemidophorus communis*	2n=46	a (2: 24:20)	48	3
		Aspidoscelis costatus	*Cnemidophorus costatus*	2n=46	a (2: 24:20)	48	3
		Aspidoscelis cozumelae	*Cnemidophorus cozumelae*	2n=49, 50	a (0: 28: 21), a (11: 19: 20)	49, 61	3, 16
		Aspidoscelis deppei	*Cnemidophorus deppei*	2n=50, 52	a (0: 26: 24), a (0: 28: 24)	50, 52	3, 16
		Aspidoscelis exsanguis	*Cnemidophorus exsanguis*	3n=69*	-	-	3, 10
		Aspidoscelis flagellicaudas	*Cnemidophorus flagellicaudas*	3n=69*	-	-	3
		Aspidoscelis gularis	*Cnemidophorus gularis*	2n=46	a (2: 24: 20)	48	3
		Aspidoscelis guttatus	*Cnemidophorus guttatus*	2n=52	a (0: 28: 24)	52	3
		Aspidoscelis hyperythrus	*Cnemidophorus hyperythrus*	2n=52	a (0: 28: 24)	52	3
		Aspidoscelis inoratus	*Cnemidophorus inoratus*	2n=46	a (2: 24: 20)	48	3, 10
		Aspidoscelis laredoensis	*Cnemidophorus laredoensis*	2n=46	a (2: 24: 20)	48	4

Subfamily	Genus	Species (*sensu* [2])	Species (initial description)	2n	Type of KF and description	FN	Reference
		Aspidoscelis lineatissima	*Cnemidophorus lineatissima*	2n=52	a (0: 28: 24)	52	3
		Aspidoscelis marmoratus	*Cnemidophorus marmoratus*	2n=46	a (0: 22: 24)	46	11
		Aspidoscelis maslini	*Cnemidophorus maslini*	2n=47	a (14: 13: 20)	49	3
		Aspidoscelis mexicana	*Cnemidophorus mexicana*	2n=46	a (2: 24: 20)	48	3
		Aspidoscelis motaguae	*Cnemidophorus motaguae*	2n=46	a (2: 24: 20)	48	3
		Aspidoscelis neomexicanus	*Cnemidophorus neomexicanus*	2n=46	a (4: 20: 22)	50	3,10
		Aspidoscelis opatae	*Cnemidophorus opatae*	3n=69*	-	-	3
		Aspidoscelis parvisocius	*Cnemidophorus parvisocius*	2n=46	a (2: 24: 20)	48	3
		Aspidoscelis rodecki	*Cnemidophorus rodecki*	2n=50	-	-	1
		Aspidoscelis sacki	*Cnemidophorus sacki*	2n=46	a (2: 24: 20)	48	3
		Aspidoscelis sptemvittatus	*Cnemidophorus sptemvittatus*	2n=46	a (2: 24: 20)	48	3
		Aspidoscelis sexlineatus	*Cnemidophorus sexlineatus*	2n=46	a (2: 24: 20), a (8: 18: 20)	48, 54	3, 5
		Aspidoscelis sonorae	*Cnemidophorus sonorae*	2n=46, 3n=69*	a (4: 20: 22)	48	2, 3, 10
		Aspidoscelis tesselatus	*Cnemidophorus tesselatus*	2n=46, 3n=69*	a (4: 20: 22)	50	3, 10, 15
		Aspidoscelis tigris tigris	*Cnemidophorus tigris tigris*	2n=46(XX/XY)	a (6: 16: 24)	52	2, 10
		Aspidoscelis tigris aethiops	*Cnemidophorus tigris aethiops*	2n=46	a (6: 16: 24)	52	3
		Aspidoscelis t. estebanensis	*Cnemidophorus t. estebanensis*	2n=46	a (6: 16: 24)	52	3
		Aspidoscelis t. gracilis	*Cnemidophorus t. gracilis*	2n=46	a (6: 16: 24)	52	3
		Aspidoscelis t. marmoratus	*Cnemidophorus t. marmoratus*	2n=46	a (6: 16: 24)	52	3
		Aspidoscelis t. maximus	*Cnemidophorus t. maximus*	2n=46	a (6: 16: 24)	52	3
		Aspidoscelis t. septentrionalis	*Cnemidophorus t. septentrionalis*	2n=46	a (6: 16: 24)	52	3
		Aspidoscelis ubiparens	*Cnemidophorus uniparens*	3n=69*	-	-	3, 10
		Aspidoscelis velox	*Cnemidophorus velox*	3n=69*	-	-	3
	Cnemidophorus	*Cnemidophorus arenivagus*	*Cnemidophorus arenivagus*	2n=50	a (2: 24: 24)	52	9, 13
		Cnemidophorus arubensis	*Cnemidophorus arubensis*	2n=50	a (2: 24: 24)	52	9, 13
		Cnemidophorus cryptus	*Cnemidophorus cryptus*	2n=50	-	52	9
		Cnemidophorus gramivagus	*Cnemidophorus gramivagus*	2n=50	-	52	9
		Cnemidophorus lemniscatus	*Cnemidophorus lemniscatus*	2n=50	a (2: 24: 24)	52	2, 3
		Cnemidophorus murinus	*Cnemidophorus murinus*	2n=50	a (2: 24:24)	52	4, 3

Subfamily	Genus	Species (*sensu* [2])	Species (initial description)	2n	Type of KF and description	FN	Reference
	Contomastix	*Contomastix lacertoides*	*Cnemidophorus lacertoides*	2n=50	a (0: 26: 24)	52	6, 17
	Kentropyx	*Kentropyx borckiana*	*Kentropyx borckiana*	2n=50	a (0: 26: 24)	50	12
		Kentropyx calcarata	*Kentropyx calcarata*	2n=50	b(gradual series of acrocentric chromosomes)	50	12, 19
		Kentropyx striata	*Kentropyx striata*	2n=50	a (0: 26: 24)	50	12
		Kentropyx paulensis	*Kentropyx paulensis*	2n=50	b(gradual series of acrocentric chromosomes)	50	18
		Kentropyx pelviceps	*Kentropyx pelviceps*	2n=50	b(gradual series of acrocentric chromosomes)	50	20
		Kentropyx vanzoi	*Kentropyx vanzoi*	2n=50	b(gradual series of acrocentric chromosomes)	50	18
	Teius	*Teius oculatus*	*Teius oculatus*	2n=54	a (8: 28: 18)	62	17
		Teius teyou	*Teius teyou*	2n=54	a (8: 22: 24)	62	2
	Crocodilurus	-	*Crocodilurus lacertinus*	2n=34	c (12M+22m)	46	2
		Crocodilurus amazonicus	*Crocodilurus amazonicus*	2n=34	c (12M+22m)	46	19
	Dracaena	*Dracaena guianensis*	*Dracaena guianensis*	2n=38	a (10:2:26)	48	2
	Tupinambis	-	*Tupinambis nigropunctatus*	2n=36, 38	a (10: 2: 24), c (16M+22m)	46, 54	2, 7
		Tupinambis quadrilineatus	*Tupinambis quadrilineatus*	2n=38	c (12M+26m)	-	19
		Tupinambis teguixin	*Tupinambis teguixin*	2n=38, 36	a (10: 0: 28), (12M+24m)	48	7, 19
	Salvator	*Salvator merianae*	*Tupinambis merianae*	2n=36, 38	a (10: 0: 26), c (12M+26m)	48, 50	7, 17, 19

* Polyploidy in triploid form (3n). 1 - Fritts 1969; 2 - Gorman 1970; 3 - Lowe et al. 1970; 4 - Robinson 1973; 5 - Bickham et al. 1976; 6 - Cole et al. 1979; 7 - de Smet et al. 1981; 8 - Navarro et al. 1981; 9 - Peccinini-Seale and Almeida 1986; 10 - Ward and Cole 1986; 11 - Porter et al. 1991; 12 - Cole et al. 1995; 13 - Markezich et al. 1997; 14 - Rocha et al. 1997; 15 - Walker et al. 1997; 16 - Manriquen-Moran et al. 2000; 17 - Veronese et al. 2003; 18 - Santos et al. 2007; 19 - Santos et al. 2008; 20 - Present work.

Table 3. Cytogenetic banding data compiled from the literature for the differential Teiidae family. Nucleolar organizer regions (NORs), constitutive heterochromatin (CH), fluorescent *in situ* hybridization (FISH). Locality: Amazonas (AM), Bahia (BA), United States (USA), Espírito Santo (ES), Goiás (GO), Mato Grosso (MT), Minas Gerais (MG), Pará (PA), Rio de Janeiro (RJ), Rio Grande do Sul (RS), Rondônia (RO), São Paulo (SP), Sergipe (SE), Tocantins (TO). For data not included in the literature, "-" is indicated.

Subfamily	Species (Current description)	Species (Initial description)	Locality	NOR	CH	FISH	Reference
Teiinae	*Ameiva ameiva*	*Ameiva ameiva*	GO, RO, MT, TO	Terminal region of the long arms of pair 7	Centromeric and terminal regions	-	8
	Ameiva ameiva	*Ameiva ameiva*	AM	Terminal region of the long arms of pair 7	Centromeric and terminal regions	18S rDNA (pair 7)	Present work
	Ameiva auberi	*Ameiva auberi*	-	-	-	45S rDNA (pair of microchromosomes)	4
	Aspidoscelis gularis	*Cnemidophorus gularis*	USA		Centromeric region	-	1
	Aspidoscelis laredoensis	*Cnemidophorus laredoensis*	USA	-	Centromeric region	-	1
	Aspidoscelis marmoratus	*Cnemidophorus marmoratus*	-	-	-	45S rDNA (pair 2)	4
	Aspidoscelis sexlineatus	*Cnemidophorus sexlineatus*	USA	-	Centromeric region	-	1
	Aspidoscelis tigris	*Cnemidophorus tigriss*	USA	-	Centromeric region	-	2
	Ameivula littoralis *Ameivula nativo* *Ameivula ocellifera*	*Cnemidophorus littoralis* *Cnemidophorus nativo* *Cnemidophorus ocellifera*	RJ ES BA, SE, MG	Terminal region of the long arms of pair 8 Multiple NORs (not indicated pairs) Terminal region of the long arms of pair 5	- - Centromeric and terminal regions	- - - -	7 5 8
	Cnemidophorus arenivagus	*Cnemidophorus arenivagus*	-	Terminal region of the long arms of pair 1	-	-	3
	Cnemidophorus cryptus	*Cnemidophorus cryptus*	-	Terminal region of the long arms of pair 1	-	-	3
	Cnemidophorus gramivagus	*Cnemidophorus gramivagus*	-	Terminal region of the long arms of pair 1	-	-	3
	Cnemidophorus lemniscatus	*Cnemidophorus lemniscatus*	-	Terminal region of the long arms of pair 1	-	-	3
	Cnemidophorus sp.1	-	AM	Terminal region of the long arms of pair 1	Centromeric and terminal regions	18S rDNA (pair 1)	Present work
	Contomastix larcetoides	*Cnemidophorus larcetoides*	RS	-	Centromeric region	-	6
	Kentropryx calcarata	*Kentropryx calcarata*	BA, TO, MT	Distal region of the long arms of pair 1	-	-	8

Subfamily	Species (Current description)	Species (Initial description)	Locality	NOR	CH	FISH	Reference
	Kentropryx calcarata	*Kentropryx calcarata*	AM	Distal region of the long arms of pair 1	Centromeric and terminal regions	18S rDNA (pair 1)	Present work
	Kentropryx paulensis	*Kentropryx paulensis*	SP	Distal region of the long arms of pair 1	Centromeric and terminal regions	-	8
	Kentropyx pelviceps	*Kentropyx pelviceps*	AM	Distal region of the long arms of pair 1	Centromeric and terminal regions	18S rDNA (pair 1)	Present work
	Kentropryx vanzoi	*Kentropryx vanzoi*	RO	Distal region of the long arms of pair 1	-	-	8
	Teius oculatus	*Teius oculatus*	RS	Multiple NORs (not indicated pairs)	-	-	6
Tupinambinae	*Crocodilurus amazonicus*	*Crocodilurus amazonicus*	PA	Distal region of the long arms of pair 2	Pericentromeric region	-	9
	Salvator meriane	*Tupinambis merianae*	TO, SP, ES	Distal region of the long arms of pair 2	Pericentromeric region	-	9
	Tupinambis quadrilineatus	*Tupinambis quadrilineatus*	GO, TO	Distal region of the long arms of the pair 2	Centromeric, pericentromeric, interstitial, proximal and terminal regions	-	9
	Tupinambis teguixin	*Tupinambis teguixin*	GO, TO	Distal region of the long arms of pair 2	-	-	9
	Tupinambis teguixin	*Tupinambis teguixin*	AM	Distal region of the long arms of pair 2	Centromeric and terminal regions	18S rDNA (pair 2)	Present work

1 - Bickhan et al. 1976; 2 - Bull 1978; 3 - Peccinini-Seale and Almeida 1986; 4 - Porter et al. 1991; 5 - Rocha et al. 1997; 6 - Veronese et al. 2003; 7 - Peccinini-Seale et al. 2004; 8 - Santos et al. 2007; 9 - Santos et al. 2008

Kentropyx pelviceps and *Tupinambis teguixin*, the presence of a secondary constriction localized in the distal region of pairs 1 and 2 was observed. The secondary constriction is absent in *Ameiva ameiva*.

Secondary constrictions are typically present in a single chromosomal pair and are very common in several lizard species (Bertolloto et al. 1996, Kasahara et al. 1996, Bertolloto et al. 2002, Srikulnath et al. 2009a). This region contain genes that produce ribosomal RNA and these regions may hold nucleoli proteins during the entire process of cellular division (Guerra 1988). In such secondary constrictions, NORs are usually placed and they are identified, indirectly, by silver nitrate impregnation of the chromosomes. Such impregnation marks only nucleoli proteins involved in the transcriptional activity of ribosomal genes of the 45S family. NORs may be located in a single chromosomal pair, a basal characteristic already reported for different lizard species (Porter et al. 1991).

In the present study, the localization of the NORs was revealed as an genus marker and this information has already been discussed for some genera in the family Teiidae, such as *Kentropyx* (*Kentropyx calcarata*, *Kentropyx paulensis* (Boettger, 1893) and *Kentropyx vanzoi* Gallagher & Dixon, 1980), *Crocodilurus* (*Crocodilurus amazonicus*), *Cnemidophorus* (*Cnemidophorus arenivagus*, *Cnemidophorus cryptus*, *Cnemidophorus gramivagus* and *Cnemidophorus lemniscatus lemniscatus*), *Salvator* (*Salvator merianae*) and *Tupinambis* (*Tupinambis quadrilineatus* and *Tupinambis teguixin*). Localization of the NORs is important for characterizing species and evolutionary studies among teiid lizards (Santos et al. 2007, 2008).

Tupinambis teguixin has a simple NOR, as evidenced by the secondary constriction of the long arm of pair 2. A common characteristic among species the subfamily Tupinambinae is the presence of such a secondary constriction in pair 2 (Gorman 1970). Four species of the subfamily Teiinae, *Ameiva ameiva*, *Cnemidophorus* sp.1, *Kentropyx calcarata* and *Kentropyx pelviceps*, also have simple NORs, but they are located in distinct chromosomal pairs. In *Cnemidophorus* sp.1, *Kentropyx calcarata* and *Kentropyx pelviceps*, a secondary constriction was seen in pair 1 while in *Ameiva ameiva* occurred in pair 7. The NOR data analyzed for *Ameiva ameiva* and *Kentropyx calcarata* in the present study corroborate previous data (Schmid and Guttenbach 1988, Cole et al. 1995, Veronese et al. 2003, Santos et al. 2007), but for *Cnemidophorus* sp.1 and *Kentropyx pelviceps* they are new data.

Two populations of *Ameiva ameiva* from the eastern Amazon showed multiple NORs involving pairs 1, 2, 6, 16, 18, 19 and some small chromosomes (Peccinini-Seale and Almeida 1986). Some authors suggest that the inter-individual variation observed in *Ameiva ameiva* may be related to the identification of active NOR sites, once the silver nitrate binds to acid nucleoli proteins involved with the transcriptional activity of the ribosomal genes (Miller et al. 1976, Howell and Black 1980, Boisvert et al. 2007). Such variability may also result from impregnation of CH regions rich in acid residues, in which the nitrate impregnates both the NORs and heterochromatic regions not bearing ribosomal sites, thereby not revealing the exact number of NORs (Sumner 2003). Moreover, this variation may be suggesting that *Ameiva ameiva* is a

specie complex, as other teiids like *Ameivula ocellifera* (Spix, 1825) (Arias et al. 2011) or *Cnemidophorus lemniscatus* (Harvey et al. 2012).

Using 45S ribosomal DNA probes and FISH, it is possible to understand the organization of the NORs and to elucidate questions concerning the chromosomal organization and karyotypic evolution. The FISH technique is a more refined method than silver nitrate impregnation to locate 45S rDNA sequences in mitotic chromosomes (Carvalho et al. 2012, Terencio et al. 2012, Schneider et al. 2013). However, for the species analyzed in the present study, the fluorescent *in situ* hybridization of the 18S ribosomal gene corroborated the results obtained with silver nitrate impregnation, confirming the existence of this ribosomal site in a single pair of chromosomes. This same pattern was identified in other species in the family Teiidae, supporting the sites seen in a microchromosome pair in *Ameiva auberi* (Cocteau, 1838). In *Aspidoscelis marmorata* (Baird & Girard, 1852), the same pattern was located in a macrochromosome pair (Porter et al. 1991). Furthermore, it was possible to observe a size heteromorphism of the sites between the homologue chromosomes in the four analyzed species, a fact also described for other lizard species (O'Meally et al. 2009, Srikunath et al. 2009b, Srikunath et al. 2011). Such a size heteromorphism is likely associated with unequal crossing-over mechanisms, rearrangements such as transpositions, deletions and/or duplications or variations in the number of rDNA copies present in such regions that would entail some changes in ribosomal sites (Gross et al. 2010, Ribeiro et al. 2008).

