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Karyotype analysis and visualization of 45S rRNA genes using fluorescence *in situ* hybridization in aroids (Araceae)

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Abstract

Karyotype analysis and FISH mapping using 45S rDNA sequences on 6 economically important plant species *Anthurium andraeanum* Linden ex André, 1877, *Monstera deliciosa* Liebmann, 1849, *Philodendron scandens* Koch & Sello, 1853, *Spathiphyllum wallisii* Regel, 1877, *Syngonium auritum* (Linnaeus, 1759) Schott, 1829 and *Zantedeschia elliottiana* (Knight, 1890) Engler, 1915 within the monocotyledonous family Araceae (aroids) were performed. Chromosome numbers varied between 2n=2x=24 and 2n=2x=60 and the chromosome length varied between 15.77 µm and 1.87 µm. No correlation between chromosome numbers and genome sizes was observed for the studied genera. The chromosome formulas contained only metacentric and submetacentric chromosomes, except for *Philodendron scandens* in which also telocentric and subtelocentric chromosomes were observed. The highest degree of compaction was calculated for *Spathiphyllum wallisii* (66.49Mbp/µm). B-chromosome-like structures were observed in *Anthurium andraeanum*. Their measured size was 1.87 times smaller than the length of the shortest chromosome. After FISH experiments, two 45S rDNA sites were observed in 5 genera. Only in *Zantedeschia elliottiana*, 4 sites were seen. Our results showed clear cytogenetic differences among genera within Araceae, and are the first molecular cytogenetics report for these genera. These chromosome data and molecular cytogenetic information are useful in aroid breeding programmes, systematics and evolutionary studies.

Keywords
Araceae, B-chromosomes, chromosome formula, cytogenetics, genome size, FISH

Introduction

The Araceae (commonly known as aroids) are a very widely distributed monocotyledonous family. Most aroids are tropical and subtropical species while some members are growing in temperate regions. There are about 117 genera and 3300 species (Mayo et al. 1997; Boyce and Croat 2013). The leaves of aroids often show broad netted venation. The inflorescence possesses a dense mass of apetalous flowers on a central 'spadix'. The flowers are generally covered in a leaf like 'spathe', which can be colored or colorless. Because of this attractive feature, aroids are commonly used as ornamentals (cut flowers and pot plants) or for landscaping in more (sub) tropical areas (Chen et al. 2005). However, more molecular cytogenetic information would be very useful for plant systematics and evolutionary studies and in plant breeding programs. In breeding programs, (cyto)genetic information of parent plants can be useful to select suitable parent combinations and to trace parental markers in putative hybrids.

In cytogenetic studies, one of the first goals is chromosome identification and karyotype construction based on microscopic morphological characteristics of the chromosomes. In addition to morphological chromosome features, by molecular cytogenetic techniques such as fluorescence *in situ* hybridization (FISH) and based on DNA sequence information, chromatin regions of individual chromosomes can be addressed (Shubert et al. 2001). FISH has become important for physical mapping of single-copy DNA sequences of interesting genes, e.g. economically important genes relevant for breeding programs. FISH is also particularly valuable for identifying the sites of highly repetitive genes, e.g. rRNA genes, which are difficult to map by other methods (Leitch and Heslop-Harrison 1992). The localization of this repetitive DNA using FISH can play a role in chromosome identification and karyotype analysis. FISH of single-copy DNA sequences and repetitive sequences has become indispensable in map-based cloning and other physical mapping strategies. rRNA genes have been isolated from many different plant species and used as probes for FISH (Schwarzacher 2003). FISH with rRNA genes can also help to detect recent polyploidization (duplication or dysploidy), since the number of 5S rDNA and 45S rDNA sites sometimes doubles with polyploidization (Souza et al. 2010). Up till now, only very little molecular cytogenetic information is known for Araceae. Cusimano et al. (2011) performed a phylogenetic study to infer Araceae chromosome evolution based on molecular data compared with morphological and anatomical data analyses. In their study, Cusimano et al (2011) distinguished 44 clades having morphological or anatomical synapomorphies as well as ecological or geographic cohesion. Chromosome numbers are available for 862 species (26% of the family), ranging from 2n=10 to 2n=168 (Cusimano et al. 2012). Cusimano et al. (2012) suggested an ancestral haploid chromosome number of 16 or 18, rather than the base number of x=7 (Larsen 1969; Marchant 1973) or x=14 (Petersen 1993)

previously hypothesized. Few karyotype studies for species distinction and relationship have been reported (Fu-Hua et al. 2001; Chen et al. 2007; Begum et al. 2009; Ghimire et al. 2012). And physical mapping of repetitive sequences such as 45S or 5S rDNA using FISH has only been reported for *Typhonium* Schott, 1829 (Sousa et al. 2014).

In our study, flow cytometric analysis for genome size measurements, karyotype construction, and FISH mapping using 45S rDNA sequences were performed for the first time on the Araceae species *Anthurium andraeanum* Linden ex André, 1877, *Monstera deliciosa* Liebmann, 1849, *Philodendron scandens* Koch & Sello, 1853, *Spathiphyllum wallisii* Regel, 1877, *Syngonium auritum* (Linnaeus, 1759) Schott, 1829 and *Zantedeschia elliottiana* (Knight, 1890) Engler, 1915. These six species were chosen for their economic importance as ornamental species.

Material and methods

Plant material

A. andraeanum '061' and *S. wallisii* 'Domino' were present in the ILVO collection; *M. deliciosa* 'Variegata', *P. scandens* and *S. auritum* were obtained from the greenhouse of Tsitsin RAS Botanical Garden, Moscow, Russia; *Z. elliottiana* '068' was provided by Sandegroup, the Netherlands. The plants used in this study are known ornamental cultivars (no hybrids). The plants were grown in greenhouse conditions (20±2 °C; 16 h/day at 30 µmol m^{-2} s^{-1} photosynthetic period, 60±3% relative humidity) in terracotta pots, filled with potting soil (Saniflor®, NV Van ISRAEL, Geraardsbergen, Belgium) and watered two days before collecting the root tips.

Genome size measurements

Genome size analysis was performed according to Dewitte et al. (2009) using young leaf material. A minimum of 5000 nuclei were analyzed per sample. Obtained data were analyzed using Flomax software on a CyFlow space of PASIII (Partec).

The following reference plants were used: *Pisum sativum* Linnaeus, 1759 'Ctirad' (9.09 pg/2C; Doležel et al. 1998) for *Spathiphyllum wallisii* 'Domino'; *Solanum lycopersicum* Linnaeus, 1759 'Stupické Polní Rané' (1.96 pg/2C ; Doležel et al. 1992) for *Philodendron scandens*, *Syngonium auritum* and *Zantedeschia elliottiana* '068'; and *Glycine max* (Linnaeus, 1753) Merrill, 1917, 'Polank' (2.5 pg/2C; Doležel et al. 1994) for *Anthurium andraeanum* '061' and *Monstera deliciosa* 'Variegata'. At least three repeats were analyzed. The genome size was calculated based upon peak position ratios of the sample plants and the reference plants. The influence of plant cytosolic compounds on fluorochrome accessibility of nuclear DNA was tested. To this end, we tested the stability of the peak positions of the reference plants, either with or without sample plants, in all tests.

Chromosome spread preparation

Actively growing root tips were collected. The root tips of *Spathiphyllum wallisii* 'Domino' were pretreated in ice-cold (4 °C) water overnight. *Anthurium andraeanum* '061', *Monstera deliciosa* 'Variegata', *Philodendron scandens*, *Syngonium auritum*, and *Zantedeschia elliottiana* '068' root tips were pretreated in a α-bromonaphthalin solution overnight at 4 °C. α-Bromonaphthalin solution was prepared dissolving 10 µL of α-bromonaphthalin in 10 mL water. After the pretreatment, the root tips were fixed in Carnoy solution (3:1 absolute ethanol-acetic acid) at least 1 h at room temperature. They were either used immediately or stored at -20 °C until use. The Carnoy solution was removed by washing the root tips three times in tap water for 20 minutes. The root tips were digested in a pectolytic enzyme mixture [0.1% (w/v) pectolyase Y23 (Duchefa, Haarlem, the Netherlands), 0.1% (w/v) cellulase onozuka RS (Duchefa, Haarlem, the Netherlands) and 0.1% (w/v) cytohelicase (Sigma-Aldrich, Steinheim, Germany)] in 10 mM citrate buffer (10mM tri sodium citrate + 10 mM citrate, pH 4.5) at 37 °C for 1 h. Chromosome preparations were made according to the spreading method of Pijnacker and Ferwerda (1984). The best slides were selected under a phase contrast microscope (Leica DM IRB).

***In situ* hybridization**

Plasmid clone pTa71 containing a 9 kb *Eco*RI fragment of the 45S rDNA from *Triticum aestivum* Linnaeus, 1753 (Gerlach and Bedbrook 1979) were used. Isolated pTa71 plasmids were labelled with a Biotin-Nick Translation Kit (Roche Diagnostics Gmbh, Mannheim, Germany) or Digoxigenin-Nick Translation Mix (Roche Diagnostics Gmbh, Mannheim, Germany), respectively, according to manufacturer's instructions.

Slides were pretreated with 4% (w/v) paraformaldehyde for 10 min at room temperature and air dried after sequential washes in 70% (-20 °C), 90% and 100% ethanol for 3 min each (Leitch and Heslop-Harrison 1994). DNA denaturation and *in situ* hybridization were done according to Schwarzacher and Leitch (1993) and Schwarzacher and Heslop-Harrison (1994). The hybridization mixture was made of 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 2x SSC (Saline Sodium Citrate buffer), 0.25% (w/v) sodium dodecyl sulphate and 2 ng/µL labelled DNA. The hybridization mixture was denatured at 80 °C for 5 min and placed on ice for 5 min. After the hybridization mixture (40 µL) was added to the slides, a 5 min denaturation process was carried out at 80 °C. Then the slides were incubated overnight in a humid chamber at 37 °C to hybridize. The slides were washed in 2x SSC at room temperature for 15 min, then transferred to 0.1x SSC at 48 °C for exactly 30 minutes to give a 78% stringent wash (Schwarzacher and Heslop-Harrison 2000). Finally, they were washed again in 2x SSC for 15 min at room temperature. To reduce non-specific binding of antibodies and thus to lower the background fluorescence, 100 µL of 1% TNB [Boeringer blocking reagent in TN buffer (0.1 M Tris-HCl, 0.15

M NaCl, and pH 7.5)] was added to the slides and incubated for 10 min at 37 °C in a humid chamber. Biotin-labelled DNA was detected with 5 µL CY3-conjugated streptavidin and amplified with 1 µL biotinylated goat-antistreptavidin (Vector Laboratories, Burlingame, CA, USA) followed by addition of CY3-conjugated streptavidin. Digoxigenin-labelled probes were detected using FITC conjugated anti-Dig antibody (0.01% FITC in TNB; Roche Diagnostics Gmbh, Mannheim, Germany) from sheep and 1µL anti-sheep FITC from rabbit diluted in TNB. These detection steps were performed at 37 °C in a humid chamber for 1 h. Biotin-labelled DNA was only used for *Zantedeschia elliottiana* '068'.