Conclusion

Our present data and those from the literature show that teiid lizards have karyotype variation with respect to diploid number, fundamental number and karyotype formula. This, reinforces the importance to increase the number of chromosomal analyses in the family Teiidae. Studies are currently underway with the chromosomal physical mapping of repetitive DNA sequences in three species of Amazonian teiids that are essential for the understanding of genome organization and karyotype evolution in this group of lizards.

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RESEARCH ARTICLE

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Ribosomal DNA in diploid and polyploid *Setaria* (Poaceae) species: number and distribution

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Abstract

Setaria Beauvois, 1812 is a genus of economically important forage species, including *Setaria italica* (Linnaeus, 1753) Beauvois, 1812 and *Setaria viridis* (Linnaeus, 1753) Beauvois, 1812, closely related species and considered as model systems for studies of C4 plants. However, complications and uncertainties related to taxonomy of other species of the genus are frequent due to the existence of numerous synonyms for the same species or multiple species with the same name, and overlapping of morphological characteristics. Cytogenetic studies in *Setaria* can be useful for taxonomic and evolutionary studies as well as for applications in breeding. Thus, this study is aimed at locating 45S and 5S rDNA sites through fluorescent *in situ* hybridization (FISH) in *S. italica*, *S. viridis* and *Setaria sphacelata* (Schumacher, 1827) Stapf, Hubbard, Moss, 1929 cultivars (cvs.) Narok and Nandi. *S. italica* and *S. viridis* have 18 chromosomes with karyotype formulas 6m + 3sm and 9m, respectively. The location of 45S and 5S rDNA for these species was in different chromosome pairs among the evaluated species. *S. viridis* presented a more symmetrical karyotype, strengthening the ancestral relationship with *S. italica*. *S. sphacelata* cvs. Narok and Nandi have 36 chromosomes, and karyotype formulas 11m+7sm and 16m+2sm, respectively. The 45S rDNA signals for both cultivars were also observed in distinct chromosome pairs; however chromosomes bearing 5S rDNA are conserved. Karyotypic variations found among the studied species are evidence of chromosomal rearrangements.

Keywords

Karyotype, FISH, forage species, chromosomal rearrangements

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Introduction

Setaria Beauvois, 1812 is a genus of the family Poaceae Barnhart including 125 cultivated, wild or weed species distributed in the warmer temperate regions worldwide (Gressel 2005). This genus includes Foxtail millet (*Setaria italica* (Linnaeus, 1753) Beauvois, 1812) and its wild ancestor Green foxtail (*Setaria viridis* Linnaeus, 1753) Beauvois, 1812), which have been considered as model systems for studies of C4 plants, stress biology and biofuel production (Muthamilarasan and Prasad 2015). *S. italica* is important for production of forage (McCartneyet al. 2009) and cereal for human consumption, especially in China, India, Japan, Russia and Nepal (Wanous 1990). *Setaria sphacelata* (Schumacher, 1827) Stapf, Hubbard, Moss, 1929, known as Golden bristle grass, is also important for its use in the formation of grasslands (Jones and Ford 1972).

Cytogenetic descriptions demonstrate that over 82% of the genus consists of polyploid species (Caponio and Pensiero 2002), with basic number x=9 (Darlington and Wylie 1955). Studies performed by Benabdelmouna et al. (2001) and Zhao et al. (2013) show that six distinct genomes are part of the evolutionary history of the genus. *S. italica* and *S. viridis* have 2n=18 chromosomes belonging to the genome A (Benabdelmouna et al. 2001) and details of chromosome morphology were presented by Croullebois and Siljak-Yakovlev (1989) and Benabdelmouna et al. (2001). The chromosome number of *S. sphacelata* varies from 2n=18 to 2n=90 (Hacker and Jones 1969), however, there are no descriptions on the chromosomal morphology.

In the genus *Setaria*, complications and uncertainties related to taxonomy are common due to the existence of numerous synonyms for the same species or multiple species with the same name, and overlapping of morphological characteristics. (Missouri Botanical Garden 2014). Taxonomic divergences represent a problem for breeders and for the exchange and conservation of genetic resources, because many plants can be wrongly incorporated in germplasm collections or incorrectly used in crosses.

Studies using molecular markers (Wang et al. 1998), isozymes (Kawase and Sakamoto 1984) and comparisons between genomes through GISH technique (Genomic *In Situ* Hybridization) (Benandelmouna et al. 2001) have been made on *S. italica* and *S. viridis*, and the results increasingly confirm the proximity between these species. The only justification for maintaining two separate taxa is the history of domestication of these species, which allowed them to become seemingly distinct groups in morphology, growth and agronomic use (Benandelmouna et al. 2001).

The chromosome number is an important datum in cytotaxonomic studies, and when combined with size, morphology, karyotype symmetry, banding patterns and satellite DNA position in the chromosome, enables a better understanding of karyotype evolution between species (Greilhuber 1995). The fluorescence in situ hybridization (FISH) technique allows the identification of specific regions in the physical mapping of chromosomes, and enables more accurate karyotypic comparisons among species, cultivars and populations (Guerra 2008), especially in the case of species with small chromosomes, since specific signals facilitate differentiation of chromosomes (Galasso et al. 1995).

rDNA probes are widely used in cytogenetic studies as they can contribute with information about homology between chromosomal segments, especially among related species (Britton-Davidian et al. 1995). Studies on rDNA of *Setaria* species began with Benabdelmouna et al. (2001), who identified signals of 18S-5, 8S-25S and 5S rDNA in chromosomes of *Setaria adhaerens* (Forsskål, 1775) Chiovenda, 1919, *Setaria faberi* Herrmann, 1910, *S. italica*, *S. verticillata* (Linnaeus, 1762) Beauvois, 1812 and *S. viridis*. However, the chromosome pairs with ribosomal DNA signals were not identified, and there are no reports on the location of 45S and 5S rDNA in *S. sphacelata*. In addition, this information was not used in cytotaxonomic and evolutionary approaches.

More detailed cytogenetic comparisons between diploid and polyploid species are important to increase the knowledge and understanding of the relationship between species of the genus. In this context, this study analyzed the karyotype using the location of 45S and 5S rDNA in the chromosomes of *S. sphacelata* (cultivars Nandi and Narok), *S. italica* and *S. viridis*. The results will contribute to understand the chromosomal/genomic organization of this genus and can produce useful information for taxonomic and breeding studies.

Material and methods

We evaluated diploid genotypes of *S. italica* variety yugul and *S. viridis* variety A10.1, from roots collected in Missouri - United States, and tetraploid genotypes of *S. sphacelata*, cultivars Nandy and Narok, from the germplasm bank of forage plants of the Department of Animal Science, Federal University of Lavras, Minas Gerais State, Brazil.

Root tips were collected and pretreated with 3 mM 8-hydroxyquinoline at 0-4 °C for 4 hours, fixed in Carnoy for 20 minutes and subsequently stored in 70% ethanol at -20 °C until use. For the preparation of the slides by the flame-drying technique, the roots were previously digested in a solution of 4% cellulase and 2% pectinase at 37 °C for about 1 hour and 20 minutes.

Mitotic chromosomes were denatured with 70% formamide in 2x SSC at 85 °C for 2 minutes and dehydrated in an increasing alcohol series: 70%; 90% and 100% ethanol at -20 °C for 5 minutes each. The hybridization mixture (50% formamide, 2x SSC (Saline-sodium citrate buffer), 10% dextran sulfate, 50-100 ng labeled probe) was denatured at 95 °C for 8 min. The hybridization took place for at least 16 hours.

The 5S (pTa794) and 45S rDNA (pTa71) probes used came from the genome of *Triticum aestivum*. Probe detection was made with streptavidin antibody conjugated with alexafluor 488 and anti-digoxigenin antibody conjugated with rhodamine (Roche Diagnostics, Indianapolis, IN) after stringent washes with 2x SSC buffer and TNT (Tri-HCl, NaCl and Tween 20). Chromosomes were counterstained with DAPI in antifade mounting medium VectorShield (Vector Laboratories, Burlingame, CA). Images were captured using a CCD (charge-coupled device) camera (Retiga EXi QImaging) coupled to an Olympus BX60 fluorescence microscope and the final contrast made with Photoshop CS5.

At least 10 metaphases were evaluated for each species/cultivar and data on chromosomal morphology were obtained from the five best which presented similar level of chromosome condensation in each species. Measurements were taken with the software Micro Measure (Colorado State University). The parameters used for karyotypic studies were CL (total chromosome length – μm); LA (long arm length – μm); SA (short arm length – μm); RL (relative length – %); AR (arm ratio) and TLHS (total length of the haploid set – μm). The nomenclatures used to describe the chromosome morphology and rDNA position are proposed by Levan et al. (1964) and Roa and Guerra (2012), respectively. The karyotype classification was based on the categories established by Stebbins (1958) and intrachromosomal (A1) and interchromosomal (A2) asymmetry indices were calculated following Zarco (1986).

Results

S. italica presented 2n=18 chromosomes, with karyotype formula 6m+3sm and *S. viridis* presented the same chromosome number, however, the karyotype formula is 9m (Figures 1A and 2A). In *S. italica*, the relative length of the largest and the smallest chromosome pairs corresponds to 15.15% and 7.43%, respectively, and in *S. viridis*, 13.25% and 8.25%, respectively (Table 1).

Satellites were identified in chromosome pair 7 for *S. italica* and pair 5 for *S. viridis*, which were confirmed by chromosome hybridization with 45S rDNA probe by FISH technique. Satellites in both species exhibited extended DNA fibers with length ranging from 0.8 to 2.42 μm in *S. italica*, and 1.14 to 1.75 μm in *S. viridis*. The extent of chromatin overestimated the total length of these chromosome pairs. Signals of 45S

Table 1. Morphometry of chromosomes of *Setaria viridis* and *Setaria italica*: CL (total chromosome length – μm); LA (long arm length – μm); SA (short arm length – μm); RL (relative length – %); AR (arm ratio) and TLHS (total length of the haploid set – μm). Metacentric (m) and submetacentric (sm) chromosomes according to Levan (1964).

S. italica							*S. viridis*						
Pair	**CL**	**LA**	**SA**	**RL**	**AR**	**Class.**	**Pair**	**CL**	**LA**	**SA**	**RL**	**AR**	**Class.**
1	4.89	2.83	2.06	15.15	1.37	m	1	3.52	1.84	1.68	13.25	1.10	m
2	4.21	2.60	1.61	13.04	1.61	m	2	3.27	1.98	1.29	12.31	1.53	m
3	4.06	2.59	1.47	12.58	1.76	sm	3	3.10	1.86	1.24	11.67	1.50	m
4	3.54	2.27	1.27	10.97	1.79	sm	4	2.87	1.76	1.11	10.81	1.59	m
5	3.23	1.97	1.26	10.01	1.56	m	5*	3.99	1.76	2.23	15.02	1.27	m
6	3.01	1.87	1.14	9.32	1.64	m	6	2.64	1.46	1.18	9.94	1.24	m
7*	4.15	2.63	1.52	12.86	1.73	sm	7	2.58	1.47	1.11	9.71	1.32	m
8	2.79	1.59	1.20	8.64	1.33	m	8	2.40	1.45	0.95	9.04	1.53	m
9	2.40	1.40	1.00	7.43	1.40	m	9	2.19	1.19	1.00	8.25	1.19	m
TLHS	32.28						**TLHS**	26.56					

* Chromosome pair with satellite showing extended chromatin.

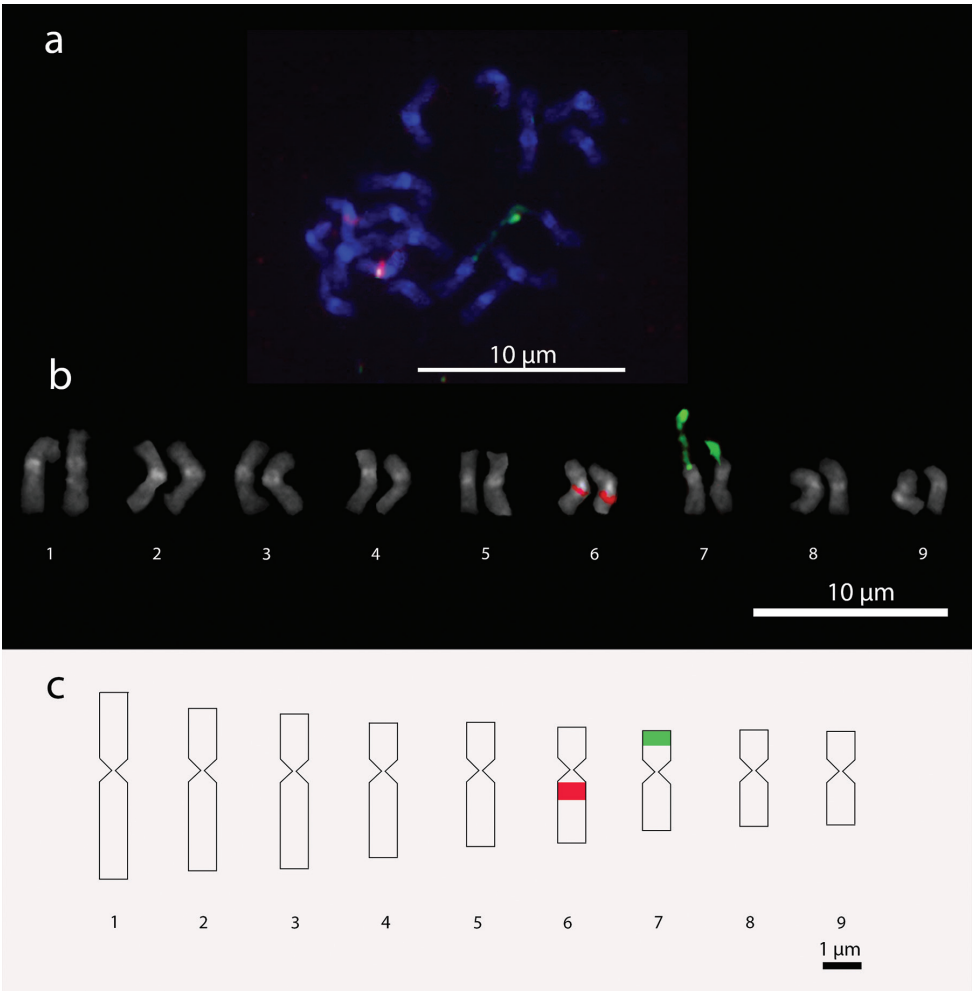

Figure 1. *Setaria italica* chromosomes. **a** Metaphase with 2n=18 chromosomes **b** Karyogram **c** Idiogram. In green, 45S rDNA signals; in red, 5S rDNA signals.

rDNA have an average distance from the centromere of 0.54 µm for *S. italica* and 0.77 µm for *S. viridis*. The presence of two proximal-interstitial 5S rDNA signals were identified in *S. italica* on par 6 with average size of 0.48 µm and average distance of 0.34 µm from the centromere. In *S. viridis*, signals of 5S rDNA were identified in pair 8, with average sized of 0.40 µm and average distance of 0.17 µm from the centromere (Figures 1B and 1C, 2B and 2C).

S. sphacelata, cultivars Narok and Nandi, presented 2n=36 chromosomes (Figures 3A and 4A). The cultivar Narok has karyotype formula 11m+7sm. The largest and smallest chromosome pairs have relative length of 7.03% and 3.59%, respectively (Table 2). Satellites were found in pairs 13 and 16, also confirmed by chromosome hybridization with 45S rDNA probe. The satellite region also showed extended DNA fibers with length ranging

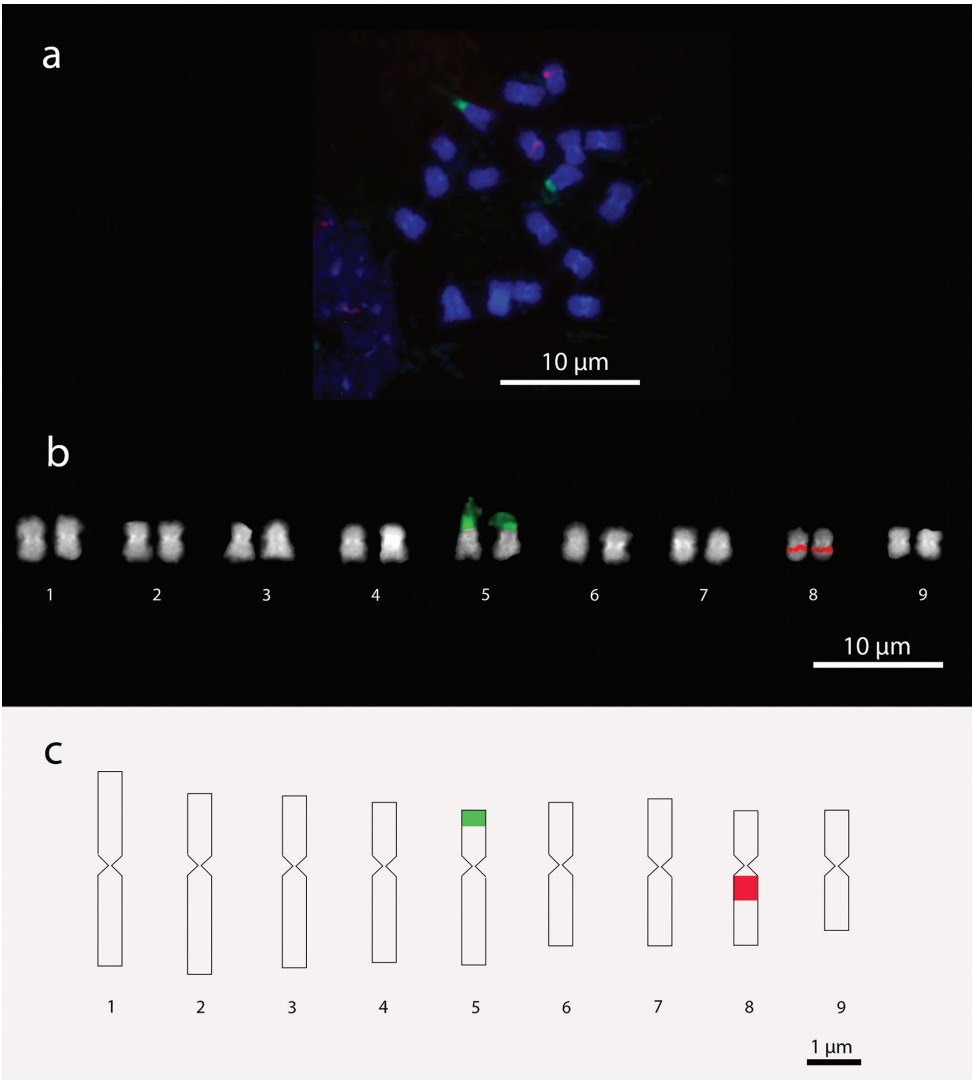

Figure 2. *Setaria viridis* chromosomes. **a** Metaphase with 2n=18 chromosomes **b** Karyogram **c** Idiogram. In green, 45S rDNA signals; in red, 5S rDNA signals.

from 0.9 to 7.71 µm, with an average distance from the centromere of 0.81 and 0.66 µm for pairs 13 and 16, respectively. The FISH technique also located two interstitial-terminal 45S rDNA signals in chromosome pair 7 with average size of 0.50 µm and average distance from the centromere of 1.02 µm. The 5S rDNA signals were located at proximal-interstitial regions in chromosome pairs 8 and 11, with respective sizes of 0.49 and 0.42 µm, with average distance from the centromere of 0.22 and 0.20 µm, respectively (Figures 3B and 3C).

S. sphacelata cultivar (cv.) Nandi presented karyotype formula 16m+2sm. The largest chromosome pair has relative length of 6.71% and the smallest,of 4.35% (Table 2).

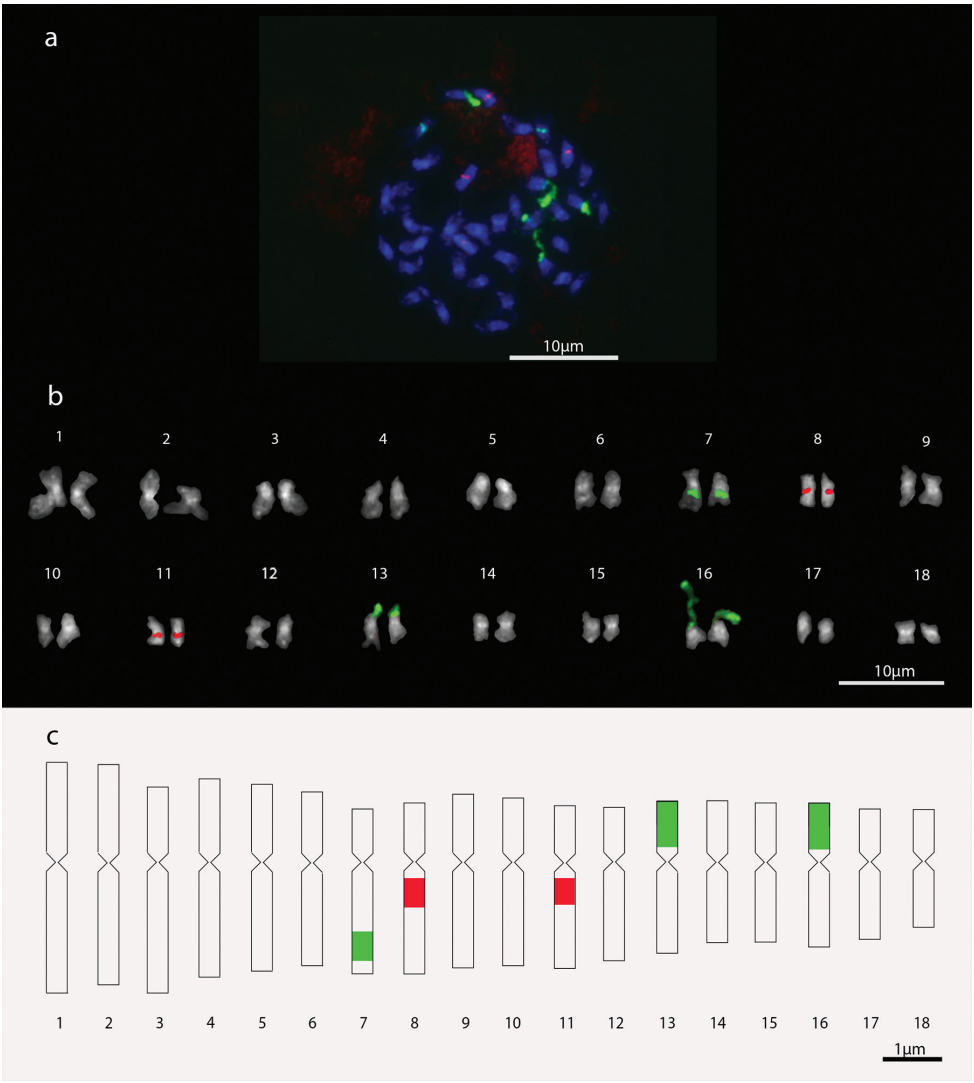

Figure 3. *Setaria sphacelata* cv. Narok chromosomes. **a** Metaphase with 2n=36 chromosomes **b** Karyogram **c** Idiogram. In green, 45S rDNA signals; in red, 5S rDNA signals.

Five rDNA signals were identified for this cultivar. In chromosome pair 1, only one of the chromosomes has a "whole arm" site of 45S rDNA in the short arm, with length of 1.54 µm, at 0.06 µm far from the centromere. In pair 4, 45S rDNA signals were observed in the terminal region with an average size 0.78 µm and average distance of 0.39 µm from the centromeres. The pair 9 showed heteromorphism for the size and position of the 45S rDNA site. One of the chromosomes presented 45S rDNA signal in the terminal region with average size of 0.57 µm and average distance from the centromere of 0.57 µm. In the homologous chromosome, the signal is positioned in

Table 2. Morphometry of chromosomes of *Setaria sphacelata* cvs. Narok and Nandi: CL (total chromosome length – μm); LA (long arm length – μm); SA (short arm length – μm); RL (relative length – %); AR (arm ratio) and TLHS (total length of the haploid set – μm). Metacentric into (m) and submetacentric (sm) chromosomes according to Levan (1964).

***S. sphacelata* cultivar Narok**							***S. sphacelata* cultivar Nandi**						
Pair	**CL**	**LA**	**SA**	**RL**	**AR**	**Class.**	**Pair**	**CL**	**LA**	**SA**	**RL**	**AR**	**Class.**
1	3.92	2.24	1.68	7.03	1.33	m	1**	2.58	1.61	0.97	6.71	1.66	m
2	3.74	2.10	1.64	6.71	1.28	m	2	2.43	1.51	0.92	6.32	1.64	m
3	3.50	2.22	1.28	6.28	1.73	sm	3	2.39	1.41	0.98	6.22	1.44	m
4	3.37	1.97	1.40	6.04	1.41	m	4***	2.33	1.43	0.9	6.06	1.59	m
5	3.16	1.86	1.30	5.67	1.43	m	5	2.31	1.31	1.00	6.01	1.31	m
6	2.98	1.79	1.19	5.34	1.50	m	6	2.3	1.39	0.91	5.98	1.53	m
7	2.95	2.05	0.90	5.29	2.28	sm	7	2.23	1.33	0.9	5.80	1.48	m
8	2.94	1.93	1.01	5.27	1.91	sm	8	2.18	1.38	0.8	5.67	1.73	sm
9	2.93	1.79	1.14	5.25	1.57	m	9****	2.16	1.31	0.85	5.62	1.54	m
10	2.87	1.77	1.10	5.15	1.61	m	10	2.15	1.32	0.83	5.59	1.59	m
11	2.77	1.80	0.97	4.97	1.86	sm	11	2.06	1.18	0.88	5.36	1.34	m
12	2.65	1.71	0.94	4.75	1.82	sm	12	2.05	1.3	0.75	5.33	1.73	sm
13*	4.85	3.32	1.53	8.70	2.17	sm	13	2.04	1.21	0.83	5.31	1.46	m
14	2.42	1.38	1.04	4.34	1.33	m	14	1.98	1.21	0.77	5.15	1.57	m
15	2.38	1.38	1.00	4.27	1.38	m	15	1.93	1.18	0.75	5.02	1.57	m
16*	4.10	2.70	1.40	7.35	1.93	sm	16	1.86	1.06	0.80	4.84	1.33	m
17	2.24	1.33	0.91	4.02	1.46	m	17	1.78	1.01	0.77	4.63	1.31	m
18	2.00	1.12	0.88	3.59	1.27	m	18	1.67	0.97	0.7	4.35	1.39	m
TLHS	55.77						TLHS	38.43					

*Chromosome pair with satellite showing extended chromatin; **Chromosome pair with satellite in hemizygous state; ***Chromosome pair with satellite; ****Heteromorphic chromosome pair for the 45S rDNA site.

the proximal-interstitial region of the short arm, with an average size of 0.46 μm and average distance from the centromere of 0.03 μm. The signals of 5S rDNA were positioned in the proximal, interstitial of pairs 8 and 11, as well as in the cultivar Narok, with respective average measures of 0.43 and 0.49 μm and average distances of 0.06 and 0.05 μm from the centromeres (Figures 4B and 4C).

Indices of intra- (A1) and interchromosomal (A2) asymmetry showed lower values for *S. viridis* and *S. sphacelata* cv. Nandi, respectively. The highest values were found for *S. sphacelata* cv. Narok (Table 3). *S. italica* and *S. sphacelata* cv. Narok had closer indices (Figure 5).

Table 3. Chromosomal asymmetry indices in *Setaria* species, where A1 = intrachromosomal asymmetry, A2 = interchromosomal asymmetry.

	S. italica	***S. viridis***	***S. sphacelata* cv. Narok**	***S. sphacelata* cv. Nandi**
A1	0.36	0.25	0.37	0.33
A2	0.22	0.19	0.23	0.11

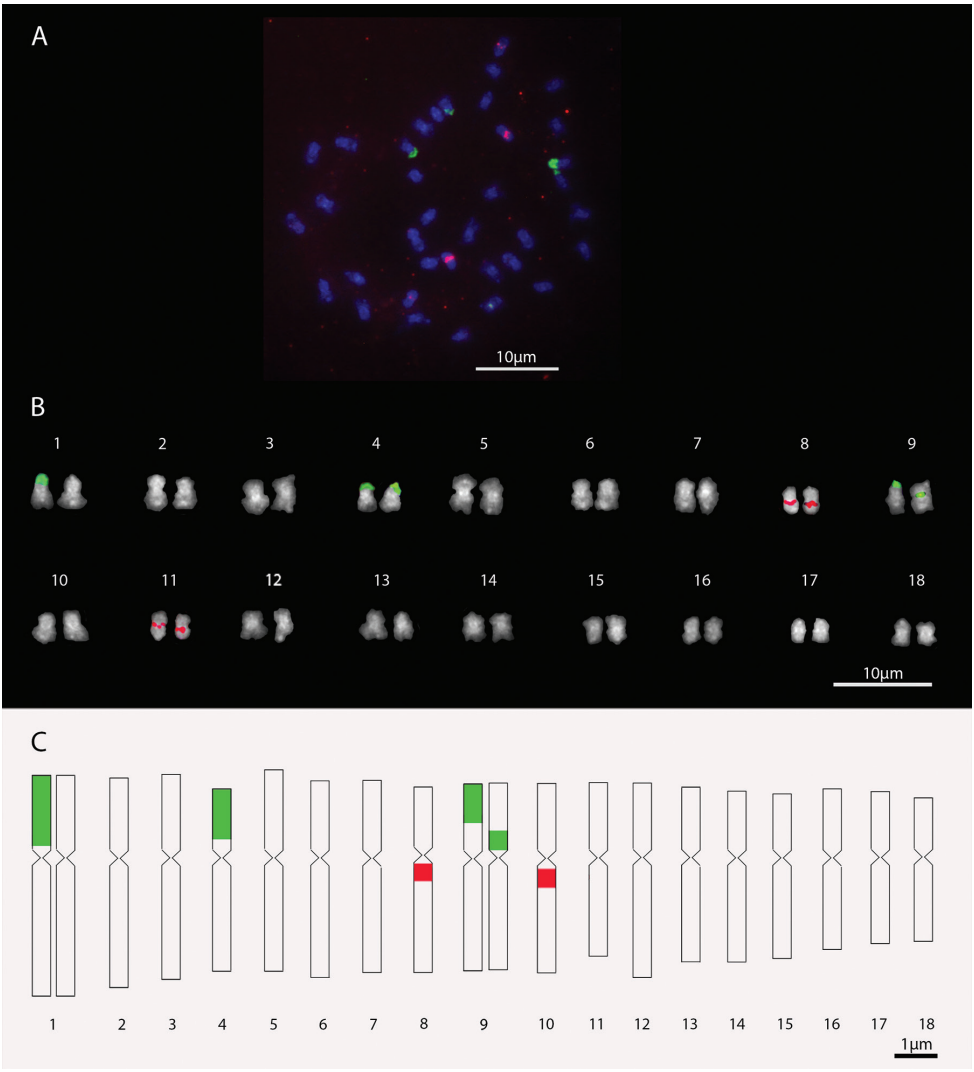

Figure 4. *Setaria sphacelata* cv. Nandi chromosomes. **a** Metaphase with 2n=36 chromosomes **b** Karyogram **c** Idiogram. In green, 45S rDNA signals; in red, 5S rDNA signals.

Discussion

The occurrence of 2n=2x=18 chromosomes for *S. italica* and *S. viridis* corroborates the description of chromosome number previously found by different authors (Sharma and Deepesh 1956; Willweber-Kishimoto 1962; Sivaraman and Ranjekar 1984; Croullebois and Siljak-Yakovlev 1989).

The morphology of chromosomes in *S. italica* coincides with the findings of Croullebois and Siljak-Yakovlev (1989) for the Chinese variety Glutineux rouge, except for pair 2, in which the authors classify it as submetacentric and, in this study, the

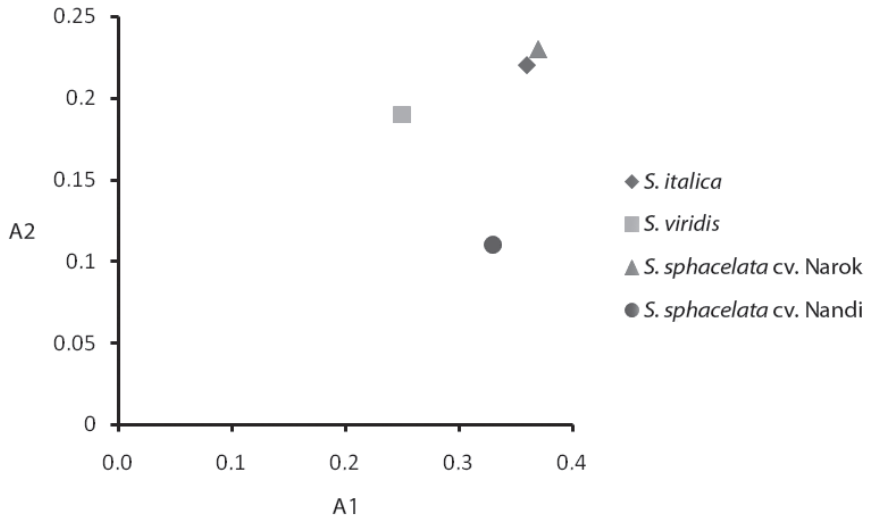

Figure 5. Scatter plot of karyotype asymmetry data of *Setaria* species. A1 = intrachromosomal asymmetry, A2 = interchromosomal asymmetry.

same pair is classified as metacentric. The varieties of *S. italica* studied by Croullebois and Siljak-Yakovlev (1989) showed differences in chromosome morphology, which were attributed to chromosomal rearrangements that have occurred during the domestication of the species, from a wild ancestor of *S. viridis* or two wild types already differentiated from *S. italica*.

In metaphases of *S. italica* studied, the satellites were located on chromosome pair 7. The variation found for the total length of this pair may be due to the late condensation of chromosomes in the terminal region, which was also reported by Croullebois and Siljak-Yakovlev (1989) for the species. The extent of chromatin in the satellite area may also be related to slide preparation with a tendency to promote detachment of the satellite. Croullebois and Siljak-Yakovlev (1989) also observed satellites in chromosome pair 7. The authors also verified, in some metaphases, the presence of a second pair of satellites, apparently located on chromosome pair 6 or 8. The presence of two chromosome pairs with satellite was previously only reported by Sharma and Deepesh (1956) in analysis on plants from Mumbai, India, however, the satellite was located on chromosome pairs 1 and 2. The differences observed for the satellite position can be attributed to the origin of the evaluated genotype, however, the satellite depends on activation/deactivation of the NOR. Therefore this cytogenetic marker is very plastic and variable.

The number of signals of 45S and 5S ribosomal genes found for *S. italica* and *S. viridis* coincides with previous analyses carried out by Benabdelmouna et al. (2001), however, the authors failed to identify in which pairs the signals were present.

The karyotypes of varieties of *S. italica* and *S. viridis* described herein are classified as symmetrical, according to Stebbins (1958), as also observed in varieties Glutineux

rouge, Burganjou of *S. italica* evaluated by Croullebois and Siljak- Yakovlev (1989) using the criteria of Arano and Saito (1980).