Microscopy and karyotyping

The slides were counterstained with 1µg/mL 4',6-Diamidino-2-phenylindole (DAPI) and mounted with Vectashield® (Vector Laboratories, Burlingame, CA, USA). Slides were examined under a Zeiss Axio Imager microscope (Carl Zeiss MicroImaging, Jena, Germany). Images were captured by AxioCam and Axiovision 4.6 software, Zeiss. Karyotype analysis was done on five well-spread, DAPI stained metaphases for *Anthurium andraeanum*, *Monstera deliciosa*, *Philodendron scandens*, *Spathiphyllum wallisii*, *Syngonium auritum* and 10 metaphases for *Zantedeschia elliottiana* using MicroMeasure (Reeves 2001) for Windows, version 3.3. Arm lengths were measured and relative chromosome length (percentage length of the individual chromosome/total length of all chromosomes in the genome at haploid level), and centromeric index (length of short arm divided by total chromosome length X 100) were calculated. The position of the hybridization signal were measured. FISH signal positions were determined analyzing 3 spreads from *Anthurium andraeanum* and *Spathiphyllum wallisii*; 5 spreads from *Monstera deliciosa* and *Philodendron scandens*; 4 for *Syngonium auritum* and 6 for *Zantedeschia elliottiana*. Characterization of chromosome type was done based on centromeric index as mentioned by Levan et al. (1964). Chromosomes were arranged in order of decreasing length. The asymmetry of the karyotype was evaluated according to Paszko (2006). The degree of chromosome compaction [Genome size 1C (Mbp) / mean total chromosome length (µm)] was calculated assuming that it is uniform along the entire chromosome.

Results

The results for genome size measurements and karyotype analysis are summarized in Table 1. Metaphases are shown in Fig. 1 and the idiograms in Fig. 2. Flow cytometric analysis showed the small genome size for *Zantedeschia elliottiana*, *Philodendron scandens* and *Syngonium auritum* while for *Spathiphyllum wallisii* the largest genome size (7.39 ± 0.04 pg/1C) was observed. *Monstera deliciosa* (2n=60) had the highest chromosome number. The lowest chromosome number was found in *Syngonium auritum*

Table 1. Summary of genome size and karyotypic data for *Anthurium andraeanum* '061', *Monstera deliciosa* 'Variegata', *Philodendron scandens*, *Spathiphyllum wallisii* 'Domino', *Syngonium auritum* and *Zantedeschia elliottiana* '068'. Data are averages ± SE. (n=5, except for *Z. elliottiana* n=10).

	Anthurium andraeanum	*Monstera deliciosa*	*Philodendron scandens*	*Spathiphyllum wallisii*	*Syngonium auritum*	*Zantedeschia elliottiana*
Genome size (pg/1C)	5.27 ± 0.08	6.36 ± 0.22	1.74 ± 0.01	7.39 ± 0.04	2.60 ± 0.04	1.35 ± 0.01
Chromosome number	2n=2x=30	2n=2x=60	2n=2x=32	2n=2x=30	2n=2x=24	2n=2x=32
Chromosome formula[z]	3m+12sm	26m+4sm	10m+2sm+3st+1t	15m	8m+4sm	16m
Total chromosome complement (μm)[y]	132.72 ± 1.39	147.14 ± 0.39	44.64 ± 0.52	107.97 ± 0.58	64.88± 1.25	73.19 ± 0.99
Length of the longest chromosome (μm)	15.77 ± 1.72	7.76 ± 0.99	3.81 ± 0.75	8.58 ± 0.02	8.71 ± 1.91	6.51 ± 2.10
Length of the shortest chromosome (μm)	6.20 ± 0.10	3.35 ± 0.40	1.87 ± 0.33	5.64 ± 1.35	3.07 ± 0.57	3.01 ± 0.85
Asymmetry index (AI)	5.49	1.90	6.58	0.70	4.31	0.90
Degree of compaction (Mbp/μm)	38.52 ± 0.19	41.98 ± 0.29	37.74 ± 0.39	66.49 ± 0.37	38.85 ± 2.02	17.84 ± 0.59
45S rRNA gene chromosome number(s)	3	19	6	9	2	5, 8

[z]m – metacentric; sm – submetacentric; st – subtelocentric; t – telocentric

[y]Total chromosome length at haploid level

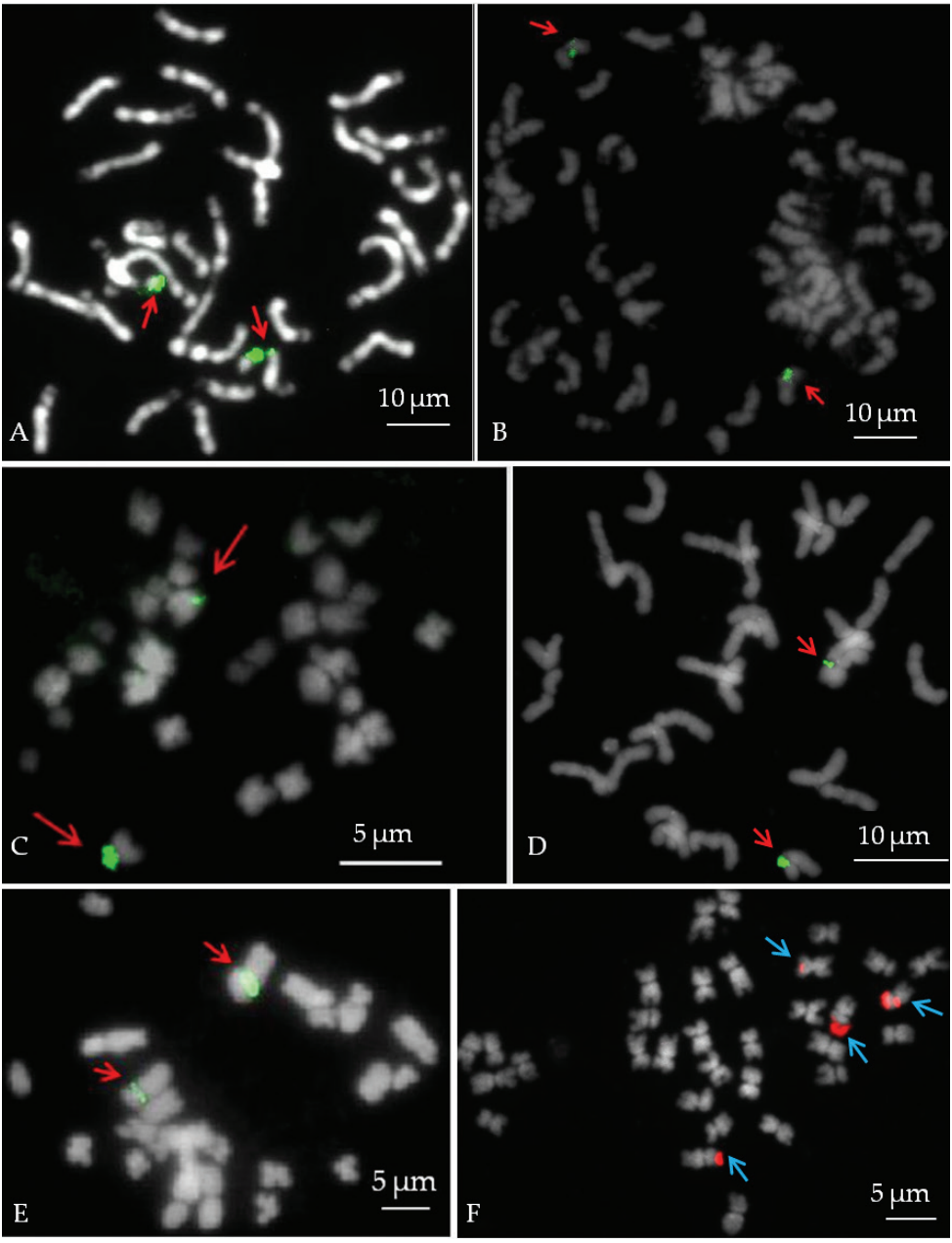

Figure 1. DAPI stained mitotic metaphases with FISH signal: **A** *Anthurium andraeanum* '061' **B** *Monstera deliciosa* 'Variegata' **C** *Philodendron scandens* **D** *Spathiphyllum wallisii* 'Domino' **E** *Syngonium auritum*; and **F** *Zantedeschia elliottiana* '068'. 45S rDNA FISH signals are indicated by arrows. 45S rDNA sites were observed using FITC (green **A–E**) and using CY3 (red **F**).

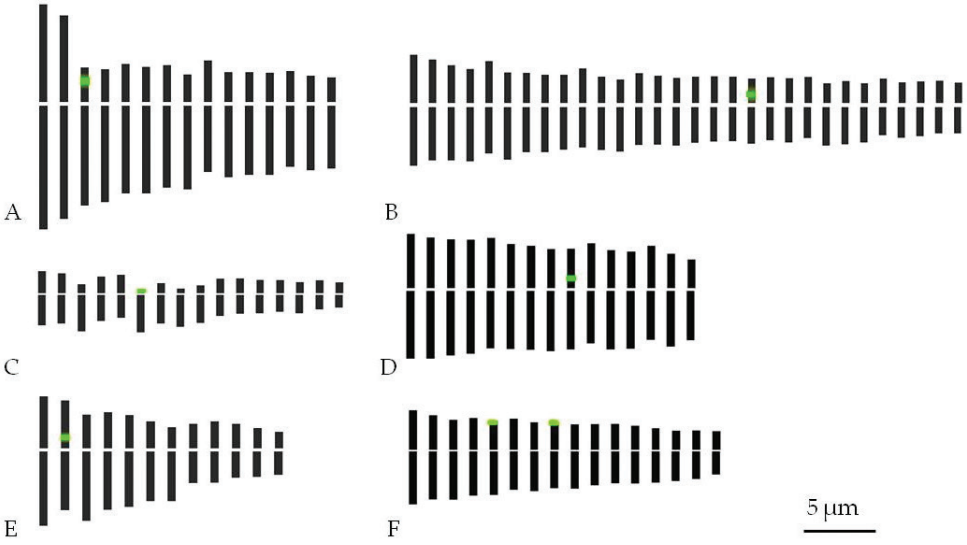

Figure 2. Idiograms with indication of 45S rDNA (green) based on observation: **A** *Anthurium andraeanum* '061' **B** *Monstera deliciosa* 'Variegata' **C** *Philodendron scandens* **D** *Spathiphyllum wallisii* Regel 'Domino' **E** *Syngonium auritum*; and **F** *Zantedeschia elliottiana* '068'.