According to the asymmetry indices patterns set by Stebbins (1958), the karyotype of *S. italica* was included in the category 1B and *S. viridis* in the category 1A. The species were included in different categories since *S. italica* have some of the chromosomes classified as submetacentric and because *S. viridis* has a higher ratio between the largest and smallest chromosomes, justifying the divergence of these species in relation to the asymmetry indices proposed by Zarco (1986). According to Stebbins (1958), less asymmetric karyotypes are characterized by the predominance of metacentric chromosomes of similar size, typical of species with less specialized and phylogenetically more basal karyotypes. Considering the criteria proposed by Zarco (1986), *S. italica* and *S. viridis* do not have the same tendencies of intra- and interchromosomal asymmetry and *S. italica* was closer to the tetraploid *S. sphacelata* cv. Narok.

S. italica is a species that was domesticated from *S. viridis* in northern China around 6000 BC (Bettinger et al. 2010). These species are apparently very similar and there are no enough characters for taxonomic separation (Kawase and Sakamoto 1984; Wang et al. 1998; Benabdelmouna et al. 2001). The morphological and chromosomal differences probably occurred due to domestication (Harlan and De Wet 1971; Benandelmouna et al. 2001). The presence of all metacentric chromosomes in *S. viridis*, a typical characteristic of basal species, reinforces the hypothesis of ancestry relationship with *S. italica*.

The analysis on karyotype asymmetry, the classification of chromosome pairs 3, 4 and 7 as submetacentric in *S. italica*, the relative length of the largest and the smallest chromosomes different between *S. italica* and *S. viridis* and differences in the position and size of 45S and 5S rDNA signals indicate chromosomal rearrangements and/or amplifications in the diversification process between these species.

Sequence data of the genome of *S. italica* and *S. viridis* done by Bennetzen et al. (2012) showed that transposable elements are abundant, newly activated and non-randomly distributed in the genome of these species. Because of the nature of promoting breaks in chromosomes, acquiring and amplifying genes or fragments of genes and serving as recombination sites (Bennetzen 2005), the transposable elements are likely candidates for participation in macro and micro chromosomal rearrangements (Bennetzen et al. 2012).

Cultivars of *S. sphacelata* presented 2n=4x=36 chromosomes. According to Hacker and Jones (1969), the species has basic chromosome number of x=9, but presents cultivars and varieties with chromosome numbers ranging from 2n=18 to 2n=90.

Details of chromosome morphology for *S. sphacelata* are first described in this paper. Differences between cultivars were found with respect to the size of the largest and smallest chromosome pairs. By comparison, the chromosome pair 3, 7, 11, 13 and 16 differ in relation to the centromere position, while the others were similar. The chromosome pairs 8 and 11, carrying the 5S rDNA signals, are preserved, but differed in the position of the centromere. The 45S rDNA signals also showed variation in size, probably by means of amplification and/or rearrangements, in addition to late condensation of the terminal region of chromosomes.

S. sphacelata cv. Narok and Nandi have symmetrical karyotypes included in categories 2A and 1A of Stebbins (1958), respectively. The cultivar Narok has higher asymmetry rates than those of Nandi, given the presence of a greater number of chromosomes classified as submetacentric, besides having a higher ratio between the largest and smallest chromosomes. According to Stebbins (1971), increased karyotype asymmetry is due to changes in centromere position and the relative size of the chromosomes. In this way, the karyotype of *S. sphacelata* cv. Narok is considered more asymmetric than that of *S. sphacelata* cv. Nandi.

A higher number of rearrangements is usually attributed to species with more specialized karyotypes (Stebbins 1971). Nevertheless, the cultivar Nandi, despite presenting a higher number of metacentric chromosomes, also showed a chromosome pair in a hemizigous state and other heteromorphic pair for 45S rDNA signals indicating rearrangements.

Moreover, in chromosome pair 1, it is possible that one of the homologous chromosome sites has been eliminated after polyploidization. In agreement with Roa and Guerra (2012), in general, recent polyploidization events result in duplication of number of sites, but in comparisons between diploid and polyploid plants of the same genus, there is a clear trend in reducing the number of sites, leading to diploidization. Thus, chromosomal duplication makes the number of copies genes greater than necessary, and the loss of some repeated sequences causes no deleterious effects on plant species (Phillips 1978, Rogers and Bendich 1987, Winterfeld and Roser 2007).

Inactive sites of 45S rDNA are more likely to be polymorphic and eventually be eliminated. The dynamic inactivation and subsequent deletion seems to neutralize the duplication and dispersion of repeated ribosomal genes, leading to the observation of a lower number of sites in the species (Roa and Guerra 2012).

The heteromorphic state found in par 9 for the 45S rDNA signal in *S. sphacelata* cv. Nandi may be due to different events, such as intrachromosomal translocation, transposable elements and inversions. Ribosomal DNA has high potential for intragenomic mobility causing chromosome polymorphisms (Schubert and Wobus 1985). Blocks of ribosomal genes can suddenly change their position without any other change in the other remaining chromosomal marks (Dubcovsky and Dvorak 1995). Nucleolus organizer regions in *Allium* Linnaeus, 1753 species can jump in the genome to apparently non-random sites, however, it is still unclear whether the unequal recombination or transposition are the mechanisms responsible for the mobility of these sites (Schubert and Wobus 1985; Schubert 2007).

The occurrence of chromosomes in hemizygous has already been reported for other grasses, such as *Lolium* Linnaeus, 1753 (Rocha et al. 2015), *Paspalum* Linnaeus, 1759 (Vaio et al. 2005) and *Hordeum* Linnaeus, 1753 (Taketa et al. 2001). The mobility of genes, polymorphism in the original population, reduction in the site size and the deletion of genes are mechanisms for karyotypic evolution that may be involved in the origin of heteromorphism of the rDNA locus (Vaio et al. 2001).

The mobility of rDNA sites caused by transposons has already been confirmed in wheat. EN/Spm transposable elements, for example, have the ability to capture entire genes and to move them to different parts of the genome (Jiang et al. 2004; Lai et al.

2005), furthermore can form clusters, associated or not with rDNA regions, which weaken the chromosome structure causing breakage and subsequent karyotypic remodeling (Raskina et al. 2004).

Another hypothesis for the polymorphism in chromosome pair 9 can be the paracentric inversion in the short arm of one of the homologous chromosomes. The change in NOR position associated with inversion is described by Schubert (2007) as an important rearrangement for karyotype evolution, and had already been reported for *Arabidopsis thaliana* (Linnaeus, 1753) Heynhold, 1842 (Lysak et al. 2006), and hybrids between *Avena insularis* Ladizinsky, 1998 and *Avena murphyi* Ladizinsky, 1971 (Ladizinsky 1999). Confirmation of inversion and analysis of consequences in *S. sphacelata* cv. Nandi should be performed with analysis in meiotic cells at pachytene.

Conclusions

The number and position of the 5S rDNA sites are stable for the species studied.

There is intraspecific and interspecific variation for the number and location of 45S rDNA sites in *Setaria*.

S. sphacelata cultivars can be distinguished by means of karyotype analysis, which revealed chromosomal rearrangements in the evolutionary process.

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RESEARCH ARTICLE

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Whole chromosome painting of B chromosomes of the red-eye tetra *Moenkhausia sanctaefilomenae* (Teleostei, Characidae)

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Abstract

B chromosomes are dispensable genomic elements found in different groups of animals and plants. In the present study, a whole chromosome probe was generated from a specific heterochromatic B chromosome occurring in cells of the characidae fish *Moenkhausia sanctaefilomenae* (Steindachner, 1907). The chromosome painting probes were used in fluorescence *in situ* hybridization (FISH) experiments for the assessment of metaphase chromosomes obtained from individuals from three populations of *M. sanctaefilomenae*. The results revealed that DNA sequences were shared between a specific B chromosome and many chromosomes of the A complement in all populations analyzed, suggesting a possible intra-specific origin of these B chromosomes. However, no hybridization signals were observed in other B chromosomes found in the same individuals, implying a possible independent origin of B chromosome variants in this species. FISH experiments using 18S rDNA probes revealed the presence of non-active ribosomal genes in some B chromosomes and in some chromosomes of the A complement, suggesting that at least two types of B chromosomes had an independent origin. The role of heterochromatic segments and ribosomal sequences in the origin of B chromosomes were discussed.

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Keywords
Chromosomal evolution, Chromosome microdissection, Chromosome painting, Genome organization, Repetitive DNA

Introduction

Supernumerary or B chromosomes are defined as nonhomologous extra genomic elements that actively recombine with chromosomes of the A complement (Jones and Rees 1982, Camacho et al. 2000). They are also characterized by their peculiar evolutionary mode in the genome of carrier species (Jones and Rees 1982, Beukeboom 1994). Additionally, B chromosomes represent dispensable genetic material and can persist in natural populations without being eliminated by natural selection (Carvalho et al. 2008). Studies on the biology of supernumerary chromosomes include analyses of their distribution among species, structure and origin, inheritance, population dynamics and evolution. They further comprise the integration of B chromosomes with chromosomes of the A complement (Camacho et al. 2000, Bugrov et al. 2007). The occurrence of B chromosomes in fish is uncommon and has been described in about 5% of species already karyotyped (Oliveira et al. 2009, Arai 2011).

The red-eye tetra *M. sanctaefilomenae* (Steindachner, 1907) provides a good model for studying B chromosomes in fishes. This species possesses (i) from 0 to 8 B chromosomes (with numbers varying intra- and inter-individually); (ii) B chromosomes with a remarkable polymorphism, morphology and structure (as evidenced by C-banding, with either euchromatic or partially or fully heterochromatic Bs); (iii) putative sex-related B chromosomes (Foresti et al. 1989, Portela-Castro et al. 2001, Hashimoto et al. 2012). However, considering the scarce data about the molecular structure, homology among different B chromosomes and inferences about the origin of *M. sanctaefilomenae*, the aim of this work was to apply physical mapping of ribosomal DNA, microdissection and chromosome painting techniques to address these questions.

Material and methods

We collected 30 individuals (21 males and 9 females) of *M. sanctaefilomenae* from Araquá stream 22°44.83'S and 48°28.5'W (DDM), 19 individuals (10 males and 9 females) from Mané-Teixeira stream 22°45.78'S and 48°15.71'W (DDM) and 6 individuals (3 males and 3 females) from Olaria stream 21°9.18'S and 50°3.06'W (DDM), all tributaries of the Tietê River, São Paulo, Brazil. Vouchers were deposited in the fish collection of Laboratório de Biologia e Genética de Peixes, UNESP, Botucatu (LBP 18986/18987/18988, LBP18983 and LBP18982, respectively). The samples were collected in accordance with the Brazilian environmental protection legislation (collection permission MMA/IBAMA/SISBIO - No 3245). The procedures for sampling, maintenance and analysis of the specimens were performed in compliance with the

Brazilian College of Animal Experimentation (COBEA) and approved by Bioscience Institute/UNESP Ethics Committee on use of animals (CEUA) (protocol 405).

The mitotic chromosomes were obtained from kidney and gill tissues using the technique described by Foresti et al. (1993). Triple fluorochrome staining with CMA_3/DA/DAPI followed the procedures described by Schweizer (1980). For DNA extraction, we used the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. Probes of 18S rDNA were obtained by PCR (Polymerase Chain Reaction) from the total DNA of *M. sanctaefilomenae* from the Araquá stream using the primers 18S F (5'CCG CTT TGG TGA CTC TTG AT 3') and 18S R (5'CCG AGG ACC TCA CTA AAC CA 3') (White et al. 1990).

Microdissection of the B chromosome which are easily identified after GC-specific triple fluorochrome staining (CMA_3/DA/DAPI), was performed using an Eppendorf Transfer Man NK2 micromanipulator coupled to a Zeiss Axiovert 100 microscope. Ten B chromosomes, previously identified as heterochromatic B chromosomes using the CMA_3/DA/DAPI staining protocol, were microdissected and transferred into a microcentrifuge tube containing a DOP-PCR mix solution. The probes, here referred to as MsB, were obtained by PCR (Polymerase Chain Reaction) using a DOP primers according to Telenius et al. (1992) and the technique described by Diniz et al. (2008).

For FISH experiments, the chromosomes were treated according to the procedures described by Pinkel et al. (1986). The probes were labeled by PCR with biotin-16-dUTP (Roche Applied Science) and the signal was detected with avidin-FITC (Roche Applied Science). Or else, they were labeled with Digoxigenin-11-dUTP (Roche Applied Science) and the signal was detected with anti-digoxigenin-rhodamine (Roche Applied Science). The images were captured with a digital camera (Olympus DP70) attached to an Olympus BX61 epifluorescent photomicroscope. Image treatment, including karyotype mounting and optimization of brightness and contrast, was performed using the Adobe Photoshop CS2 program.

Results

Hybridization with the MsB probe on metaphase chromosomes of individuals of the Araquá stream resulted in the complete painting of one B chromosome (Fig. 1a), present in 17% of all specimens analyzed. Additionally, fluorescent signals were observed in the pericentromeric region of 16 chromosome pairs in the A complement. Nevertheless, in some cases a single chromosome of a pair was stained as observed in pair 1 (Fig. 1a). FISH experiments with 18S rDNA probes showed positive signals in pairs 7, 12, 15 and 17 of the A complement and in one B chromosome (Fig. 1a), present in 100% of all specimens analyzed.

Individuals from the Mané Teixeira stream showed hybridization signals in the pericentromeric region of 12 chromosome pairs in the A complement and hybridization signals in only one of the homologous of pairs 15 and 22 with the MsB probe. Signals with the 18S rDNA probe were identified in pairs 7, 10, 14, 15 and 17 (Fig. 1b).

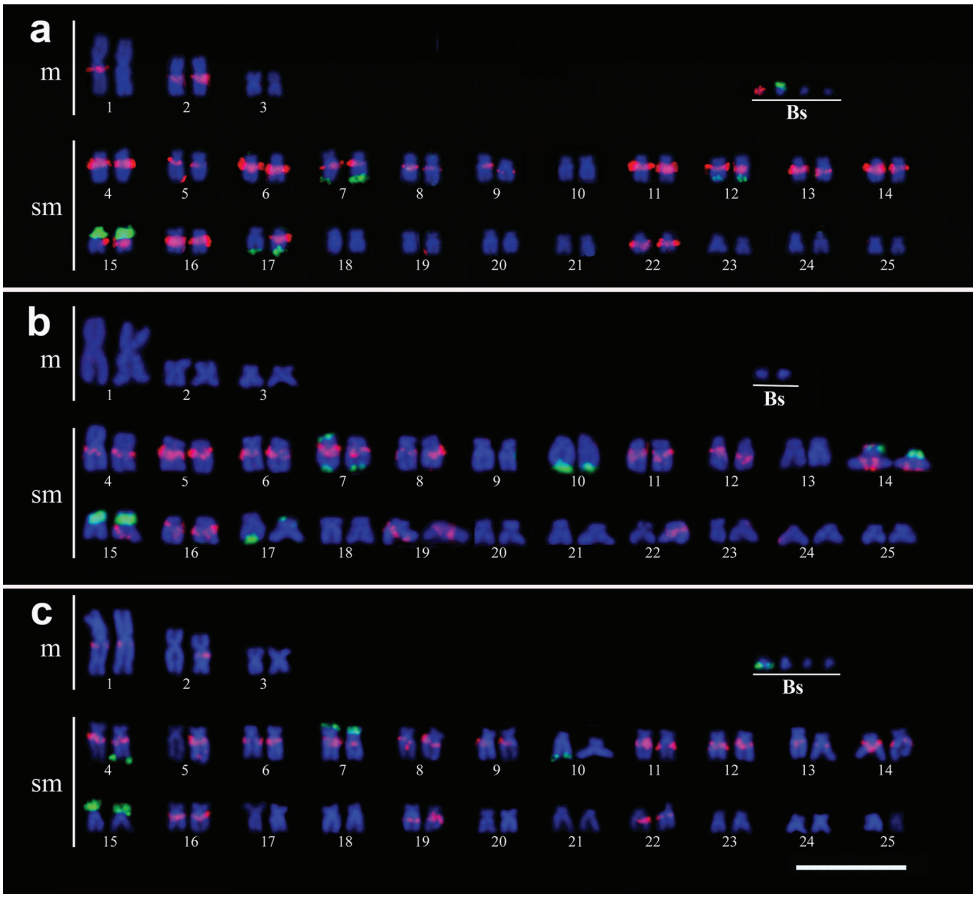

Figure 1. Karyotypes of *Moenkhausia sanctaefilomenae* from the Araquá (**a**), Mané Teixeira (**b**) and Olaria (**c**) streams, arranged from chromosomes after double-FISH. Chromosome painting with MsB-probe (red) and 18S rDNA (green). Bar =10 μm.

In pair 7, one chromosome occured with positive signals in the terminal position of both short and long arms. In pair 17 one chromosome appeared with positive signals in the terminal position of the long arm and the putative homologous with positive signals in the terminal position of the short arm (Fig. 1b). In this population, the MsB and 18S probes did not hybridize with the B chromosomes (Fig. 1b).

Hybridization with the MsB probe in the individuals collected in the Olaria stream showed signals in the pericentromeric region of chromosome pair 15 in the A complement. Notwithstanding, in some cases, a single chromosome of a pair was stained, as observed in pairs 2 and 5 in (Fig. 1c). No hybridization signal was observed in the B chromosomes. FISH with 18S rDNA probes showed positive signals in pairs 4, 7, 10 and 15, but only one of the chromosomes of pairs 4 and 10 were stained (Fig. 1c). B chromosome with 18S sequences were observed in 100% of all specimens analyzed (Fig. 1c).

Discussion

The B chromosomes observed in the populations of *M. sanctaefilomenae* were all very small and characterized as B microchromosomes by Foresti et al. (1989). The occurrence of inter- and intra-individual variation was observed in relation to the number of B chromosomes in the cells of individuals carrying as many as six Bs. Such variation is with accordance with the study by Foresti et al. (1989), who also examined a population of the Tietê River Basin. On the other hand, the individuals from the Paraná River studied by Portela-Castro et al. (2001) showed karyotype differences due to the presence of 0-2 B chromosomes, which were reported to occur only in males. Study by Hashimoto et al. (2012) also showed an inter- and intra-individual variation of the number of B chromosomes in a population from the Batalha River, another tributary of the Tietê River, with individuals carrying up to eight B microchromosomes with a modal number between 2 and 3. All these results showed that the variation in number of B chromosomes in *M. sanctaefilomenae* is very high and that different populations have different modal number of B chromosomes.

Our results of chromosome painting with the DNA probes obtained from the B heterochromatic chromosome from the Araquá Stream population (here referred to as MsB) showed that specific hybridization signals were observed in the pericentromeric region of many chromosomes of the A complement and in only one type of B chromosome. In *M. sanctaefilomenae,* those pericentromeric regions are heterochromatic (Foresti et al. 1989; present study not showed), the hybridization signals found in most chromosomes of the A complement imply that the microdissected B chromosome may have originated from the heterochromatic region of chromosomes of the A complement, as was also shown by Teruel et al. (2009). This finding suggests an intra-specific origin for this B chromosome. Interestingly, putative homologues of A chromosomes inside populations and in different populations have a slightly different pattern of staining with the MsB probe (Fig. 1). This may be caused by small differences involving the MsB sequences in the A chromosomes occur.

The presence of B chromosomes with no signals of hybridization with the specific B chromosome probe pointed to an independent origin of these elements. Alternatively, it is possible to suppose that: (i) in some B chromosomes, the number of the MsB sequences were too small to be detectable by the technique used here; (ii) the loss of some specific chromosome segments during the dynamic process of modification occurred in the independent evolution of different B chromosome lineages; (iii) the B chromosomes that did not stain with the MsB probe may represent B chromosomes derived from different heterochromatic regions; and (iv) the B chromosomes that did not stain with the MsB probe may represent chromosomes recently originated from euchromatic segments.

Sharing of repetitive DNA sequences between B chromosomes and the elements of the A complement is considered a common feature in the chromosomes of different organisms, such as in the mammals *Vulpes vulpes* (Linnaeus, 1758) (Yang et al. 1999) and *Apodemus peninsulae* (Thomas, 1907) (Rubtsov et al. 2004) and in the insects

Podisma sapporensis Shiraki, 1910 (Bugrov et al. 2004), *Podisma kanoi* Storozhenko, 1994 (Bugrov et al. 2007) and *Locusta migratoria* Linnaeus, 1758 (Teruel et al. 2009). Among fish, we also have some reports of sequence sharing between A and B complements, as in the case of *Prochilodus lineatus* (Valenciennes, 1836) in, whose SATHI satellite DNA is shared by both B chromosomes and components of the A set (Jesus et al. 2003; Artoni et al. 2006). However, not all heterochromatic segments in *Prochilodus lineatus,* are composed of SATHI satellite DNA, indicating that other families of repetitive DNA could participate in the structure of the chromosomes in this species. The intra-specific origin of B chromosomes of *Astyanax scabripinnis* (Jenyns, 1842) was also corroborated by Vicari et al. (2011), with the use of chromosome painting.

FISH with 18S probes revealed high variability in the *M. sanctaefilomenae* samples studied here, involving seven chromosome pairs (Fig. 1). However, the number of active NORs is always three: one chromosome pair (pair 15) and a single chromosome of a pair, as observed by Foresti et al. (1989) for this species. These observations are similar with the results obtained for *Triportheus venezuelensis* Malabarba, 2004 by Nirchio et al. (2007), in which the sequences of 18S rDNA were distributed in nine pairs of chromosomes, but the number of chromosomes with active NORs always remained lower. Studies conductd by Daniel et al. (2012) and Silva et al. (2013) detected up to eight sites of 18S rDNA genes in populations of *Astyanax bockmanni* Vari & Castro, 2007, reinforcing the idea that in this species, sites and location of 18S rDNA are usually multiple and variable.

The presence of ribosomal sites in B chromosomes of *M. sanctaefilomenae* suggests that these chromosomes had 18S sequences. Nevertheless, as they are not silver-stained, they may not correspond to active NORs (Foresti et al. 1989, Portela- Castro et al. 2001, Dantas et al. 2007). On the other hand, in fish from the Olaria Stream, ribosomal sites were observed in one B chromosome after silver staining (data not shown here). Mitchell-McGrath and Helgeson (1998), studying hybrids of *Solanum brevidens* Phil. and *Solanum tuberosum* L. and Houben et al. (1997), studying the genus *Brachycome* Cass. suggest that NOR sites are prone to chromosome breakage in plants supporting the hypothesis that B chromosomes could be generated by chromosome fragments coming from these regions. The present results reinforce the hypothesis that chromosome rearrangements involving NOR-bearing chromosomes may be related to the origin of B chromosomes.

According to Lim and Simmons (1994) and Dimitri et al. (1997), the accumulation of repetitive DNA sequences, including transposable elements in specific areas of the chromosomes can render such sites prone to chromosomal rearrangements. Additionally, the molecular structure analysis shows that these B chromosomes may be subject to gene silencing, accumulation of repetitive DNA and also to heterochromatinization processes (Leach et al. 2004). Therefore, the presence of large heterochromatic segments in the A and in some B chromosomes of *M. sanctaefilomenae*, and the reduced number of active ribosomal genes in these chromosomes may corroborate the above hypothesis about the gene silencing due to their close relationship with heterochromatin.

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RESEARCH ARTICLE

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First cytogenetic report in *Cichlasoma sanctifranciscense* Kullander, 1983 (Perciformes, Cichlidae) from northeastern Brazil with inferences on chromosomal evolution of Cichlasomatini

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Abstract

Even though genetic aspects of some cichlids have been widely studied over the last decades, little is known about the genomic structure of Cichlidae when compared to the large number of species in the family. In this paper, the first chromosomal data for *Cichlasoma sanctifranciscense* Kullander, 1983 are presented and discussed based on cytotaxonomic and karyoevolutionary inferences on Cichlasomatini. All individuals shared a diploid number of 2n=48 distributed as 10sm+28st+10a and Ag-NORs on short arms of a submetacentric pair. Heterochromatin was detected at pericentromeric regions of most chromosomes and at terminal sites of a few pairs. GC-rich regions were observed on short arms of two biarmed pairs, including the pair bearing Ag-NORs. Double-FISH with ribosomal probes revealed 18S rDNA clusters coincident with GC-rich regions in two biarmed pairs and 5S rDNA at interstitial location of an acrocentric pair. *C. sanctifranciscense* shares some symplesiomorphic traits described in Cichlidae (2n=48 and pericentromeric C-bands) while other chromosomal features diverge from the common trend reported in Cichlasomatini, such as multiple 18S rDNA sites combined with high FN values. Finally, the present results are useful to support taxonomic identification once species-specific markers have been provided in *C. sanctifranciscense*.

Keywords

Chromosomes, Cichlasomatini, Cytotaxonomy, Ichthyofauna

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Introduction

Cichlids are one of the largest families within vertebrates, including more than 1600 species (Froese and Pauly 2015) and have been regarded as model organisms for evolutionary, genetic and ecological studies. In the Neotropical region, this group is represented exclusively by the monophyletic subfamily Cichlinae that stands out as the third most predominant group of freshwater fish (Reis et al. 2003).

Because of their explosive adaptive radiation (Smith et al. 2008), comparative cytogenetic studies in cichlids are particularly interesting for inferences on chromosomal evolution and cytotaxonomy. Yet, the number of karyotyped species in Cichlidae is small when compared to the remarkable diversity of this family, comprising only about 8% of described species (Feldberg et al. 2003, Valente et al. 2012). Moreover, most karyotypic reports in this fish group include only conventional chromosomal analyses, while detailed information such as mapping of specific genes or regions are restricted to a few species (e.g. Perazzo et al. 2011, Schneider et al. 2012, Schneider et al. 2013).

A compilation of the chromosomal dataset in this family revealed that more than 60% of karyotypes in Cichlidae follow the plesiomorphic condition proposed for the order Perciformes, i.e. 48 chromosomes, mostly acrocentric (Thompson 1979, Feldberg et al. 2003, Poletto et al. 2010, 2012). On the other hand, cichlids with highly divergent karyotypes have been recently reported in this family, like that observed in genus *Symphysodon* Heckel, 1840 whose species are characterized by 2n=60, several biarmed chromosomes and meiotic chains (Gross et al. 2009, 2010).

A relatively high number of cytogenetic reports is available in cichlids of the tribe Cichlasomatini (35 species). These data (see Suppl. material 1: Table S1) indicate a remarkable chromosomal variation (mainly pericentric inversions) that contrasts with the narrow ecomorphological diversity of Cichlasomatini in relation to other tribes like Geophagini and Heroini (López-Fernándes et al. 2013). Such discrepancy between genome organization and variation in external morphology reinforces the potential of cytogenetic data to assess evolutionary trends and speciation processes in this tribe.

Therefore, cytogenetic studies based on distinct banding methodologies and mapping of ribosomal genes were performed in populations of *Cichlasoma sanctifranciscense* Kullander, 1983 along isolated hydrographic basins in northeastern Brazil. Besides increasing the chromosomal data in Cichlidae, these results have proved to be informative to evolutionary and cytotaxonomic inferences in Cichlasomatini.

Material and methods

Twenty-one specimens of *Cichlasoma sanctifranciscense* were collected along three rivers from two large coastal hydrographic basins in Bahia, northeastern Brazil. The sampled rivers were: Contas River (eight males, three females and three juveniles) and Preto do Crisciúma River (two males), both within the Contas River Basin; and Itapicuru-

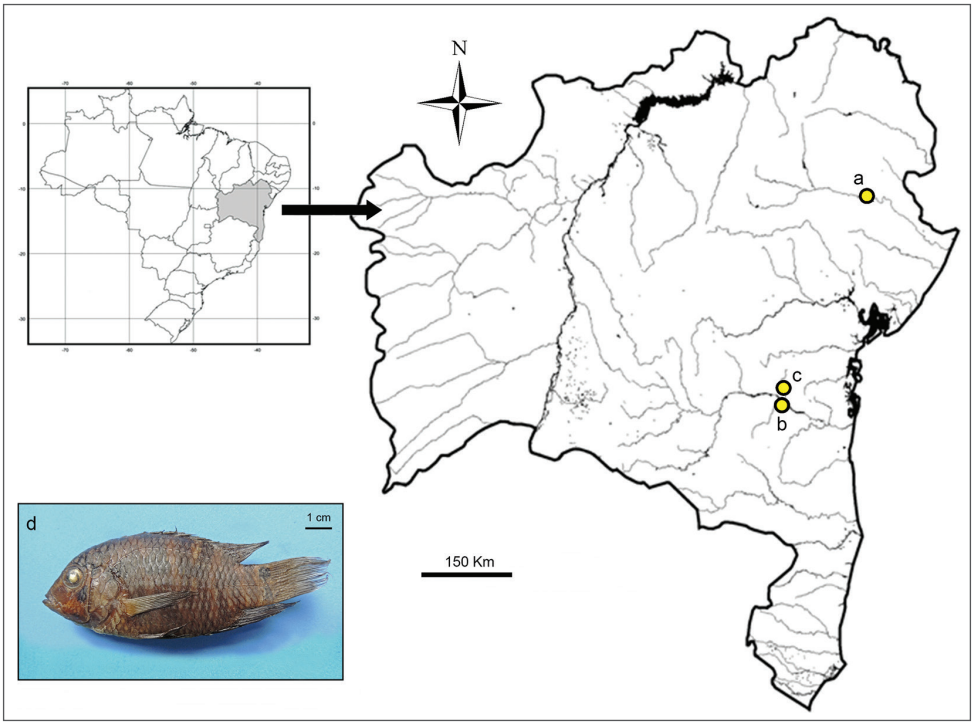

Figure 1. Map of state of Bahia, northeastern Brazil indicating the collection sites in Itapicuru-mirim (**a**), Contas (**b**) and Preto do Crisciúma (**c**) rivers of *Cichlasoma sanctifranciscense* specimens (**d**).

mirim River (four females and one male) in the Itapicuru River basin (Fig. 1). Voucher specimens are deposited in the fish collection from the Zoology Museum at Universidade de Sro Paulo (MZUSP 95173).

Direct metaphase preparations were obtained from kidney cells (Bertollo et al. 1978) after immunostimulation of collected specimens for 48–72 h (Molina et al. 2010). Prior to this procedure, all individuals were euthanized by immersion in tap water at 0–4 °C up to complete interruption of gill movements (Blessing et al. 2010). Chromosomes were stained with 5% Giemsa in phosphate buffer (pH 6.8) for karyotyping, taking into account that metacentric (m), submetacentric (sm) and subtelocentric (st) are biarmed and acrocentric (a) chromosomes are one-armed (Levan et al. 1964).

C-banding (Sumner 1972) was performed to detect heterochromatic regions while silver nitrate staining was carried out to reveal active nucleolus organizer regions (Ag-NORs) as proposed by Howell and Black (1980). Chromosomes were stained with base-specific fluorochromes to detect GC-rich and AT-rich regions by using chromomycin A_3 (CMA_3) and 4'6-diamidino-2-phenylindole (DAPI), respectively, with addition of Distamycin A (DA) as counterstain (Schmid 1980).

Fluorescence *in situ* hybridization using simultaneous 18S and 5S rDNA probes (double-FISH) followed the procedure reported by Pinkel et al. (1986) under high stringency conditions (77%). The 18S rDNA probe from *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka and Galetti 2004) was labeled with 16-dUTP–biotin (Roche) while the 5S rDNA probe obtained from *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti 1999) was labeled with digoxigenin-11-dUTP by nick translation.

The hybridization mix comprised 1 μg of each DNA probe, 10 mg/ml dextran sulfate, 2xSSC, and 50% formamide to a final volume of 30 μl. The mix was dropped onto previously denaturated chromosomes in 70% formamide/2xSSC. Hybridization was carried out overnight at 37 °C in a dark moist chamber. The hybridization signal of 18S and 5S rDNA probes was detected with fluorescein isothiocyanate-avidin conjugate (Sigma-Aldrich®) and anti-digoxigenin-Rhodamine conjugate (Roche®), respectively. Chromosomes were counterstained using DAPI (0.2 mg/mL) in Vectashield Mounting Medium (Vector®) and slides were stored in a dark chamber up to analyses.

All metaphases were photographed by using an Olympus BX-51 epifluorescence microscope equipped with digital camera. Chromosomal images were digitalized in the software IMAGE-PRO PLUS® 6.2.

Results

All specimens of *C. sanctifranciscense* shared similar chromosomal features independently of collection sites or hydrographic basins. Both males and females presented a modal diploid number of 2n=48 with a karyotype formula of 10sm+28st+10a and a fundamental arm number of FN=86 (Fig. 2a). Heterochromatin segments were invariably more conspicuous in the pericentromeric region, even though some terminal C-bands could be observed at short and long arms of a few chromosomal pairs (Fig. 2b). Active NORs, as revealed by silver nitrate staining, were observed on short arms of a submetacentric pair (equivalent to pair 1), indicating a single active NOR system (Fig. 2c).

On the other hand, GC-rich regions, i.e. repetitive sequences positively stained by CMA_3 and negatively stained by DAPI, were identified at terminal regions on short arms of four chromosomes, including the sm pair bearing active NORs and a st pair (Fig. 2d). Unfortunately, this additional st pair could not be precisely defined because of the subtle size differences among chromosomes, but it was putatively equivalent to pair 6. Similarly to CMA_3 staining, double-FISH revealed two pairs bearing 18S rDNA clusters in *C. sanctifranciscense*, coincident with Ag-NORs in the first sm pair and another on short arms of a st pair (probably the 6th pair), thereby characterizing a multiple NOR system in this species (Fig. 2e).

Furthermore, the simultaneous hybridization of 18S and 5S rDNA probes showed that 5S rRNA genes are non-syntenic to NORs, occupying the interstitial region of two large acrocentric chromosomes (probably pair 20) (Fig. 2e).

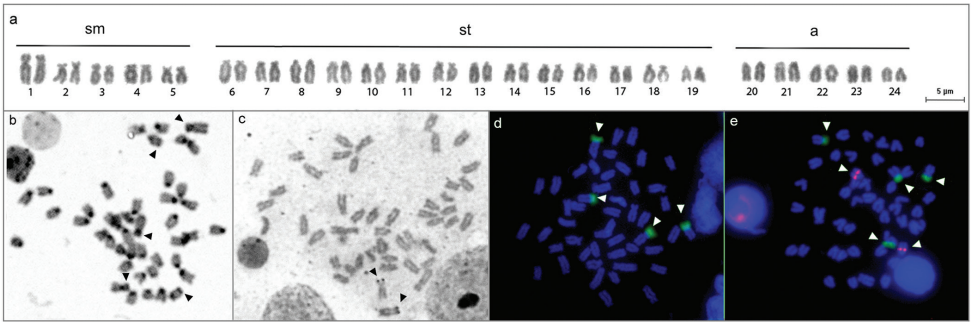

Figure 2. Giemsa-stained karyotype (**a**) and metaphases of *Cichlasoma sanctifranciscense* after C-banding highlighting some non-pericentromeric heterochromatic segments (**b**), silver nitrate staining with single Ag-NORs (**c**), base-specific fluorochrome staining with four CMA_3^+ sites (**d**) and FISH with 18S rDNA (green) and 5S rDNA (pink) probes (**e**), as indicated by arrows.