(2n=24). The total chromosome length at haploid chromosome level varied between 147.14 ± 0.39 µm and 44.64 ± 0.52 µm. Compared to the other species *Anthurium andraeanum* and *Spathiphyllum wallisii* had the biggest chromosomes. *Philodendron scandens* possessed the smallest chromosomes and was the only species that contains subtelocentric and telocentric chromosomes besides metacentric and submetacentric chromosomes (Fig. 2). This is reflected in the asymmetry index (AI) for *Philodendron scandens* (Table 1). The degree of compaction was the highest in *Spathiphyllum wallisii* and the lowest in *Zantedeschia elliottiana*.

B-chromosome-like structures were observed in *Anthurium andraeanum* metaphase spreads (Fig. 3). Approximately 19.75% cells possessed two B-chromosomes-like structures, 34.57% spreads showed one and 45.68% showed none. The size of B-chromosome-like structures was 3.32 ± 0.12 µm (measurement on 15 B chromosome like structures), which is about 1.87 times less than the size of the shortest chromosome in the complement (6.20 ± 0.10 µm). B-chromosome structures were not seen in any other of the studied plants.

45S rRNA genes were visualized using FISH (Fig. 1). In all genera, two 45S rDNA sites were visualized except in *Zantedeschia elliottiana* which had four 45S rDNA sites (Table 1). 45S rDNA sites were seen in a distal position of *Anthurium andraeanum* and *Zantedeschia elliottiana* short arms and on the proximal position of the short arms in other species (Figs 1 and 2). In *Philodendron scandens,* signals were observed at the terminal position of the telocentric chromosome (Fig. 1 C).

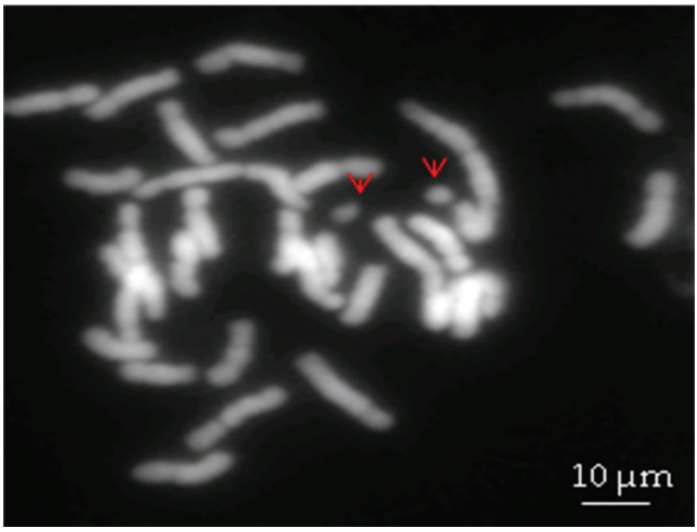

Figure 3. DAPI stained chromosome spreads of *Anthurium andraeanum* '061' with presumable B-chromosomes (indicated by arrows).

Discussion

The success of interspecific or intergeneric crosses using traditional breeding mainly depends on how closely the parental species are (cyto) genetically related. Moreover, differences between parent plants concerning chromosome number, genome size and morphology of pairing chromosomes decide the fate of hybrid chromosome pairing during meiosis. According to Cusimano et al. (2011), the six genera we tested belong to different groups. Among them *Anthurium* Schott, 1829, *Monstera* Adanson, 1763 and *Spathiphyllum* Schott, 1832 are closer to each other by sharing some morphological and anatomical features while *Philodendron* Schott, 1829, *Syngonium* Schott, 1829 and *Zantedeschia* Sprengel, 1826 are very distantly related. In our study, we also detected many cytogenetic differences among them.

The first things we noticed were different chromosome numbers and genome sizes among the six genera. The commonly known chromosome number for *Anthurium andraeanum* is 2n=30 (Marutani et al. 1993; Cusimano et al. 2011) which is in agreement with our results. However, there is also a report of 2n=32 for *Anthurium andraeanum* (Sheffer and Croat 1983). An equal chromosome number of 30 was reported for *Spathiphyllum* (Marchant 1973; Cusimano et al. 2011) and confirmed in our study. Also for *Philodendron scandens* and *Zantedeschia* our results are in agreement with earlier findings of 2n=32 (Marchant 1971a; 1971b). The chromosome number (2n=24) for *Syngonium auritum* in our study and *Syngonium wendlandii* Schott, 1858 are similar. However, other *Syngonium* species have varying chromosome numbers (Marchant 1971b; Cusimano et al. 2011). Different authors have reported different *Monstera deliciosa* chromosome numbers (Petersen 1989). Our counts for *Monstera deliciosa*

'Variegata' (2n=60) agree with the counts of Marchant (1970) and Cusimano et al. (2012). The varying chromosome numbers within genera might be explained by aneuploid derivations such as chromosome losses or gains after meiotic irregularities leading to the formation of aneuploid gametes (Petersen 1989; Sousa et al. 2014). We might conclude that the higher chromosome numbers in *Monstera deliciosa* compared to other Araceae plants, might be due to either an ancient polyploidization origin of the genus or a high basic chromosome number.

Araceae genome sizes are described to vary between 0.33 (*Lemna* Linnaeus, 1753; Wang et al. 2011) and 24.05 pg/1C (*Zamioculcas* Schott, 1856; Zonneveld et al. 2005). The six genera we analyzed also showed significant differences in genome sizes. *Anthurium*, *Spathiphyllum* and *Monstera* had higher genome sizes. For *Anthurium* and *Spathiphyllum*, our results were consistent with earlier reported genome sizes of 4.49 pg/1C for *Anthurium andraeanum* (Bliss et al., 2012), and 7.11 pg/1C for *Spathiphyllum* (Zhao et al., 2012). For *Zantedeschia elliottiana*, the total genomic content calculated in our study (1.35 pg/1C) was clearly higher than the 0.59 pg/1C mentioned by Ghimire et al. (2012). Therefore, we also repeated flow cytometrical analysis using *Pisum sativum* L. 'Citrad' as the reference plant. This additional analysis confirmed *Zantedeschia elliottiana* genome size as 1.30 pg/1C.

Although *Spathiphyllum wallisii* 'Domino' had the largest genome size, the total chromosome complement was lower than in *Anthurium andraeanum* and *Monstera deliciosa*. *Zantedeschia elliottiana* had a higher chromosome length than *Philodendron scandens* and *Syngonium auritum* although its DNA content was lower. A direct correlation between total chromosome complement and genomic content is reported (Cerbah et al. 2001; Zonneveld 2004). However, also negative correlations have been reported (Van Laere et al. 2008).

Karyotypic symmetry varies according to the presence of different chromosome types. A symmetrical karyotype mainly possesses metacentric and submetacentric chromosomes of approximately equal size whereas asymmetric karyotypes arise by shifts in centromeric position towards the telomere, and/or by addition or deletion of chromatin in some chromosomes, which gives rise to size differences (Stebbins 1971). The most common chromosome morphology type was metacentric, followed by submetacentric. Subtelocentric and telocentric chromosomes were only observed in *Philodendron scandens*, which showed also the highest asymmetry index. The karyotype we found for *Anthurium andraeanum* is comparable to the one published by Kaneko and Kamemoto (1979) for *Anthurium warocqueanum* Moore, 1878: 2 pairs of large chromosomes, 1 pair of satellite chromosomes and 12 pairs of small chromosomes. However, the size of the chromosomes differed between both species. Additionally, the choice of the pretreatment, fixating agents and chromosome preparation techniques considerably influence the chromosome structure (Sharma and Bhattacharyya 1961).

Zantedeschia elliottiana karyotypic data differed from those published by Ghimire et al. (2012). Various factors might affect karyotypic results of which chromosome fixation, slide preparation or chromosome staining method are very important and different in the study of Ghimire et al. (2012) compared to our study. Moreover,

DAPI staining (fluorescent) is preferred over other staining methods as it can provide a stronger signal (Maluszynska 2003; Van Laere et al. 2008).

Supernumerary or putative B chromosomes have been reported in some gerera of Araceae, such as *Anthurium* (Sharma and Bhattacharyya 1961; Kaneko and Kamemoto 1979; Marutani et al. 1993), *Apoballis* Schott, 1858, *Arisaema* Martius, 1831, *Asterostigma lividium* (Loddiges, 1830) Engler, 1930 *Philodendron radiatum* Schott, 1853, *Piptospatha burbidgei* (Brown, 1882) Hotta, 1965, *Schismatoglottis* Zollinger & Moritzi, 1846 and *Typhonium* Schott, 1829 (Sousa et al. 2014). The size of the B-chromosomes in *Anthurium ochranthum* Koch, 1853 was smaller than the smallest chromosome in the karyotype while in *Anthurium garagaranum* Standley, 1940 the B-chromosome had the same size as the smallest regular chromosome (Marutani et al. 1993). However, none of these studies used meiotic analysis for a more detailed understanding. B-chromosomes are unnecessary components in the karyotypes of some plants, fungi and animal species. They are present in some individuals of a population and absent in others. They do not pair or recombine with any chromosomes (A-chromosomes) of the standard diploid (or polyploid) at meiosis and their inheritance is non-mendelian and irregular (Jones and Houben 2003). In our study, we observed one or two B-chromosome-like structures in almost 50% of the spreads of *Anthurium andraeanum*. Our experiments, of course, are insufficient to establish the presence of B chromosomes. Meiotic stage analyzes are needed to confirm their presence and to exclude that they are broken chromosome arms or satellites.

The six genera we analyzed showed different chromosome condensation indices. DNA condensation variation is also described in other plant genera. For instance, in onion condensation is six times higher than in tomato (Khrustaleva and Kik 2001). Van Laere et al. (2008) and Lysak et al. (1999) even reported varying genomic condensation differences among genera and subspecies as well as among accessions. They also proposed the geographical origin of the plants, even within species, as a probable cause for the differences. However, there is no clear proof yet that geographical origin plays a major role in DNA condensation. In our studies, *Zantedeschia elliottiana*, having less condensed chromosomes, is the only South African species, whereas all other genera in this study originated in tropical America.

Finally, we applied FISH in order to localize the 45S rDNA chromosome markers. No secondary constriction could be distinguished in the DAPI stained spreads. DAPI binds to AT rich heterochromatic regions, whereas the nucleolus organizing region (NOR) is composed of GC rich tandem repeats (Lima-de-Faria 1976). Generally, 45S rDNA is associated with a NOR in eukaryotes and NOR is often positioned with a secondary constriction such as satellites (Roa and Guerra 2012). Sometimes, these secondary constrictions are lost during slide preparation. There are few reports of 45S rDNA signal without visible satellites (Ricroch et al. 1992, Van Laere et al. 2008). In our study, 45S rDNA signals were observed in the short chromosome arms as it was reported by Lima-de-Faria (1976). All 45S signals were localized on the short arms; for *Anthurium andraeanum* at intercalary position, for *Monstera deliciosa*, *Philodendron scandens*, *Spathiphyllum wallisii* and *Syngonium auritum* near the centromere, and for

Zantedeschia elliottiana at the distal end. Five of the six investigated genera had 2 45S rDNA signals. Only *Zantedeschia* had 4 45S rDNA signals. This increase of rDNA sites might indicate an ancient polyploidization, although they had a similar chromosome amount as e.g. *Spathiphyllum* which had only 2 45S rDNA signals. Known polyploid angiosperms commonly show an increased number of rDNA sites. Alternative explanations involve jumping NOR regions, perhaps mediated by transposable elements (Raskina et al. 2008).

In conclusion, our results give a clear first view on the cytogenetic differences among six genera within Araceae which is a valuable addition to the phylogenetic differences demonstrated by Cusimano et al. (2011; 2012). All these data constitute a basic knowledge of genetic resources, resulting in an advantageous feature for facilitating molecular approaches to study taxonomic relationships, evolutionary events such as past chromosome number changes, chromosomal aberrations and cellular functions. Moreover, for plant breeding purposes, the choice of species for interspecific hybridization is sometimes critical. When parental species differ sufficiently in nuclear DNA content and chromosome morphology, it is easier to detect interspecific hybrids by intermediate values of DNA or by FISH techniques. Cytogenetic markers can then be used to trace back chromosomal behaviour of parental plants in the hybrids. On the other hand, when DNA differences are too large, e.g. very different karyotype, large sequence divergence, interspecific crosses will not be successful. Therefore, our results can be used by breeders to select suitable parents for interspecific or intergeneric crosses in aroids in future aroid breeding programs and can be a first step for establishing Genomic *In Situ* Hybridization (GISH) in Araceae. Finally, the physical localization of the rRNA genes can provide a first link between (future) physical and genetic maps, which can lead to a better understanding of the genetic structure of the Aroids.

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Ribosomal DNA clusters and telomeric (TTAGG)n repeats in blue butterflies (Lepidoptera, Lycaenidae) with low and high chromosome numbers

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Abstract

Ribosomal DNA clusters and telomeric repeats are important parts of eukaryotic genome. However, little is known about their organization and localization in karyotypes of organisms with holocentric chromosomes. Here we present first cytogenetic study of these molecular structures in seven blue butterflies of the genus *Polyommatus* Latreille, 1804 with low and high chromosome numbers (from n=10 to n=ca.108) using fluorescence *in situ* hybridization (FISH) with 18S rDNA and $(TTAGG)_n$ telomeric probes. FISH with the 18S rDNA probe 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. We discuss evolutionary trends and possible mechanisms of changes in the number of ribosomal clusters. We also demonstrate that *Polyommatus* species have the classical insect $(TTAGG)_n$ telomere organization. This chromosome end protection mechanism probably originated *de novo* in small chromosomes that evolved via fragmentations.

Keywords

Lycanidae, ribosomal DNA, chromosome, taxonomy, karyotype evolution, telomeres

Introduction

Most studied butterfly families and genera share the modal chromosome number of n=30 or n=31 (Robinson 1971) and this, most likely ancestral chromosome number is maintained in the Lepidoptera karyotype evolution (Suomalainen 1979, Lukhtanov 2000, 2014). The vast majority of Lepidoptera species have also similar karyotype structure with all the chromosomes being of a similar size or forming gradually increasing size series (Lukhtanov and Dantchenko 2002). The uniformity of karyotypes does not imply that chromosome rearrangements were not involved in genome evolution in butterflies and moths. Numerous inter- or intrachromosomal rearrangements such as translocations and inversions, can contribute to karyotype evolution without significant changes in chromosome number and size. However, detecting these rearrangements is difficult due to several specific properties of Lepidoptera karyotype. *Lepidoptera* and their sister group, caddisflies (Trichoptera), have holocentric chromosomes, i.e. chromosomes without localized centromeres (Wolf et al. 1997), and this makes impossible using the centromere as a marker. Attempts to use differential banding techniques have appeared but were inefficient (Guerra et al. 2010).

These are the reasons explaining why the karyotype evolution is still poorly understood in Lepidoptera, though some data regarding karyotype organization and genome rearrangements are present for *Bombyx mori* (Linnaeus, 1758) (Yoshido et al. 2005), *Heliconius melpomene* (Linnaeus, 1758) (Pringle et al. 2007), *Bicyclus anynana* (Butler, 1879) (Van't Hof et al. 2008), *Samia cynthia* (Drury, 1773) (Yoshido et al. 2011), *Biston betularia* (Van't Hof et al. 2013), and *Melitaea cinxia* (Linnaeus, 1758) (Ahola et al. 2014).

A molecular hybridization technique, such as fluorescence *in situ* hybridization (FISH), is a very useful method for studying molecular organization of chromatin and for tracing individual chromosomes in different species (Pinkel et al. 1986). FISH markers, specifically rDNA clusters, were proposed for some insects (Colomba et al. 2000, Grozeva et al. 2010, 2011, Gokhman et al. 2014, Panzera et al. 2012, 2014) including butterflies (Nguyen et al. 2010). Ribosomal gene clusters consist of rDNA arrays and as a part of nucleolus organizer regions (NORs) form the nucleolus during interphase (Scheer and Hock 1999).

The sparse data available have contributed to generalizations about the pattern and mode of the major rDNA cluster evolution in Lepidoptera. According to Nguyen et al. (2010) rDNA distribution in Lepidoptera is a result of dynamic evolution with the exception of Noctuoidea, which showed the static rDNA pattern. In a compilation with previous data they also hypothesize multiplication of rDNA clusters as a trend in the Lepidoptera karyotype evolution. Using specimens with dramatically different high and low chromosomal numbers we aim to examine the association between karyotype and rDNA cluster number. Thus, as a model we have chosen blue butterflies of the subgenus *Agrodiaetus* Hübner, 1822, which includes about 130 described species within the genus *Polyommatus* Latreille, 1804 (Lepidoptera, Lycaenidae) (Talavera et al. 2013a). This subgenus exhibits a wide diversity of karyotypes, with haploid chromosome numbers of different species ranging from 10 to 134 (Lukhtanov et al.

2005, 2014, Vershinina and Lukhtanov 2010). The variability is not associated with polyploidy and is caused by multiple chromosome fusions and fissions (Kandul et al. 2007). We investigated distribution of ribosomal clusters in karyotypes by mapping 18S ribosomal DNA probe on chromosomes of *P.* (*A.*) *caeruleus* (Staudinger, 1871), *P.* (*A.*) *hamadanensis* (de Lesse, 1959), *P.* (*A.*) *karindus* (Riley, 1921), *P.* (*A.*) *morgani* (Le Cerf, 1909), *P.* (*A.*) *peilei* (Bethune-Baker, 1921), *P.* (*A.*) *pfeifferi* (Brandt, 1938) and *P.* (*A.*) *sennanensis* (de Lesse, 1959) which are drastically different in their chromosome numbers (from n=10 to n=108).

Additionally, we analyzed molecular organization of telomeric repeats in *Polyommatus* (subgenus *Agrodiaetus*). In animals there are three main types of telomeric tandem repeats: TTAGGG, TTAGGC, and TTAGG. The TTAGGG motif is probably ancestral for all Metazoa and has been found in all multicellular animals, except round worms and arthropods (Traut et al. 2007). TTAGGC repeats are specific for nematodes (Wicky et al. 1996), whereas the TTAGG motif prevails in most arthropod groups providing support for a common origin (Vítková et al. 2005, Lukhtanov and Kuznetsova 2010). The $(TTAGG)_n$ telomeric structure has been demonstrated in several lepidopteran species, such as the silkmoths *Bombyx mori* (Linnaeus, 1758) and *B. mandarina* (Moore, 1872) (Bombicidae, Okazaki et al. 1993, Sahara et al. 1999); saturniid moths *Antheraea pernyi* (Guérin-Méneville, 1855), *A. yamamai* (Guérin-Méneville, 1861) and *Samia cynthia* (Drury, 1773) (Okazaki et al. 1993); the vapourer *Orgyia antiqua* (Linnaeus, 1758) (Lymantriidae, Rego and Marec 2003); the wax moth *Galleria mellonella* (Linnaeus, 1758) and the flour moth *Ephestia kuehniella* (Zeller, 1879) (Pyralidae, Sahara et al. 1999, Rego and Marec 2003). Thus, TTAGG telomeric structure is expected in other butterfly and moth families. However, several exceptions from the $(TTAGG)_n$ motif are known for insects (for additional information see Frydrychová et al. 2004, Lukhtanov and Kuznetsova 2010, Kuznetsova et al. 2011; Gokhman et al. 2014). Exceptions in the telomere structure occur at different taxonomic levels, not only at the level of order but also on the level of infraorder in Heteroptera (Kuznetsova et al. 2012) and Hymenoptera (Gokhman et al. 2014), at the level of family in Curculionidae (Sahara et al. 1999), and even within Curculionidae (Frydrychová and Marec 2002). So far nothing is known about telomeres in Lycaenidae butterflies. Here we study the structure of telomeres in *Polyommatus* (subgenus *Agrodiaetus*) butterflies by using FISH with $(TTAGG)_n$ probes.

Material and methods

Butterfly species were collected from 2005 to 2011 by V. Lukhtanov, A. Dantchenko and N. Shapoval in Iran (Table 1). Only male adult specimens (from 1 to 5 individuals for each population) were analyzed. In field, gonads were fixed in a solution of absolute alcohol and glacial acetic acid (3:1) and then stored at -4 °C; meiotic chromosomes were obtained from testes, according to the standard protocol for squash preparation (Lukhtanov and Dantchenko 2002, Lukhtanov et al. 2008; Vila et al. 2010). Tissues were prepared in

Table 1. List of *Polyommatus* (subgenus *Agrodiaetus*) populations used in the present study and their haploid chromosome numbers (n) according to original data.