Discussion

The modal number (2n=48) in *C. sanctifranciscense* follows the plesiomorphic pattern reported in the majority of studied cichlids (Feldberg et al. 2003), suggesting a conservative chromosomal evolution in relation to diploid values (Affonso and Galetti 2005). On the other hand, the high number of biarmed chromosomes in spite of the predominance of 2n=48 in Cichlasomatini (Suppl. material 1: Table S1) reveals that pericentric inversions have played a major role in the cytogenetic diversification of this tribe. Indeed, Cichlasomatini is characterized by a remarkable variation in arm number, even though chromosomal condensation and author's criteria on chromosome morphology might lead to some bias in karyotype formulae differences (Bitencourt et al. 2012). Moreover, some representatives in Cichlasomatini diverge from the general trend observed in most cichlids and Perciformes in general, since some cases of centric fusions or fissions have been described, determining diploid values lower or higher than 48, respectively (Roncati et al. 2007, Schneider et al. 2012, Hodaňová et al. 2014 among others).

Another chromosomal peculiarity of *C. sanctifranciscense* refers to 18S rDNA cistrons, since multiple sites were observed by FISH (Fig. 2e). With a few exceptions, cichlids are characterized by a single NOR-bearing pair, usually the largest one (Feldberg et al. 2003).

It should be pointed out that most cytogenetic reports in cichlids describe only silver-stained NORs (e.g. Molina et al. 2014), thereby hindering the actual number of ribosomal cistrons when inactive rDNA regions are present. On the other hand, the hybridization *in situ* with ribosomal probes allows detection of different patterns of NOR distribution in some cichlids (Poletto et al. 2010, Schneider et al. 2012). Similarly, the number of 18S rDNA in *C. sanctifranciscense* after FISH was higher than that observed by conventional silver nitrate staining (Ag-NORs) (Fig. 2). Multiple NORs have also been detected in other Cichlasomatini like *Cichlasoma amazonarum* Kullander, 1983 (Salgado et al. 1995) as well as *Aequidens* C. H. Eigenmann & W. L.

Bray, 1894 and *Laetacara* Heckel, 1840 (Poletto et al. 2010). This unusual 18S rDNA distribution places this tribe as a divergent group within Cichlidae (Gornung 2013) and further studies using, for instance, mapping of retrotransposons interspersed to NORs might elucidate the dispersal mode of ribosomal cistrons.

Furthermore, the $CMA_3^+/DAPI^-$ signals observed in *C. sanctifranciscense* were coincident to 18S rDNA sites, reinforcing that NORs in fishes are usually associated with GC-rich heterochromatin (Verma et al. 2011). In the present study, the base-specific fluorochrome staining was more precise than Ag-NOR to detected 18S rRNA genes. This is an atypical situation in fish and raises the question whether the additional NORs on pair 6 (Fig. 2d) correspond to intact ribosomal cistrons or pseudogenes (Affonso and Galetti 2005).

Differently from 18S cistrons, the 5S rDNA seems to be highly conserved in Cichlidae being primarily located at interstitial region of a single chromosomal pair and non-syntenic to NORs (Gross et al. 2010). The same pattern is described for *C. santifranciscense*, indicating a basal condition for most fish groups (Martins and Galetti 1999). A putative explanation for the uniformity in both number and location of 5S rRNA genes is the lack of association of these cistrons with heterochromatin observed in most species (Poletto et al. 2010), including the species herein analyzed.

In addition to cytogenetic results, this is the first report about the presence of *C. sanctifranciscense* in the Contas River and Itapicuru River basins. Initially, this species was described as endemic to the São Francisco River basin but further studies reported populations of this species in other basins such as Parnaíba, Capivara (Kullander 2003), Tocantins (Lima and Caires 2011) and Recôncavo Sul (Burger et al. 2011). The natural occurrence of *Cichlasoma sanctifranciscense* in other coastal and isolated drainages such as those herein sampled might reflect several headwater captures during evolutionary history of each basin. This process can be caused by vicariant events such as geophysical uplift, landslide followed by isolation of streams or watershed erosion (Albert and Crampton 2010). Moreover, endemic tropical fish species to large riverine systems such as São Francisco River basin should be interpreted with caution since the ichthyofauna composition of smaller and isolated basins in northeastern Brazil remain poorly studied.

In conclusion, we provide the first cytogenetic report in *Cichlasoma sanctifranciscense,* adding new data about the trends of chromosomal evolution of Cichlidae. The present results are also useful to cytotaxonomic studies since peculiar species-specific cytogenetic features combined with absence of interpopulation differences are described. Based on the available karyotypic data in Cichlasomatini, which includes structural and numerical rearrangements as well as dynamic organization of ribosomal cistrons, this tribe can be characterized by high chromosomal evolutionary rates. This evidence, as corroborated by recent reports (Hodaňová et al. 2014) challenges the traditional view that cichlids fish are cytogenetically conserved. Finally, further investigations should be carried out to determine the reason(s) why additional 18S rDNA clusters remain silenced in *C. sanctifranciscense*.

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Supplementary material 1

Table S1. Cytogenetic data in Cichlasomatini

Authors: Leandro A. Argôlo, Paulo Roberto Antunes de Mello Affonso

Data type: **cytogenetic data**

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SHORT COMMUNICATIONS

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The blue butterfly *Polyommatus* (*Plebicula*) *atlanticus* (Lepidoptera, Lycaenidae) holds the record of the highest number of chromosomes in the non-polyploid eukaryotic organisms

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Abstract

The blue butterfly species *Polyommatus* (*Plebicula*) *atlanticus* (Elwes, 1906) (Lepidoptera, Lycaenidae) is known to have a very high haploid number of chromosomes (n= *circa* 223). However, this approximate count made by Hugo de Lesse 45 years ago was based on analysis of a single meiotic I metaphase plate, not confirmed by study of diploid chromosome set and not documented by microphotographs. Here I demonstrate that (1) *P. atlanticus* is a diploid (non-polyploid) species, (2) its meiotic I chromosome complement includes at least 224-226 countable chromosome bodies, and (3) all (or nearly all) chromosome elements in meiotic I karyotype are represented by bivalents. I also provide the first data on the diploid karyotype and estimate the diploid chromosome number as 2n=ca448-452. Thus, *P. atlanticus* is confirmed to possess the highest chromosome number among all the non-polyploid eukaryotic organisms.

Keywords

Acipenser, *Amoeba proteus*, *Astacus*, *Aulacantha scolymantha*, chromosome number, karyotype evolution, linkage group, Lycaenidae, *Ophioglossum*, *Pacifastacus*, *Plebicula*, *Polyommatus*, vizcacha rat

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Introduction

Trends and mechanisms of chromosome number and chromosome structure changes are currently a matter of a sharp discussion (Qumslyeh 1994, Imai et al. 2002, Eichler and Sankoff 2003, Schubert 2007, Lukhtanov et al. 2005, 2011, 2015a, Vila et al. 2010, Dincă et al. 2011, Bureš and Zedek 2014, Fleischmann et al. 2014, Lukhtanov 2014, Vershinina et al. 2015). These changes are important in evolution of eukaryotic organisms since they can trigger speciation via hybrid-sterility or/and via suppressed-recombination mechanisms (Faria and Navarro 2010). Fixation of these changes plays a serious role in maintaining postzygotic isolation between well-established species and protects hybridizing lineages from merging (Kandul et al. 2007). Change of chromosome number results in change of linkage groups and thus affects rate of meiotic recombination (Dumont and Payseur 2011).

Comparative analysis of chromosomal data is a promising way for understanding the patterns of karyotype evolution (Vershinina and Lukhtanov 2013), and this analysis requires accurate and precise data on chromosome complements of species under study. The blue butterfly *Polyommatus* (*Plebicula*) *atlanticus* (Elwes, 1906) is mentioned in many publications devoted to chromosome number evolution since it is supposed to possess the highest chromosome number (n= *circa* 223) among all the non-polyploid metazoan animals (e.g. White 1973, Imai et al. 2002, Bureš and Zedek 2014). However, this approximate count made by Hugo de Lesse 45 years ago was based on analysis of a single meiotic I metaphase plate, not confirmed by studies of diploid chromosome set and not documented by microphotographs (de Lesse 1970).

The aim of this study is cytogenetic reinvestigation and documentation of *P. atlanticus* karyotype with a special consideration of diploid chromosome set of this species.

Material and methods

The studied species is often mentioned in the literature as a member of the genus *Lysandra* Hemming, 1933 (e.g. de Lesse 1970, White 1973). However, according to the last revision of the tribe Polyommatina, it should be transferred to the genus *Polyommatus* Latreille, 1804 (Talavera et al. 2013a). The adult male samples used for chromosomal analysis (NK02A032, NK02A033 and NK02A035) were collected in Morocco (Atlas range, Col du Zad pass, 2200 m alt., 27 June 2002) by Roger Vila, Santiago Ramirez and Nikolai Kandul. The methods of chromosomal analysis were described previously (Lukhtanov and Dantchenko 2002, Lukhtanov et al. 2008, 2014, Vershinina and Lukhtanov 2010, Talavera et al. 2013b, Przybyłowicz et al. 2014). Haploid (n) chromosome numbers were analyzed in meiotic I (MI) and meiotic II (MII) cells. Diploid (2n) chromosome numbers were analyzed in asynaptic meiotic cells that can be observed in so called atypical meiosis (see Lorković 1990 for more details on atypical meiosis in Lepidoptera).

Results

The haploid chromosome number n=ca 224–226 was found in MI cells of three studied individuals (Fig. 1a, b). This count was based on analysis of 12 selected MI plates with best quality of chromosome spreading. The meiotic karyotype included one large bivalent, one medium bivalent and 222–224 small chromosome bodies. Multiple MII cells were also observed. The MII cells demonstrated one large and one medium chromosome and multiple dot-like elements, however the precise count of these elements was impossible. The diploid chromosome set was observed in male atypical (asynaptic) meiosis (Fig. 1c, d) in three studied individuals (20 cells were analysed). At this stage at least 434 chromosome entities could be observed: one pair of large chromosomes, one pair of medium chromosomes and at least 430 (most likely more) very small, dot-like chromosomes. Combination of chromosome number count at MI and diploid stages results in conclusion that all (or nearly all) chromosome elements in MI karyotype are represented by bivalents. This assumption results in diploid chromosome number estimation of 2n=ca 448–452.

Discussion

Previously, the chromosome number was estimated in *P. atlanticus* as n=ca217-223 (de Lesse 1970). This number has later been interpreted as 2n=446 (e.g. see Bureš and Zedek 2014). However, interpretation of all chromosome bodies visible at MI stage as bivalents should be considered with caution. As it was mentioned by White (1973), "there seems to be no means of distinguishing between univalents, bivalents and multivalents in lepidopteran spermatogenesis – they all look like small spheres or isodiametric bodies in which no structure is observable". For example, multiple B-chromosomes (which can be often represented by univalents in meiosis) can sometimes accumulate through processes of mitotic or meiotic drive (Jones 2008). Therefore, I believe that analysis of diploid karyotype is indispensable prerequisite for inferring the diploid chromosome number. In my research the combination of chromosome number counts at MI and diploid stages results in conclusion that all (or nearly all) chromosome elements in MI karyotype are represented by bivalents. This assumption leads to conclusion that diploid chromosome number can be estimated in *P. atlanticus* as 2n=ca 448–452, and the haploid number can be estimated as n=ca 224–226.

In eukaryotic organisms the highest number of chromosomes has been so far reported in radiolarian species, e.g. in *Aulacantha scolymantha* Haeckel, 1862 (Cercozoa, Aulacanthidae) there are more than 2000 chromosomes (Lecher 1978). This high number is an output of polyploidization (Lecher 1978, Parfrey et al. 2008), which includes 7 or 8 cycles of endomitosis resulting in each chromosome represented by 128 or 256 copies (Lecher 1978).

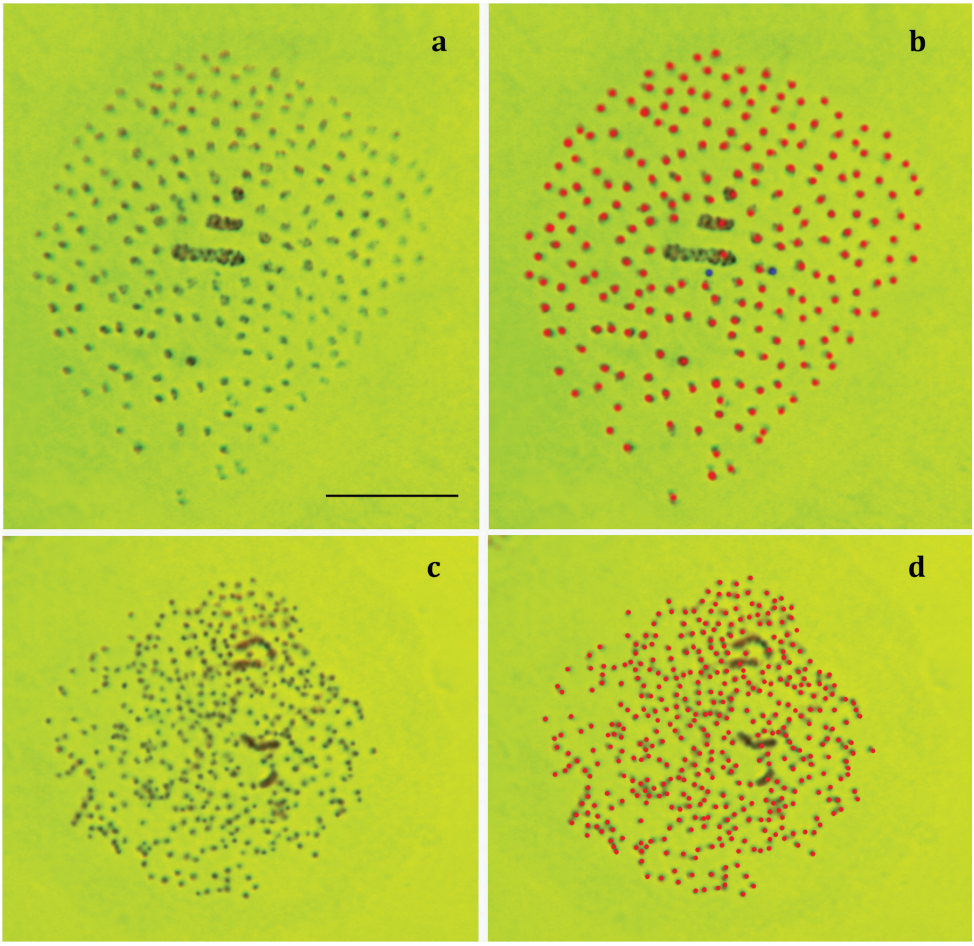

Figure 1. Male karyotype of *Polyommatus* (*Plebicula*) *atlanticus*, sample NK02A032. **a** MI plate **b** chromosome count in MI plate: red dots indicate distinct separate entities, blue dots indicate doubtful entities, n=224 red dots + 2 blue dots **c** diploid chromosome set observed in male asynaptic meiosis **d** chromosome number count in diploid chromosome set; at least 434 entities can be distinguished. Bar = 10 μm.

500 chromosomes were reported for asexual lobose amoebae, *Amoeba proteus* (Pallas, 1766) (Amoebozoa, Amoebidae) (Parfrey et al. 2008). This high number is also considered to be polyploid although the questions about the precise number of chromosomes and the ploidy level are still unanswered despite the fact that cytology of this well-known species has been under study for about 200 years (Podlipaeva et al. 2013).

Very high chromosome numbers are known in some plants, e.g. in ferns of the genus *Ophioglossum* Linnaeus, 1753 (Pteridophyta, Ophioglossaceae) n=120–720 (Shinohara et al. 2013). However, this genus is also characterized by a high degree of polyploidization with x=120 as a basic chromosome number and with the highest n=720 in hexaploid species *Ophioglossum reticulatum* Linnaeus, 1753 (Khandelwal 1990, Barker 2013, Shinohara et al. 2013).

In vertebrate animals the highest chromosome number (372 elements in mitotic cell divisions) is known in sturgeon *Acipenser brevirostrum* Lesueur, 1818 (Acipenseriformes, Acipenseridae) (Kim et al. 2005), however this species is hexaploid one, too (Kim et al. 2005). In mammals the highest chromosome number 2n=102 is found in vizcacha rat *Tympanoctomys barrerae* (B. Lawrence, 1941) (Rodentia, Octodontidae) (Suárez-Villota et al. 2012).

According to White (1973), the highest haploid chromosome number recorded in invertebrate animals (except for *P. atlanticus*) is n=191 in the butterfly *Polyommatus nivescens* (Keferstein, 1851) (Lepidoptera, Lycaenidae) (de Lesse 1970, White 1973). The next highest haploid numbers were reported in crayfish, *Pacifastacus leniusculus trowbridgii* (Stimpson, 1857) (Crustacea, Astacidae) (n=188, Niiyama 1962) and *Astacus leptodactylus* (Eschscholtz, 1823) (Crustacea, Astacidae) (n=184, Silver and Tsukersis 1964). The last two counts were even erroneously cited as the records for the highest chromosome numbers in the animal kingdom (Fetzner and Crandall 2002). However, the numbers in crayfish are, first, lower than the numbers discovered in the blue butterflies. Second, they were disputed in the more recent publications (e.g. n=93 was mentioned in *P. l. trowbridgii*, Murofushi 1999, Imai et al. 2002 and n=90 was mentioned in *A. leptodactylus*, Mlinarec et al. 2011). All these haploid numbers are essentially lower than numbers found in *P. atlanticus*.

The data obtained indicate that *P. atlanticus* is a diploid (not polyploid) species since it possesses double (not multiple) number of chromosomes that can be individually recognized: one pair of large and one pair of medium chromosomes. Thus, *P. atlanticus* is confirmed to have the highest chromosome number among all the non-polyploid eukaryotic organisms.

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RESEARCH ARTICLE

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Analysis of meiotic chromosome structure and behavior in Robertsonian heterozygotes of *Ellobius tancrei* (Rodentia, Cricetidae): a case of monobrachial homology

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Abstract

Synaptonemal complex (SC) chains were revealed in semisterile intraspecific F1 hybrids of *Ellobius tancrei* Blasius, 1884 (2n = 49, NF=56 and 2n=50, NF=56), heterozygous for Robertsonian (Rb) translocations. Chains were formed by Rb submetacentrics with monobrachial homology. Chromosome synapsis in spermatocytes of these hybrids was disturbed, apparently because of the problematic release of the chromosomes from the SC chains. These hybrids suffer from low fertility, and our data support the opinion that this is because a formation of Rb metacentrics with monobrachial homology within different races of the same species might be an initial event for the divergence of chromosomal forms.

Keywords

Meiosis, synaptonemal complex, SC multivalent, hybrid, fertility

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Introduction

Many authors have described the significant impact of chromosomal rearrangements as reproductive barriers in the speciation process (White 1945, Matthey 1951, 1953, Capanna 1982, Baker and Bickham 1986, Sites and Moritz 1987, King 1987, 1993, Vorontsov 1989, Nunes et al. 2011, etc.). W. Robertson first described centric fusions, which join two acrocentrics into one metacentric chromosome, in grasshoppers (Robertson 1916). Named Robertsonian (Rb) translocations, they are widespread among animals, including mammals, and might result in chromosome polymorphism and/or species diversification (Moses et al. 1979, Lyapunova et al. 1980, Searle et al. 1991, Stanyon et al. 2002, Volobouev et al. 2007, and others).

Heterozygosity for the Rb translocations may seriously affect the segregation of chromosomes during meiosis. In heterozygotes, the first division of meiosis will take place without complications only if both acrocentrics, homologous to Rb metacentric arms, move into one pole, whereas the metacentric moves to the opposite pole of the nucleus. Otherwise, if the Rb metacentric and one of the homologous acrocentrics go to the same pole of the nucleus, an inadequate segregation of chromosomal arms will take place and aneuploid gametes will develop (Cappanna et al. 1976, Demin et al. 1983, Redi et al. 1985, Britton-Davidian et al. 1990, Bogdanov and Kolomiets 2007).

A study of the structure and behavior of the synaptonemal complex (SC) is crucial for understanding chromosome synapsis, particularly in heterozygous animals. SC is a skeleton of meiotic bivalents, which form between homologous chromosomes in prophase I of meiosis. The SC analysis allows us to trace all details of the formation of its axial elements, from the beginning of pairing and synapsis at the zygotene, especially synapsis and its correction in heterozygotes for chromosome rearrangements at the pachytene and desynapsis dynamics at diplotene.

The eastern mole vole *Ellobius tancrei* (Cricetidae, Rodentia) is an interesting example of the wide variability of chromosome numbers (from 2n = 30 to 2n = 54 with a stable NF = 56) (Vorontsov and Radzhabli 1967, Lyapunova et al. 1980, 2010, Bakloushinskaya et al. 2013). Unlike *Mus musculus domesticus* Schwarz & Schwarz, 1943 (Capanna et al. 1976, Pialek et al. 2005) and *Sorex araneus* Linnaeus, 1758 (Searle et al. 1991, Wojcik et al. 2003), in which the variability due to Rb translocations is observed over the entire species range, for the eastern mole vole, such an area is limited to a ~150 km territory in the Pamiro-Alay mountains.

Applying comparative chromosome painting (Zoo-FISH) helped to prove the occurrence of non-homologous Rb translocations in different populations of *E. tancrei* (Bakloushinskaya et al. 2010). To evaluate the role of such chromosomal aberrations in the formation of reproductive barriers between intraspecific forms, we investigated the meiotic prophase I of hybrids that cross chromosomal forms with monobrachial homology (MBH) of Rb chromosomes (2n=48 and 2n=50; 2n=50 and 2n=50).

Materials and methods

Animals

The specimens studied by us were obtained from laboratory colonies. Hybrid animals were bred in the laboratory from crosses between animals, homozygous for the diagnostic fusions. Animals were kept under standard conditions with free access to food at the facility of the Koltzov Institute of Developmental Biology RAS. Animals were treated according established international protocols, such as the Guidelines for Humane Endpoints for Animals Used in Biomedical Research, and Regulations for Laboratory Practice in Russian Federation, and under the supervision of the Ethics Committee for Animal Research of the Koltzov Institute of Developmental Biology.

As a control we study SCs of two adult males with 2n=54, obtained from two points in Tajikistan: near Miskinobod 38° 39.78' N; 69° 33.29' E, 1780 m above sea level, and Panchkotan valley, the left bank of the Sorbo River 38° 45.27' N; 69° 17.6' E, 1265 m above sea level. Parental forms for F1 hybrids originated from the right bank of the Surkhob River, close to the airport Garm, 39° 0.28' N; 70° 17.77' E, 1310 m above sea level (2n=48), and from the opposite bank of the Surkhob River near the Voydara settlement 38° 58.9' N; 70° 14.71' E, 1440 m above sea level (2n = 50). Eight F1 hybrids (2n = 49), five newborn females and three adult males (not younger than 1 year), were investigated. For the second intraspecific crossing we use animals from Voidara (2n=50) and from the Varzob Valley, near the Khodzha-Obi-Garm settlement 38° 53.53' N; 68° 46.52' E, 2020 m above sea level (2n = 50). Five adult males of F1 hybrids (2n = 50) were investigated.

Laboratory analysis

Chromosomes from bone marrow (Ford and Hamerton 1956) were prepared from all animals; tissues of four specimens of all parental forms were used for tissue culturing. Fibroblast cell lines were prepared as previously described (Sitnikova et al. 2007). Full sets of paints derived from flow-sorted chromosomes of the field vole *Microtus agrestis* Linnaeus, 1761 (Sitnikova et al. 2007) were used. FISH was performed according to previously published protocols (Yang et al. 1999). G-banding was carried out for all metaphase chromosomes prior to FISH using trypsin treatments (Seabright 1971).

The suspensions of oocytes and spermatocytes were prepared by the method described by Kolomiets et al. (2010), and spreads were prepared and fixed using the technique of Navarro et al. (1981) with some modifications. Slides coated with poly-L-lysine were used for immunostaining and slides coated with plastic Falcon for electron microscopic study.

The slides were stained with 50% $AgNO_3$ in a humid chamber at 56 °C for 3 hours after which they were washed four times in distilled water and air dried. The stained slides were observed by light microscope and suitably spread cells were selected. Plastic circles were cut out with a diamond tap and transferred onto grids. The slides were then examined under a JEM 100B electron microscope.

The slides were washed in PBS. Whole mount SCs were blocked with HB (holding buffer: PBS, 0.3% BSA, 0.005% Triton X-100). The slides were incubated overnight at 4 °C with rabbit polyclonal antibodies against the human lateral element protein SCP3 (Abcam, 15093Ab, UK Cambridge, UK) diluted to a concentration of 1:200 in ADB (Antibody Dilution Buffer: PBS, 3% BSA, 0.05% Triton X-100), human anti-centromere antibodies ACA, 1:200 (Antibody Incorporated, California, USA), and mouse monoclonal antibodies to human mismatch repair protein MLH1, 1:50 (Abcam, Cambridge, UK). The slides were washed in PBS and incubated with goat anti-rabbit Alexa Fluore 488 conjugated antibodies (1:800, Abcam, Cambridge, UK) and goat anti-human Alexa Fluore 546 conjugated antibodies (1:800) at 37 °C for 60 min. The slides were washed with PBS, rinsed briefly with distilled water, dried and mounted in Vectashield with DAPI (Vector Laboratories).

Meiotic cellular suspensions from testes for post-pachytene stages analysis were prepared using the air-drying method (Evans et al. 1964).

The slides were analyzed with an Axioimager D1 microscope CHROMA filter sets (Carl Zeiss, Jena, Germany) equipped with a Axiocam HRm CCD camera (Carl Zeiss), and image-processing AxioVision Release 4.6.3. software (Carl Zeiss, Germany). Images were processed using Adobe Photoshop CS3 Extended. Measurements of autosomal bivalents and their ranking in each cell were made in order to determine relative lengths (MicroMeasure 3.3, Colorado, USA) using the STATISTICA 8.0 software (StatSoft, Tulsa, OK, USA).

Results

Heterozygous karyotypes of *E. tancrei*

Karyotypes of parental forms from opposite banks of the Surkhob River were published earlier (Bakloushinskaya et al. 2010) and were described according to the nomenclature of the *E. tancrei* chromosomes (2n = 54) (Bakloushinskaya et al. 2012). The form with 2n = 48 had six Rb metacentrics [2Rb(2.11), 2Rb(5.9), and 2Rb(3.18)] while the form with 2n = 50, "Voidara," had four Rb metacentrics [2Rb(2.18 and, 2Rb(5.9)]. There were five Rb metacentrics, three of them with MBH in the F1 hybrids [Rb(2.11), Rb(2.18) and Rb(3.18)] (Fig. 1a).

We identified the Rb translocations in the form with 2n = 50, "Khodza Obi-Garm", which had four Rb metacentrics [2Rb(4.12) and, 2Rb(9.13)]. F1 hybrids of crossings "Khodza Obi-Garm" with 2n = 50, "Voidara" had four different Rb metacentrics, two of them with MBH [Rb(5.9) and Rb(9.13)] (Fig. 3a).

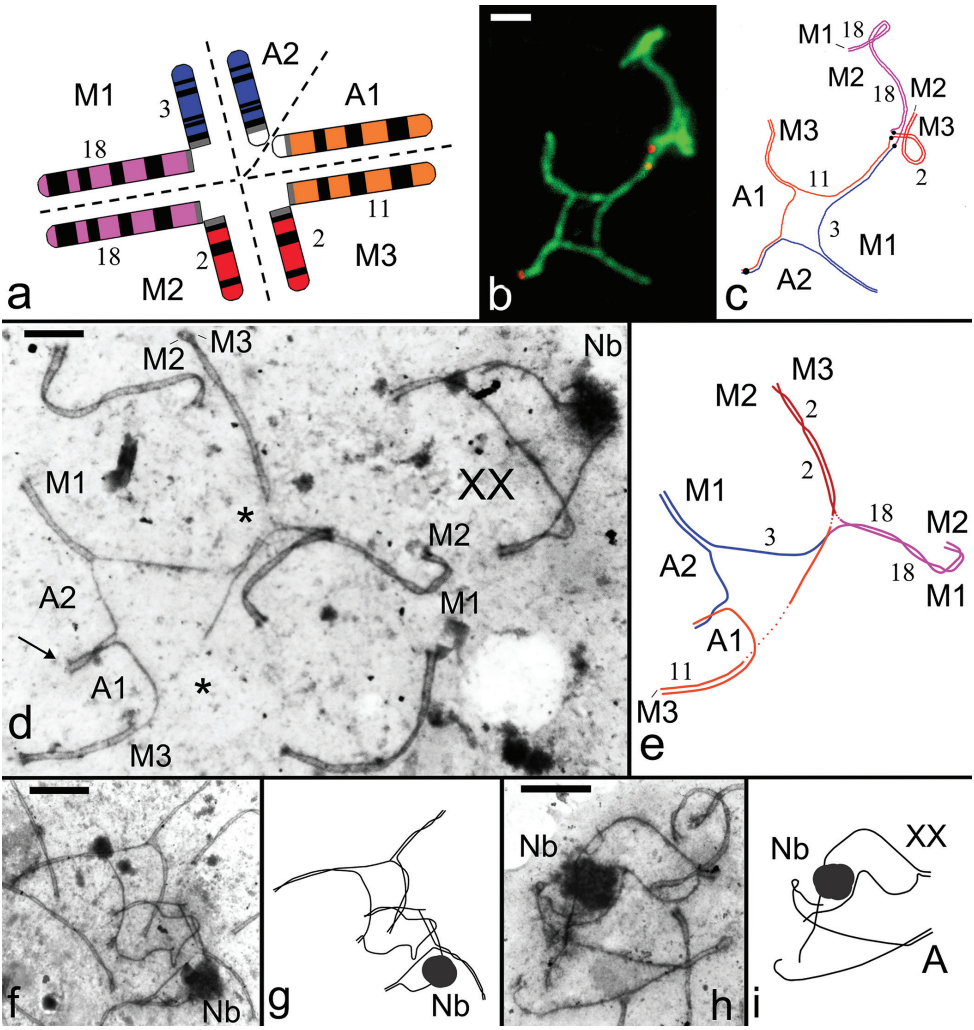

Figure 1. Chromosome synapsis in pachytene spermatocytes of F1 hybrid *E. tancrei* (2n = 49, NF = 56). **a** The scheme reflects a prognosis for chromosome synapsis in prophase I of meiosis [M1=Rb(3.18), M2=Rb(2.18), and M3=Rb(2.11)] **b** Closed SC pentavalent. Immunostaining with antibodies to SC protein 3 (green) and to the centromere ACA (red) **c** The scheme of chromosome synapsis in the structure of SC pentavalent (see Fig. 1b). Black dots mark centromere positions **d** Electron micrograph of spread spermatocyte from F1 hybrid. Closed SC pentavalent is formed from three metacentrics with monobrachial homology (M1, M2, and M3) and two acrocentrics (A1, A2). The arrow shows the fragment of SC between the short arms of homologous acrocentrics. Gaps are marked with asterisks. A sex bivalent (XX) does not associate with the multivalent. Nb – nucleolus-like body **e** The scheme of chromosome synapsis in the structure of SC pentavalent (see Fig. 1d) **f** A complex case of the association of sex bivalent (XX) **g** The scheme of the association of sex bivalent (XX) with an autosome (see Fig. 1f) **h** An association of sex bivalent (XX) **i** The scheme of the association of sex bivalent (XX) with an autosome (see Fig. 1h). Scale bars: 1 μm (**b, d, i**); 2 μm (**e, f, h**).

Intraspecific hybridization

As a control, we used *E. tancrei* with 2n=54, which is typical for the species. Their fertility was 2.37±0.22 (71 litters, 168 pups). In crossing type I (48 × 50), 24 litters and 62 hybrids F1 were obtained. Litter size was 2.58 ± 1.02. In inbred crosses 11 litters with 16 hybrids F2 were obtained. A litter size was estimated as 1.27±0.47, and the mean litter size was lower comparing the control and parents ($p<0.01$).

In crossing type II (50 × 50), 35 litters and 106 hybrids F1 were obtained. Litter size was 2.94 ± 0.63. In inbred crosses, 44 litters with 71 hybrids F2 were obtained. A litter size was estimated as 1.61±0.84, with the mean litter size was lower when comparing the control and parents ($p<0.01$).

Spermatocytes and oocytes of hybrids with 2n=49

We analyzed a total of 106 spermatocytes of the hybrids (Table 1), including 88 at the pachytene stage. We identified a closed SC pentavalent, formed by three metacentrics and two acrocentrics, at the pachytene stage (Fig. 1a, b, d). A short SC fragment was formed between the pericentromeric regions of the nonhomologous acrocentrics, which are determined by the presence in these chromosome regions of large C-heterochromatic blocks (Fig. 1c, e).

The number of spermatocytes, for which the structure of the sex bivalent and SC multivalent were defined, was less than the total number of cells. This may occur due to weak or partial staining of the axial elements of SC and XX bivalents, which was previously observed in *E. talpinus* (Kolomiets et al. 2010). It may also be caused by strong tensions and numerous gaps in SC structure of multivalent (Fig. 1e). A closed XX bivalent was identified in a significant part of pachytene nuclei (0.84±0.03SE (standard error)). An electrondense nucleolus-like body (Nb) was formed on one of the axial elements of the sex bivalent (Fig. 1). High rates of sex bivalent associations with SC autosomes were observed in the hybrids; the occurrence was significantly higher ($p<0.05$) than that found in the 54-chromosome form (0.48±0.06 and 0.17±0.03, respectively). This association could have arisen due to heterosynapsis of the axial elements of the sex bivalent with axial elements of the SC bivalents (Fig. 1f, g). As associations, we classified the cases of single and multiple overlapping SC bivalents on the sex bivalent, which occurred in slightly more than half of all associations (Fig. 1h, i).

We studied 59 oocytes in total. According to our observations, in cell suspensions of ovaries, the number of oocytes was lower in hybrids (three to six nuclei per slide) than in ordinary of *Ellobius talpinus* Pallas, 1770 females (40–60 nuclei per slide) (Kolomiets et al. 2010).

There are 21 autosomal SC bivalents in pachytene oocytes. One SC bivalent was formed by metacentric chromosomes, another SC bivalent appeared due to single pair of non-Rb submetacentrics, and the other 19 SC bivalents were formed by acrocentrics. The rest of chromosomes presented as SC pentavalent and sex (XX) bivalent (Fig. 2a–c).

Table 1. Synaptic characteristics of multivalents and sex bivalents in the spermatocytes and oocytes of homozygotes and heterozygotes of *E. tancrei*.