Species	n	Province	Locality	altitude	date
P. (*A.*) *caeruleus*	10	Golestan	Shahkuh	2700–3100 m	2005.VII.22
P. (*A.*) *hamadanensis*	19	Lortestan	Sarvand, 33°22.38'N/ 49°10.25'E	2070 m	2009.VII.22
P. (*A.*) *hamadanensis*	21	Esfahan	Kuhe-Tamandar Mts, 33°12.72'N/ 49°56.43'E	2336 m	2011.VII.16
P. (*A.*) *karindus*	ca.68	Kurdistan	40 km SW Saqqez, 36°04.39'N/ 45°59.06'E	1869 m	2009.VII.29
P. (*A.*) *morgani*	25	Kurdistan	14 km N of Chenareh, 35°42.12'N/ 46°22.35'E	2025 m	2009.VII.28
P. (*A.*) *peilei*	39	-	14 km N of Chenareh, 35°42.127'N/ 46°22.35'E	2025 m	2009.VII.28
P. (*A.*) *pfeifferi*	ca.108	Fars	Barm-i-Firuz Mts, 30°23'N/ 51°56'E	2900 m	2002.VII.19
P. (*A.*) *sennanensis*	27	Qom	Qom-Qamsar, 33°43.80'N/ 51°29.53'E	1862 m	2009.VII.16

a drop of 45% acetic acid and then fixed on a slide by freezing on a dry ice and following dehydration in a series of ethanol solutions (70-80-96%, 2 minutes each). Prior to DNA hybridization karyotypes were examined by phase contrast microscopy.

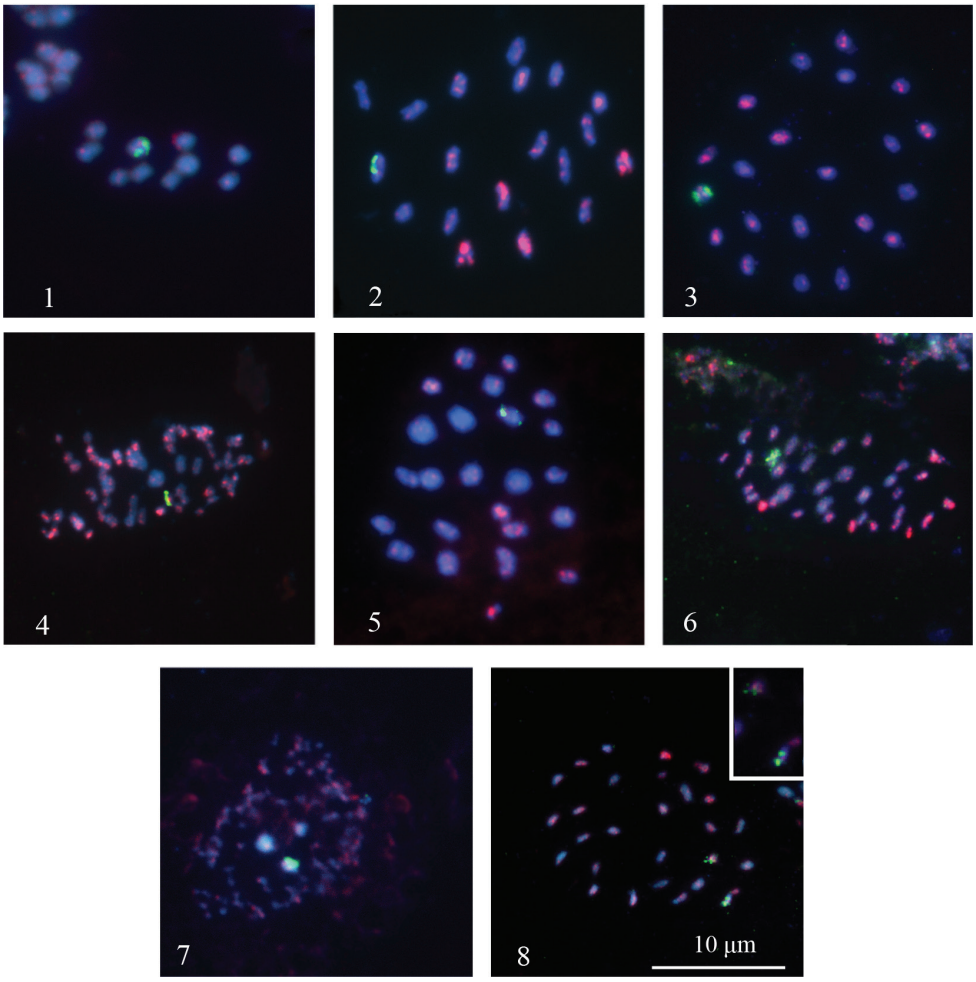
18S rDNA and $(TTAGG)_n$ probe preparation and hybridization were carried out as described in Grozeva et al. (2011). In brief, chromosome preparations were treated with 100 μg/ml RNaseA and 5 mg/ml Pepsin solution to remove excess RNA and proteins. Chromosomes were denatured on a slide in hybridization mixture with biotinylated 18S rDNA probe from the genomic DNA of *Pyrrhocoris apterus* and rhodaminated $(TTAGG)_n$ probes with addition of salmon sperm DNA blockage and then hybridized for 42 h. 18S rDNA loci 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. Images were taken with a Leica DFC 345 FX camera using Leica Application Suite 3.7 software.

Abbreviations

ca. (circa) approximately.
FISH fluorescence *in situ* hybridization.
MI meiotic metaphase I.
MII meiotic metaphase II.
NOR nucleolus organizer region.

Results

In all karyotypes weak and strong telomeric signals were present (Figs 1–8). The chromosomes of blue butterflies are very small and some of them are at the limit of the resolving



Figures 1–8. Localization of FISH signals on telomeres (red) and rDNA clusters (green) in squash chromosome preparations of seven species of *Polyommatus* (subgenus *Agrodiaetus*). Chromosomes are counterstained with DAPI. Note telomeric signals of different intensity. **1–7** one 18S rDNA cluster is found **8** two 18S rDNA clusters are found **1** *P.* (*A.*) *caeruleus*, MII **2–3** *P.* (*A.*) *hamadanensis*, MI cells from two different populations with different karyotypes (n=19 and n=21 accordingly) **4** *P.* (*A.*) *karindus*, MII **5** *P.* (*A.*) *morgani*, MI **6** *P.* (*A.*) *peilei*, MII **7** *P.* (*A.*) *pfeifferi*, MII **8** *P.* (*A.*) *sennanensis*, MII. The inset in the upper right corner shows twice enlarged image of rDNA-carrying chromosomes.

power of light microscopy. For this reason, TTAGG signals in some cases could not be distinguished from background noise. Unlike the telomere probes, rDNA probes produced strong signals of different intensity. The chromosomal distribution pattern of telomeric repeats was similar in all seven species, the exact location of telomeres (terminal or interstitial) was impossible to identify since the meiotic chromosomes were extremely contracted. The distribution pattern of 18S rDNA signals varied markedly showing two different variants – with one or two rDNA-carrying bivalents in MI karyotype and, correspondingly,

with one or two rDNA-carrying chromosomes in MII karyotype. All chromosome numbers were found to coincide with previously published karyotype data for seven studied species (Lukhtanov et al. 2005, Kandul et al. 2007). In two *P.* (*A.*) *hamadanensis* populations intraspecific chromosomal polymorphism has been discovered.

P. (*A.*) *caeruleus* had n=10 with one rDNA cluster localized in one of the chromosome pairs (Table 1, Fig. 1). In MII cells this cluster appeared as a combination of two signals, localized on sister chromatids on one of the chromosomes. Weak $(TTAGG)_n$ signals were found in all chromosomes.

In *P.* (*A.*) *hamadanensis*, the haploid chromosome number of n=19 was found in MI cells of one studied individual from Lorestan province. In the specimens from another population (Esfahan province) the number of n=21 was found in MI cells (Table 1, Figs 2–3). The karyotype had no especially large or small bivalents; all bivalents were nearly equal in size and formed a gradient size series. In both specimens, one rDNA cluster was found. In MI cells this cluster appeared as a combination of two signals, localized on homologous chromosomes of one of the bivalents (Figs 2–3). $(TTAGG)_n$ signals of different intensity were found in all bivalents.

In *P.* (*A.*) *karindus*, the haploid chromosome number of n= ca.68 was found in MII cells (Table 1, Fig.4). One rDNA cluster was found on one of the chromosomes. Numerous $(TTAGG)_n$ signals of different intensity were found in all chromosomes. The karyotype had three large chromosomes while the other chromosomes had a relatively equal small size.

In *P.* (*A.*) *morgani*, the haploid chromosome number of n=25 was found in MI cells of a single individual (Table 1, Fig. 5). One bivalent was found to carry the rDNA site. $(TTAGG)_n$ signals of different intensity were found in all bivalents.

In *P.* (*A.*) *peilei*, the haploid chromosome number of n=39 was found in MII cells (Table 1, Fig. 6). Strong 18S rDNA signals were observed on one of the chromosomes. $(TTAGG)_n$ signals of different intensity were found in all chromosomes.

In *P.* (*A.*) *pfeifferi*, the chromosome number was only approximately established and was n=ca.108 (Table 1, Fig. 7). The karyotype had two large, one medium-sized and more than 100 very small chromosomes. In MII cells, a single rDNA cluster was found on one pair of relatively large chromatids. Numerous weak $(TTAGG)_n$ signals were observed in all chromosomes, but their number and localization were difficult to estimate due to the background noise.

In *P.* (*A.*) *sennanensis*, the haploid chromosome number of n=27 was found in MII cells (Table 1, Fig. 8). In contrast to other studied species, *P.* (*A.*) *sennanensis* had two distinct rDNA clusters localized on different, non-homologous chromosomes. $(TTAGG)_n$ signals of different intensity were found in all chromosomes.

Discussion

Previous investigations by Nguyen et. al. (2010) examined ribosomal clusters in 18 species of different taxonomic groups of Lepidoptera. Discussing evolutionary dynam-

ics of rDNA clusters these authors suggest several concepts. One of them implies origin of one interstitial ribosomal cluster on rDNA-bearing chromosome as a result of a fusion between two NOR-bearing chromosomes (Nguyen et. al. 2010). However, their own table (fig. 3 in Nguyen et. al. 2010) shows a different picture: nearly all species with n=31 and haploid chromosome number less than 31 have one (mostly interstitial) rDNA cluster. Our data based on the study of diverse karyotypes in *Polyommatus* (subgenus *Agrodiaetus*) butterflies show a similar pattern. All studied species except for *P.* (*A.*) *sennanensis* have one rDNA cluster in haploid karyotype regardless of their chromosome number. Therefore, we cannot consider rDNA cluster number reduction via fusion as a common trend in the evolution of Lepidoptera genomes. Rather they tend to preserve the single rDNA cluster, the state which seems to be an ancestral one.

Specifically for blue butterflies (Lycaenidae), Nguyen et al. (2010) suggested the mechanism of rDNA cluster multiplication via chromosome fissions. This hypothesis is based on the facts that *P. icarus* (Rottemburg, 1775) which has ancestral for Lycaeninae n=23-24 (Robinson 1971) also has a single interstitial NOR whereas *Lysandra bellargus* (Rottemburg, 1775) has two NORs, therewith the chromosome number in *L. bellargus* was increased to n = 45 most likely via fragmentations (Kandul et al. 2007, Talavera et al. 2013b). Thus, Nguyen et al. (2010) hypothesized that the single ancestral NOR-chromosome was likely to split into two fragments resulting in two NOR-chromosomes. According to our data this hypothetical mechanism is, at least, not a general one in Lycaenidae since all the studied species with increased number of small chromosomes (*P.* (*A.*) *peilei*, n=39; *P.* (*A.*) *karindus*, n=68 and *P.* (*A.*) *pfeifferi*, n=ca108) have only one rDNA cluster per haploid genome.

Chromosome fissions lead to strong decrease in size of fragmented chromosomes (Lukhtanov and Dantchenko 2002). However, there is an empirical rule that in Lepidoptera one (or few) chromosome is evolutionary stable and protected from fragmentation; therefore it preserves its ancestral relatively large size whereas the rest of chromosomes are fragmented and small (White 1973). In our results, 18S rDNA probe in *P.* (*A.*) *pfeifferi* (in which the majority of chromosomes are extremely fragmented) is located on the largest chromosome (Fig. 7) suggesting possible evolutionary stability of rDNA-carrying chromosome.

The third possible mechanism which can change the number of rDNA clusters is the formation of a hybrid lineage or a homoploid hybrid speciation (hybridization without a change in chromosome number, Arnold 1996). Most likely this scenario was realized in *Pinus* (Pinaceae) and freshwater fishes (Cyprinidae) homoploid hybrids (Liu et al. 2003, Pereira et al. 2013). In the case of *Pinus*, *P. densata* has nine major rDNA clusters in haploid karyotype as a combination of rDNA clusters inherited from the paternal genomes. Similarly, homoploid cyprinid hybrids have rDNA patterns within the range of possible combinations of parental contributions.

On the basis of rDNA evolutionary dynamics and the repetitive structure of rDNA in Lepidoptera Nguyen et al. (2010) proposed ectopic recombination as a possible mechanism of rDNA repatterning. According to this mechanism, non-allelic homologous recombination may take place between homologous rDNA loci located on non-homologous

chromosomes. Species with more than one rDNA cluster in combination with an ancestral chromosome number state n=30-31 (*Colias hyale* (Linnaeus, 1758) and *Inachis io* (Linnaeus, 1758) as described in Nguyen et al. 2010) are likely to show evidence for recombination leading to rDNA cluster rearrangements. Thus, karyotype reorganizations which affect the number of rDNA-bearing chromosomes can occur without changes in chromosome number and be a result of ectopic recombination. To conclude, karyotype reorganizations which affect the number of rDNA-bearing chromosomes may occur by multiple mechanisms: chromosome fissions and fusions, hybrid formation and ectopic recombination.

In our study, FISH with telomeric $(TTAGG)_n$ probe conclusively demonstrate that *Polyommatus* (subgenus *Agrodiaetus*) blue butterflies have classical insect telomere organization. On small chromosomes of *P.* (*A.*) *peilei*, *P.* (*A.*) *karindus* and *P.* (*A.*) *pfeifferi*, originated by fragmentations, telomeric signals are also detected. Generally, fissions lead to breakdown in chromosome structure because after this reorganization the newly originated fragmented chromosomes lack telomeres and their chromosome ends need to be protected from degradation (de Lange 2009). Our data indirectly suggest that in *Polyommatus* (subgenus *Agrodiaetus*) this protection system arises after fragmentations *de novo* on the basis of TTAGG repeats.

Appearance of a new telomere seems to be a highly important event in genome evolution, however its proximate and ultimate mechanisms are still unknown. *Polyommatus* (subgenus *Agrodiaetus*) butterflies with their diverse karyotypes represent a good model system for studying these processes.

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RESEARCH ARTICLE

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Karyotypical characteristics of two allopatric African populations of anhydrobiotic *Polypedilum* Kieffer, 1912 (Diptera, Chironomidae) originating from Nigeria and Malawi

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Abstract

The African chironomid *Polypedilum vanderplanki* Hinton, 1951 is the only chironomid able to withstand almost complete desiccation in an ametabolic state known as anhydrobiosis. The karyotypes of two allopatric populations of this anhydrobiotic chironomid, one from Nigeria and another from Malawi, were described according to the polytene giant chromosomes. The karyotype from the Nigerian population was presented as the reference chromosome map for *P. vanderplanki*. Both populations, Nigerian and Malawian, showed the same number of chromosomes (2n=8), but important differences were found in the band sequences of polytene chromosomes, and in the number and the arrangement of active regions between the two populations. Such important differences raise the possibility that the Malawian population could constitute a distinct new species of anhydrobiotic chironomid.

Keywords

Chironomidae, *Polypedilum vanderplanki*, allopatric populations, Nigeria, Malawi, anhydrobiosis, polytene chromosomes

Introduction

The African non-biting midge, *Polypedilum vanderplanki* Hinton, 1951, is the only species among the family Chironomidae and also among all insects showing tolerance to almost complete dehydration, although another *Polypedilum* species was also suggested to exhibit similar desiccation tolerance (Hinton 1951, 1960a,b, Watanabe 2006, Cranston 2014). Larvae of this species live in small rock pools on granite outcrops in the semi-arid regions of Africa. During the dry season, water evaporates rapidly and the larvae are capable of surviving in a quiescent desiccated state in dry mud for several months. After rain falls, the dried larvae rehydrate rapidly, returning to active life, and resume normal development. Such an ability to survive severe desiccation in an ametabolic quiescent state is known as anhydrobiosis and the sleeping chironomid is the largest anhydrobiotic animal known to date (Watanabe 2006).

During the last decade, extensive physiological and molecular studies were performed to understand the mechanisms underlying anhydrobiosis in *P. vanderplanki* larvae (Cornette et al. 2010, Cornette and Kikawada 2011, Watanabe et al. 2002). During the desiccation process, larvae accumulate a large amount of trehalose, a nonreducing sugar that replaces water in cells and eventually forms a glassy matrix protecting biological molecules during anhydrobiosis (Watanabe et al. 2002, Watanabe et al. 2003, Kikawada et al. 2007, Sakurai et al. 2008). During anhydrobiosis, protective proteins such as heat shock proteins or late embryogenesis abundant proteins are also abundantly produced in order to prevent protein aggregation due to desiccation (Kikawada et al. 2006, Gusev et al. 2011, Hatanaka et al. 2013). In addition, *P. vanderplanki* larvae have to face oxidative stress during anhydrobiosis and express a large array of antioxidants (Cornette et al. 2010, Gusev et al. 2010). However, nuclear DNA experiences severe damage just after rehydration of dry larvae and DNA repair occurs slowly during the few days following rehydration (Gusev et al. 2010). Recently, comparative genome analysis showed that the genome of *P. vanderplanki* presents specific islands containing clusters of genes involved in anhydrobiosis (Gusev et al. 2014).

Taken together, all these data make *P. vanderplanki* an important model to study the phenomenon of anhydrobiosis in animals. Furthermore, this chironomid species was subjected to several studies with gamma- and ion beam irradiation, and the high radiotolerance of *P. vanderplanki* is now well characterized (Watanabe et al. 2005). As a consequence, *P. vanderplanki* was selected for several space experiments onboard and outside the International Space Station, including long-term exposure. This African chironomid is now recognized as an important anhydrobiotic model in the field of astrobiology. All these studies on anhydrobiosis, radiotolerance and exposure to space environment imply DNA damage, repair and possible chromosomal rearrangements. However, no cytogenetic data were available for *P. vanderplanki* to check the aforementioned effects on nuclear DNA. In addition, sequencing of the genome of *P. vanderplanki* raised the need for a chromosomal description in order to establish a physical map of the genome data.

Thus, the present study reports a detailed description of polytene chromosomes of *P. vanderplanki* larvae. The goal of this work was to establish a reference map of the *P. vanderplanki* karyotype and to estimate the cytological differences between two distant populations.

Anhydrobiotic chironomid larvae from two distant African populations originating from Nigeria and Malawi were investigated. The reference karyotype for *P. vanderplanki* was obtained from the Nigerian population. Analysis of the polytene chromosomes showed that the diploid number of chromosomes (2n=8) and their ratio were identical in both populations. However, considerable cytogenetic differences were observed between both populations in the band sequences of polytene chromosomes, and in the number and the arrangement of active regions. The results of our research raise the possibility that the Malawian population may constitute a distinct new species of anhydrobiotic chironomids from Africa.

Material and methods

Chironomids were reared in the laboratory at NIAS (Japan) as described in Watanabe et al. (2002). Briefly, chironomid larvae were reared on milk-agar under a 13:11h light:dark photoregime at 27–28 °C. Anhydrobiosis was induced by slow desiccation of the larvae as described previously (Watanabe et al. 2002, Kikawada et al. 2005).

The Nigerian strain of *P. vanderplanki* kept in the laboratory was an inbred line originating from different populations collected in small rock pools on granite outcrops around Zaria, close to the original collection points of the *P. vanderplanki* type specimen as described by Hinton (1951). Larvae from the Malawian population were collected in similar rock pools on granite outcrops in the vicinity of Mandala (14°06'044S, 33°59'517E) about 30 km South East from Lilongwe. Larvae were artificially desiccated and stored in desiccators at <5% relative humidity. Under such conditions, anhydrobiotic larvae remain viable for several years.

Larvae from both strains were placed in water at room temperature to rehydrate. Within a few hours, larvae were able to move and eat, i.e. came back to usual way of existence. The recovered larvae were maintained for 6–7 days, fed with a hay meal. After maintaining some of the larvae in good condition, they grew up and were ready to be used in the preparation of karyological slides.

Larvae were fixed in Clark's liquid: 96% of ethyl alcohol and glacial acetic acid (3:1). Fixed material was stored at low temperature (4–6 °C). Twenty six larvae from the Nigerian population and 28 from the Malawian population were suitable for preparations.

For the preparation of the karyological slides of the polytene chromosomes, dissected salivary glands were stained in a 2% solution of acetoorcein. After short maceration into 50% lactic acid, the giant cells were separated from the secretion. Squash preparations were made following the routine method described previously (Chubareva and Petrova 1982). The photographs of the chromosomes were made at the magnification 100×.