Name	Number of cells	Sex (XX) bivalent associations (±SE)	SC multivalent (±SE) (Penta-/tetravalent)		
			With gaps	Closed	Open
E. tancrei 2n=54, male	102	0.17±0.03 [1]	–	–	–
E. tancrei hybrid 2n=49, male	106	0.48±0.06 [1,2]	0.10±0.07 [3]	0.69±0.09	0.31±0.02
E. tancrei hybrid 2n=49, female	59	Sex bivalent behaves as autosomes.	0.29±0.03 [3,4]	0.62±0.04	0.38±0.03 [5]
E. tancrei hybrid 2n=50, male	94	0,08±0,04 [2]	0.11±0.04 [4]	–	0.96±0.06 [5]

SC – synaptonemal complex, [1, 2, 3, 4, 5] Significant difference ($p<0.05$)

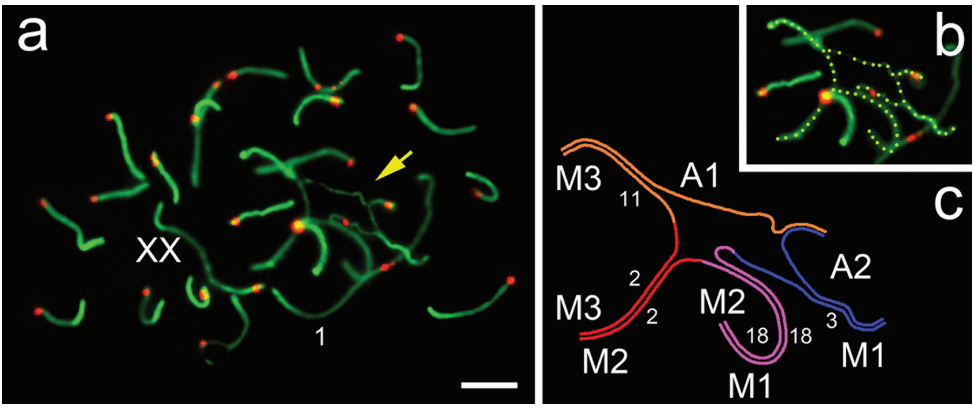

Figure 2. Spreads of oocytes from intraspecific F1 hybrid *E. tancrei* (2n= 49, NF = 56). Immunostaining with antibodies to SCP 3 (green) and to the centromere (ACA, red). The yellow arrow marks the SC pentavalent **a** Early-mid pachytene oocyte: 21 SC bivalents, SC pentavalent, XX bivalent **b** SC pentavalent (yellow dotted line) from Fig. 2a **c** Scheme of SC pentavalent (see Figs 2a, b). The chromosomes forming the SC pentavalent shown in Fig. 1a. Scale bar: 5 μm.

The XX bivalent that was formed between the two largest acrocentrics was reliably identified via immunostaining of centromeric proteins. Sex chromosomes form the SC, which is indistinguishable from autosomal SCs.

The SC pentavalent was formed by three submetacentrics with MBH, as well as two acrocentrics (Fig. 2a–c), as in spermatocytes (Fig. 1c, e). The SC pentavalent was usually closed due to the non-homologous synapsis of the short arms of the acrocentrics (Table 1). Apparently, nuclear architecture changes in hybrid oocytes, resulting in extension of chromosomes and formation of gaps in the SC axial elements of multivalens (Table 1), similar to what happens in spermatocytes. The formation of SC multivalents in the male and female meiocytes was identical with the only difference in the ability to associate with sex bivalents.

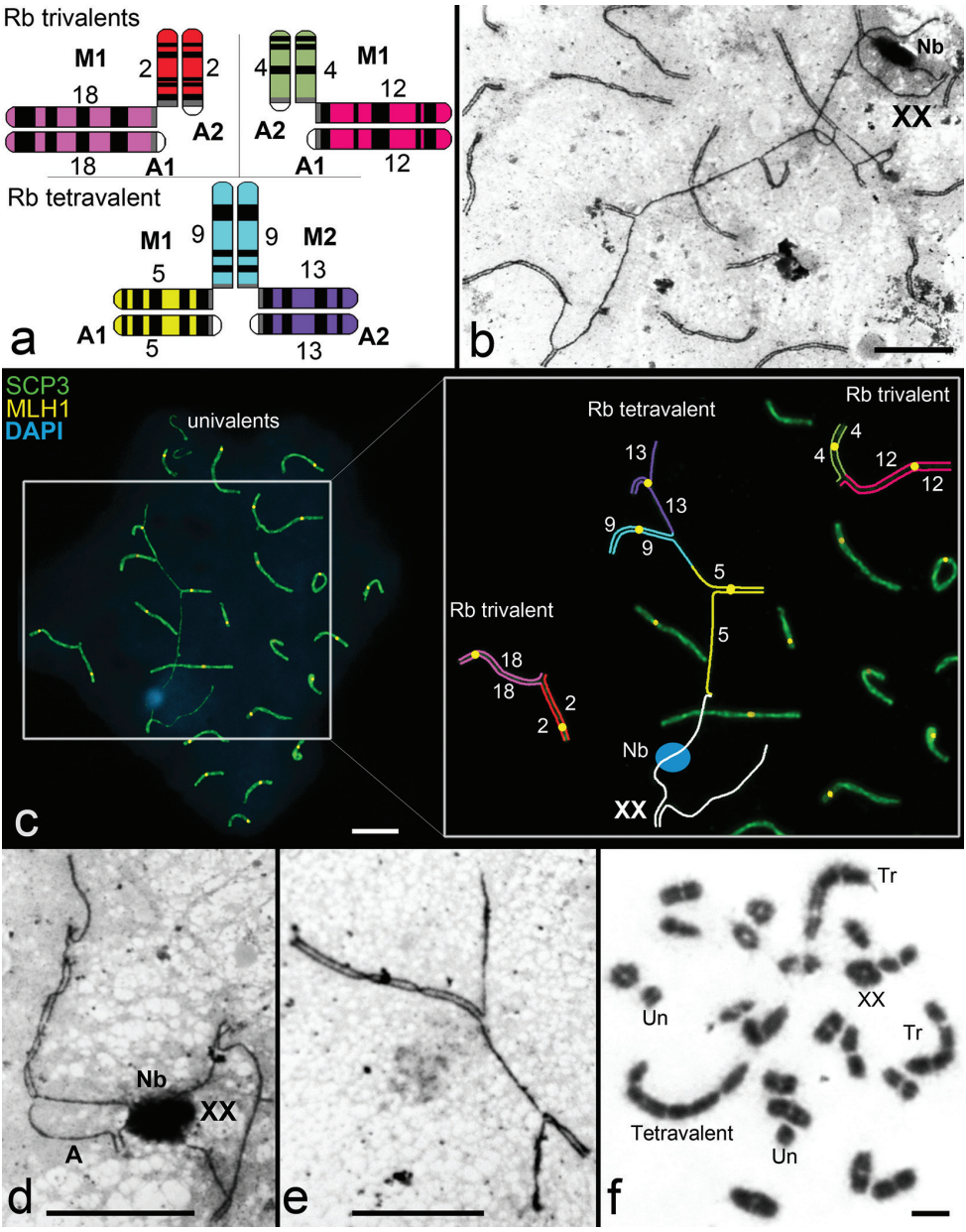

Figure 3. Chromosome synapsis in pachytene spermatocytes and diakinesis/metaphase I cell of F1 hybrid *E. tancrei* (2n = 50, NF = 56). **a** The scheme reflects a prognosis for chromosome synapsis in prophase I of meiosis [Trivalent №1 Rb(2/2.18/18), trivalent №2 Rb(4/4.12/12), and tetravalent Rb(5/5.9/9.13/13)]. M – metacentric, A – acrocentric **b** Electron micrograph of part of the spread spermatocyte. Nb – DAPI-positive nucleolus-like body **c** Spermatocyte is stained with DAPI (blue). Immunostaining with antibodies to SCP 3 (green) and to MLH1 (yellow) **d** Autosome's axial element (A) stiking to Nb of sex bivalent (autosome – sex chromosome association) **e** Open SC trivalent **f** Diakinesis/Metaphase I cell showing 18 pairing elements, XX bivalent, two univalents (Un), two trivalents (Tr) and tetravalent. Scale bar: 5 μm.

Spermatocytes of hybrids with 2n=50

We analyzed 94 spermatocytes of the F1 hybrid (Table 1). At the pachytene nuclei we distinguished 19 SC and XX bivalents, one SC tetravalent and two SC trivalents. The sex XX bivalent was closed in most of the nuclei (0.87±0.04). Typical SC was formed at the telomeric zones, and usually did not participate in associations with other chromosomes (0.08±0.04). This characteristic was lower compared to the typical *E. tancrei* with 2n=54 (Table 1). One of the few spermatocytes with the autosome – XX association is shown in Fig. 3d. One or two DAPI-positive and electrondense Nbs are formed on one of the axial elements of the sex bivalents (Fig. 3).

The SC tetravalent was usually open (0.96±0.06). It was formed by two Rb metacentrics with MBH and two acrocentrics (Fig. 3). Recombination nodules were detected in synaptic sites of the tetravalent. As a rule, the SC tetravalent did not associate with the sex bivalent, but sometimes we observed end-to-end association of the XX axial elements and the acrocentric bivalents (Fig. 3c).

Each SC trivalent was formed by one Rb metacentric and two acrocentrics (Fig. 3c). Arms of SC trivalents had one recombination nodule. In the most of the nuclei, SC trivalents were closed (0.71±0.03 and 0.63±0.03 for two trivalents, $p>0.05$). However, the open trivalents (Fig. 3e) sometimes associated with the tetravalents (Fig. 3b).

Small acrocentrics did not undergo synapsis in some pachytene nuclei, and univalents were discovered (Fig. 3c). These data correlated with diakinesis samples: for 92 cells studied, 45 univalents were identified. The diakinesis/metaphase I cell demonstrated 18 pairing elements, XX bivalent, two univalents, two trivalents and tetravalent (Fig. 3f). Mature sperm were frequently seen in meiotic preparations of both types of the heterozygous mole vole (Fig.4).

Discussion

Previous studies support the suggestion that the single and multiple Rb fusions may be involved in the process of speciation because accumulation of Rb translocations (including MBH) in different populations of the species can lead to reproductive isolation between them (Gropp and Winking 1981; Baker and Bickham 1986; Hauffe and Searle 1998). The complex compounds, which formed in meiocytes of heterozygous animals, can disturb the synapsis and dissociation of chromosomes during meiosis, leading to the death of spermatocytes, or the formation of unbalanced gametes, and eventually to the hybrid's sterility.

The impact of simple and complex SC configurations on the progression of spermatogenesis and fertility varied among different groups of animals. Simple and complex SC configurations are well studied in different races of shrews (Narain and Fredga 1998, Pavlova et al. 2008, Matveevsky et al. 2012, etc.). Trivalents in *Cricetulus* hybrids spermatocytes (Matveevsky et al. 2014) and pentavalent in heterozygous *Allocricetulus* spermatocytes led to only partial germ cell death (Gureeva et al. 2015).

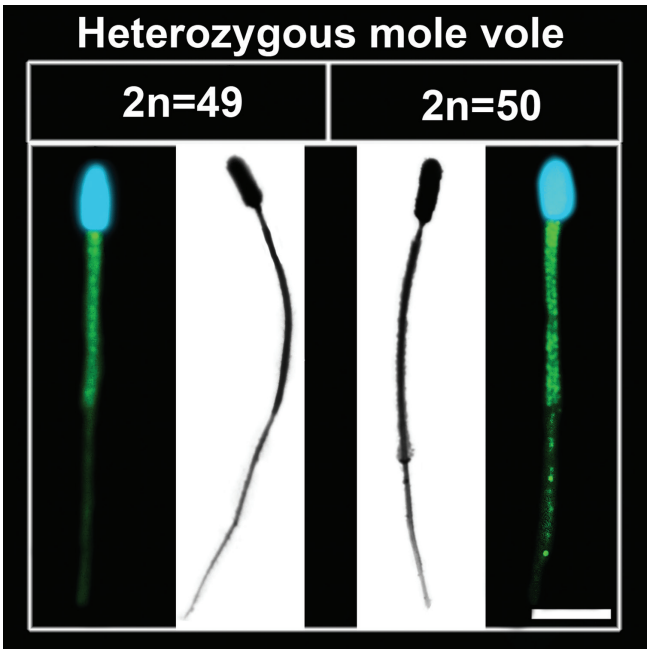

Figure 4. Spermatozoa in meiotic slides of heterozygous *E. tancrei*. Images obtained by $AgNO_3$-staining (black and white photo, electron microscopy) and nonspecific immunostaining (color photo, light microscopy). Scale bar: 2 μm.

In *Mus musculus domesticus*, the impact of Rb translocation in meiotic events and, in general, the formation of gametes, may be insignificant or significant, depending on the number of Rb fusions in the heterozygotes and, therefore, the characteristics and organization of SC chains in the meiotic nucleus. Heterozygous mice with four and seven trivalents had abnormal pairing such as SC trivalent associations with each other and with XY bivalent that might be the cause of infertility (Wallace et al., 2002). Hybrids between monobrachially homologous Rb races of the house mouse from the island of Madeira had two MBH Rb metacentrics, and thereafter in meiosis chromosomes, they formed one chain-of-four configuration (tetravalent). In such semi-sterile hybrids aneuploidy and germ cell death were higher than in homozygous mice (Nunes et al. 2011). It is interesting, that two types of hybrids with MBH Rb metacentrics and SC tetravalents, had a different fertility. Mice heterozygous by Rb(6/6.15/15.4/4) were fertile, but mice with Rb(4/4.6/6.15/15) were sterile (Mahadevaiah et al. 1990). The last type of hybrids demonstrated a high index of asynapsis in SC tetravalents, and a low number of spermatocytes. A study of the SC in *M. domesticus* hybrids obtained by crossing forms from Campobasso and Cittaducale is of particular interest (Johannisson and Winking 1994). In these hybrids, a closed ring multivalent formed which included 16 Rb metacentrics. In hybrids, obtained by crossing forms from Sicily and Alpie Orobie, an open SC multivalent formed which included 15 Rb metacentrics. Johannisson and Winking (1994) noticed that the formation of closed SC multivalents

(SC rings) due to spermatogenic normality. At the same time, the open SC multivalents (SC chains) were often associated with sex bivalent. The mice had a complete spermatogenic breakdown at the spermatocyte I level.

Fertility of lemurs was also variable depending on the number of Rb fusions. Hybrids with three to six SC trivalents were usually fertile. Heterozygous lemurs with eight trivalents in spermatocytes were viable, but had reduced fertility. Hybrids with complex SC configurations, including ones formed by Rb metacentrics that shared monobrachial homology, were sterile (Ratomponirina et al., 1988). In spermatocytes of such hybrids defects of synapsis and the association of sex chromosomes with multivalent were revealed. In spermatocytes of lemurs heterozygous for two MBH, Rb metacentrics found an open SC tetravalent with the terminal association with the XY (Djlelati et al. 1997; Rumpler 2004). The authors suggested that this may lead to a partial loss of meiocytes. The case is similar to associations in *E. tancrei* F1 hybrids with 2n=50. At the same time, Dutrillaux and Rampler (1977) found a small number of spermatocytes with pentavalents in fertile hybrids of lemurs.

Studied heterozygous *E. tancrei* had approximately half the level of fertility in comparison with parental individuals and animals with 2n=54 (see also Lyapunova et al. 1990). Despite the similar parameters of hybrid fertility in spermatocytes, chromosome synapsis with different scenarios was observed. Hybrids with 2n = 49 had an increased level of XX association with other chromosomes, while heterozygotes with 2n = 50 had surprisingly low indices, even in comparison with the animals with 2n = 54. Such associations, a failure of displacement of sex bivalent to the nuclei periphery, and SC configurations are signs of hybrid spermatocytes' pachytene arrest.

Previously, we have studied other variants of intraspecific hybrids. In the nuclei of spermatocytes of F1 (2n = 44), hybrids from crossing 2n = 54 and 2n = 34 were expected to have six SC bivalents, the sex bivalent, and 10 SC trivalents, each of which was formed as a result of the synapsis of one Rb metacentric and two acrocentrics. However, the expected pattern could be observed only in a single nucleus at the late pachytene. It was found that chromosome synapsis in forming trivalents in most cases was slower than was bivalent synapsis (Kolomiets et al. 1985, 1986, Bogdanov et al. 1986). The process of forming the axial elements slows down in the central part of the Rb metacentrics from SC trivalents, which were highly extended between the points of attachment to the nuclear envelope. There were gaps in the pericentromeric area of the metacentric's axis until the middle pachytene.

Formation of complex SC configurations (SC chains and rings) in heterozygotes for multiple Rb translocation is accompanied by extended preservation zones of asynapsis in centromeric regions of acrocentrics and metacentrics (Homolka et al. 2007, Mahadevaiah et al. 2008). However, it is known that asynapted chromatin sites undergo transcriptional inactivation, which in fact leads to a block (arrest) of meiosis. According to our observations (unpublished data), the DAPI-positive Nbs of mole vole XX bivalents are chromatin inactivation sites. The apparently unsynapsed site of the SC bivalent anchored to the Nb of XX (Fig. 3d), is a transcriptionally silent region. If the genetically important part of the genome undergoes inactivation, it can lead to germ cell death.

Signs of transcriptional inactivation of asynapted chromatin involving the proteins gamma-H2AX, ATR, and SUMO1 were detected in mice heterozygous for eight Rb translocations. Such inactivation, however, did not lead to disturbances in the formation of the sex body in most of the spermatocytes nuclei (Manterola et al. 2009). This points out the low activity of the pachytene arrest. However, apoptosis of spermatocytes was observed during the metaphase due to defects of the spindle apparatus. The low efficiency of the pachytene checkpoints in relation to the Rb translocations apparently determined the circulation of the Rb metacentrics in natural populations and their roles in the evolution of karyotypes. Perhaps there is low genetic significance of the silenced asynapted centromeric chromatin regions of the SC multivalents, and the SC chains reduce the activity of the checkpoint in the pachytene.

Baker and Bickham (1986) proposed a model of chromosomal speciation by monobrachial centric fusion. Fixation of metacentric chromosomes with homology formed as a result of independent fusion of acrocentric chromosomes can entail reproductive isolation of the population and further speciation due to the accumulation of genetic differences (Capanna 1982, Baker and Bickham 1986). Our data support the possibility of speciation by monobrachial centric fusions in *E. tancrei* as suggested by previous studies for other animals (Capanna et al. 1976, Baverstock et al. 1983, Baker et al. 1985, see review by King 1993). This evolutionary mechanism is likely widely distributed in nature and, along with other chromosomal rearrangements, led to species differentiation.

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