The cytophotomaps of the polytene chromosomes from *P. vanderplanki* are published for the first time. Cytophotomaps were obtained for the representatives of both Nigerian and Malawian populations. Division of the chromosomes into sections was performed arbitrarily. Arms of the chromosomes were designated: I – AB, II – CD, III – EF, IV – G, according to the standard accepted for *Polypedilum nubifer* by Porter and Martin (1977), which was inferred from the system of nomenclature for Chironomidae (Keyl, 1960). This system of nomenclature does not imply homology with the arms A to G in the genus *Chironomus* (Porter & Martin, 1977).

Results and discussion

Karyotype of larvae from Nigeria

Salivary glands consisted of 16–20 cells. On the anterior end of the gland, there were 4 especially large cells, which contained the supergiant chromosomes. They were characterized by a high degree of polytenization and with clear morphology of bands. The best sample was selected for mapping. In other salivary gland cells, polytene chromosomes formed meandric breaks and did not show a perfectly clear picture of the bands.

The diploid chromosome number coincided with the modal diploid number of the genus *Polypedilum*: 2n=8 (Fig. 1) (Tavcar 1967, Porter and Martin 1977, Petrova et al. 1981, Kiknadze et al. 1991, Gavrikova and Belyanina 1993, Kerkis et al. 1996, Michailova 1989). Chromosomes were designated according to their respective lengths - I, II, III and IV, with the length ratio I=II>III>IV. The combination of arms in chromosomes was AB, CD, EF and G (Keyl 1960). It should be noted that these designations do not mean a homology with arms in the genus *Chironomus*. Chromosome I was metacentric, chromosomes II and III submetacentrics, and chromosome IV telocentric. The putative centromeres were clearly visible (specified by arrows in Figure 1) and looked like conspicuous heterochromatic bands, wider than the average width of the chromosome. The karyotype of the population is multinucleolar, with two nucleoli (N).

A simplified reference map of the *P. vanderplanki* lab strain is presented in Fig. 2.

Chromosome I was arbitrarily divided into 29 sections. The putative centromere was localized in sec. 16. The puff located in sec. 23 looked like a facultative nucleolus (designated (N_3) in Figs 1 and 2). This puff was not observed in all cells.

However, in some supergiant cells, this puff was in an active state and looked as a normal large nucleolus (designated (N_3) in Fig. 3).

The marker for arm A was the dark block consisting of composite bands near the telomere. In addition, the groups of bands in sec. 4–5 and 9 can also serve as markers of this arm. Narrowing of chromosome width was observed in sec. 7 and 9. The arm B may easily be distinguished by a narrowing on the border of sec. 18–19 and by three thick heterochromatic bands almost identical near this narrowing in sec. 19. The next

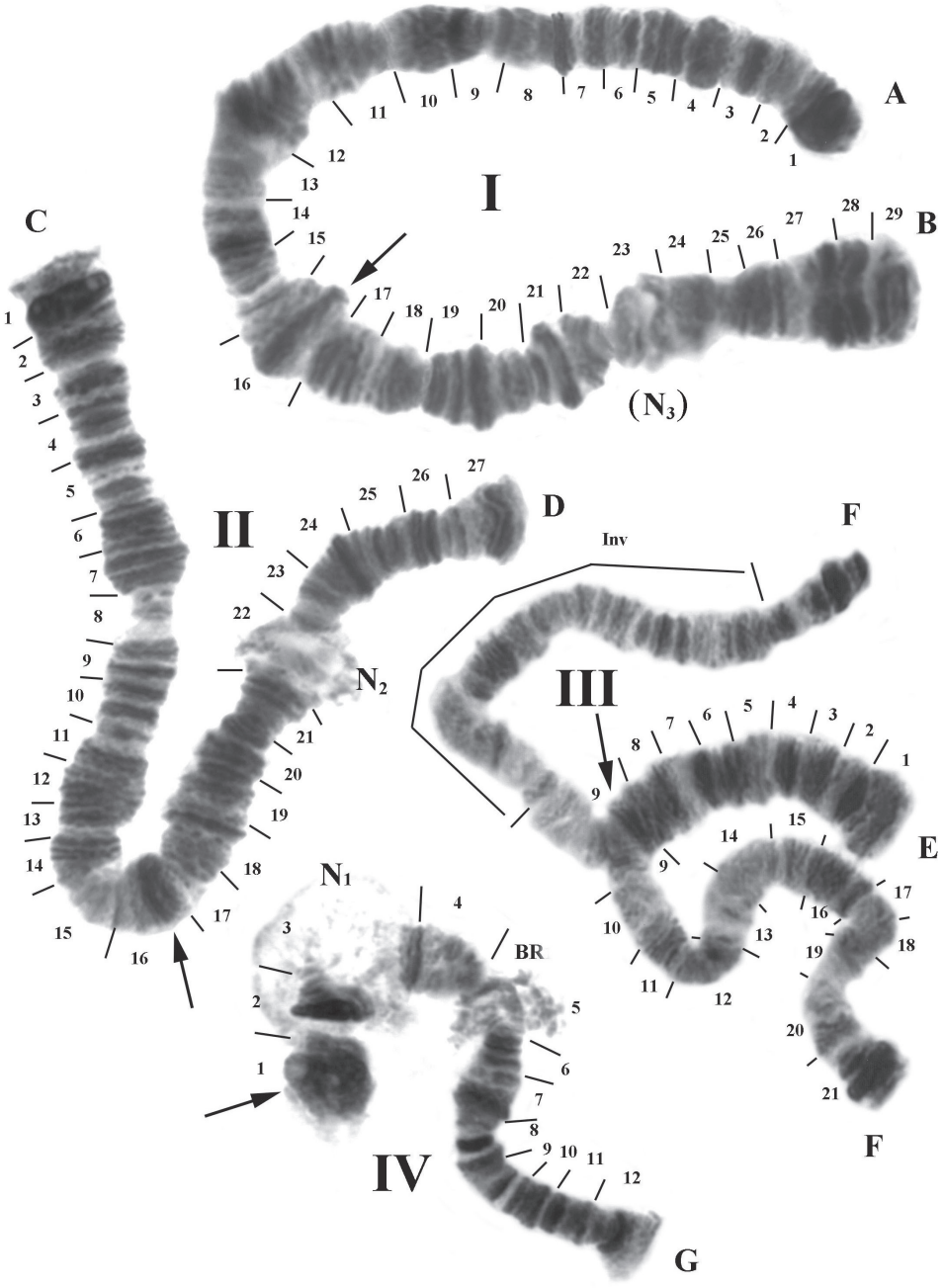

Figure 1. Representative karyotype of the *P. vanderplanki* population from Nigeria. Chromosome numbers are indicated as **I, II, III** and **IV**. Chromosome arms are labeled **A–B, C–D, E–F,** and **G**. The expected locations of the centromeres are indicated by arrows and each section is numbered and delimited by short lines. N_1, N_2, (N_3): nucleoli, BR: Balbiani ring, Inv: inversion.

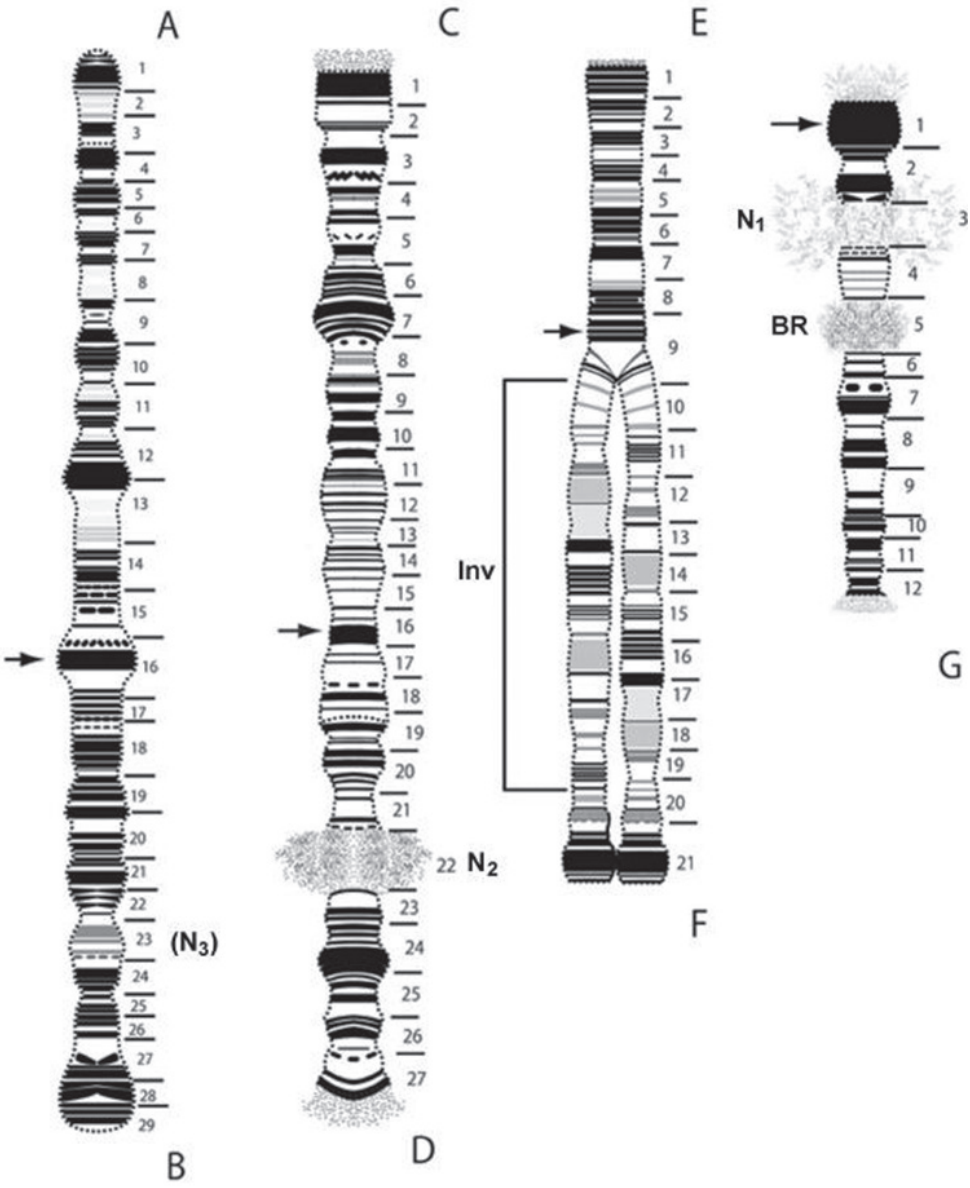

Figure 2. A simplified reference map for the *P. vanderplanki* Nigerian population. Chromosomes **I, II, III** and **IV** are shown in order from the left to the right. Numbering and abbreviations as described in Figure 1.

narrowing was conspicuous and observed on the border of sec. 22 and 23, before the facultative nucleolus N_3. A wide dark heterochromatic block, consisting of 5 composite bands, was located at the telomere of arm B (Figs 1, 2).

Chromosome II was divided into 27 sections. The putative centromere was apparently localized in sec. 16. Markers for arm C were the wide dark heterochromatic block

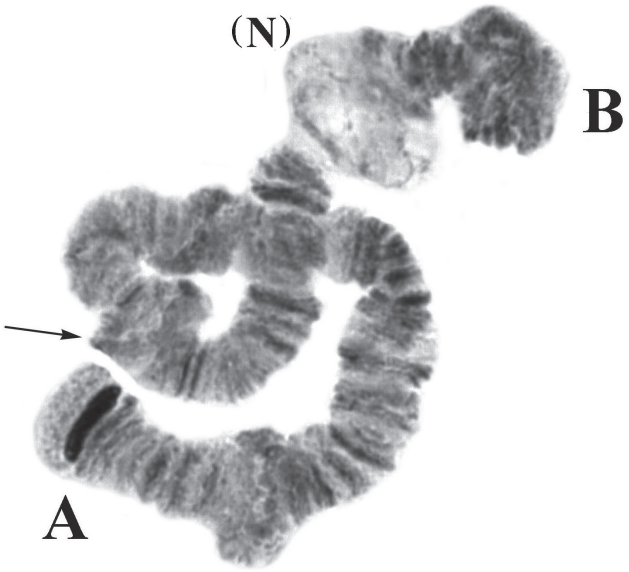

Figure 3. Chromosome I from the Nigerian *P. vanderplanki* population. Chromosome arms are labeled **A** and **B**. Arrow: location of the putative centromere, (N): activated (N_3) nucleolus.

in sec. 1 near the telomere, and the narrowing in sec. 8, which was bordered with easily recognizable groups of bands in sec. 6–7 and in sec. 9–10. In the arm D, the main nucleolus N_2 (sec. 22) was active and constantly present in all cells. The large dark block in sec.24 and groups of conspicuous bands in sec. 18, 20–21 and 26 constituted good markers for this arm (Figs 1, 2).

Chromosome III was divided into 21 sections (Figs 1, 2). The putative centromere was apparently in sec. 9. In all studied individuals, this chromosome presented only homologues of arm E conjugated, whereas in arm F the homologues were constantly uncoupled, due to a large inversion in sec. 11–19. Sometimes, sites near the telomere of arm F did conjugate, owing to an ectopic attraction of large heterochromatic blocks. Uncoupled homologues formed a large number of the meandric breaks and torsions. Arm E was well distinguished on evenly repeating groups of bands in sec. 2–4, 6–7, just as observed in the arm F in species of the genus *Chironomus* (Panis et al. 1994). In arm F, the main marker was the conjoint heterochromatic block in sec. 21, near the telomere. However, groups of bands in sec. 11 and 16 were also easily identified.

Chromosome IV was divided into 12 sections (Figs 1, 2). The putative centromere was localized in sec. 1. The nucleolus N_1 was well developed and localized in chromosome IV, near the centromere (sec. 3). The morphology of this chromosome was slightly different from the other elements of the karyotype: it was half the thickness of any of the long chromosomes. It is possible to assume that the homologues of the chromosome IV are characterized by a lower degree of polytenization, compared to other chromosomes. Such a phenomenon has been observed in some species from the subfamily Chironominae (genus *Sergentia*) and Diamesinae (genus *Sympotthastia*)

(Kerkis 1992, Proviz and Proviz 1992). Despite the prevalence of this phenomenon, it is impossible to be certain of the reason for its appearance. Next markers were the accurate dark bands located from both sides of N_1 and a well developed Balbiani ring (BR) in sec. 5. Besides these markers, the band sequence in arm G formed an easily recognizable picture.

The population was inbred in the laboratory and this explains the low variability observed for chromosomal rearrangements. The only inversion – InvF (10–20) on chromosome III was found with a frequency of 100%.

Karyotype of larvae from Malawi

As a whole, the morphology of the salivary glands was similar to those from the Nigerian population. They also contained 16–20 cells. However, the distinction between populations of cells with different sizes was not so obvious. The diploid chromosome number was also 2n=8 (Fig. 4). The combination of chromosomal arms was AB, CD, EF and G. Chromosomes were denominated from their respective lengths: I=II>III>IV. Chromosomes I and II were metacentrics, chromosome III submetacentric, and chromosome IV telocentric. Putative centromeres appeared as distinct dark heterochromatic blocks. The karyotype was mononucleolar: one obligatory nucleolus (N) was well developed in sec. 3, near the centromere and the telomere in arm G. Apart from the nucleolus N, four Balbiani rings (BR) were localized in chromosome IV. BR_1 was close to N in sec. 4, and the three other BR followed one after another: BR_2 in sec. 7, BR_3 in sec. 8, BR_4 in sec.9.

Chromosome I was arbitrarily divided into 22 sites. The putative centromere was localized in sec. 12 (Fig. 4). The narrowing in sec. 8 and also a group of five dark distinct bands in sec. 9–10 constituted Characteristic markers on the arm A. The arm B was easily distinguished due to the block of almost identical dark bands in sec. 19–20, and the narrowing in sec. 16.

Chromosome II was divided into 21 sections. The putative centromere was located in sec. 12 (Fig. 4). The arm C was easily distinguished due to the light area (sec. 5) bordered on both sides with groups of dark bands (sec. 5–6). In the arm D, we observed a narrowing in sec. 13 near the centromere, and groups of dark blocks in sec. 17–19, including the largest block of the karyotype in sec. 17.

Chromosome III was divided into 18 sections (Fig. 4). The putative centromere was localized in sec. 8. Both telomeres were fanlike. In both arms of E and F, dark blocks containing 4–5 bands were localized near the telomere (sec. 2. and sec. 16–17). These groups were separated from the telomere by conspicuous constrictions. A dense dark band was located between the centromere and sec. 9 in the arm F.

Chromosome IV was divided into 10 sections and the putative centromere was located in sec. 1 (Fig. 4). In this chromosome were localized: the nucleolus N in sec. 3 and four Balbiani rings (BR), which considerably differed on their degree of activity.

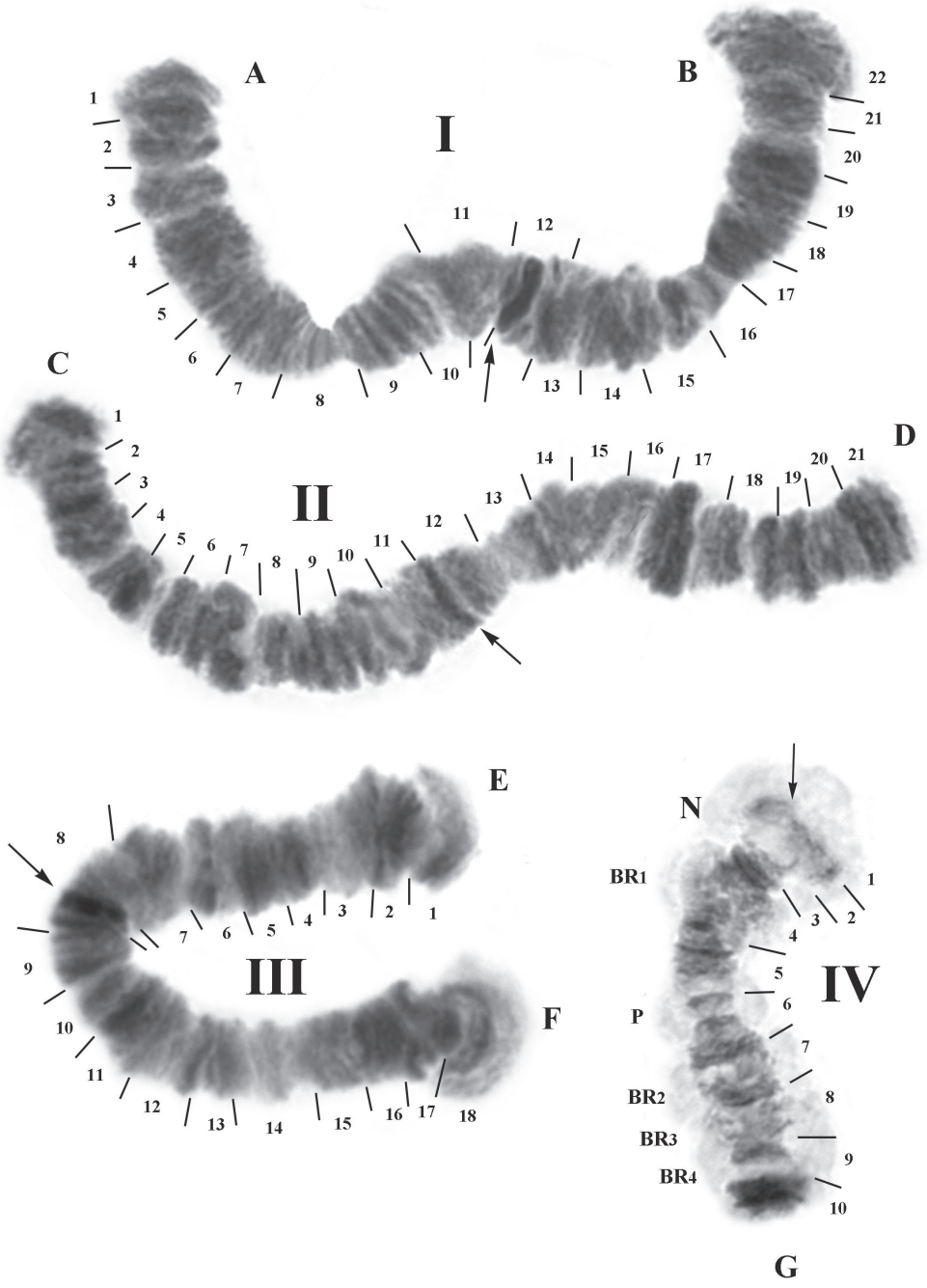

Figure 4. Representative karyotype of the population from Malawi. Chromosome numbers are indicated as **I, II, III** and **IV**. Chromosome arms are labeled **A–B, C–D, E–F,** and **G**. The expected locations of the centromeres are indicated by arrows and each section is numbered and delimited by short lines. N: nucleolus. BR_1, BR_2, BR_3, BR_4: Balbiani rings, P: puff.

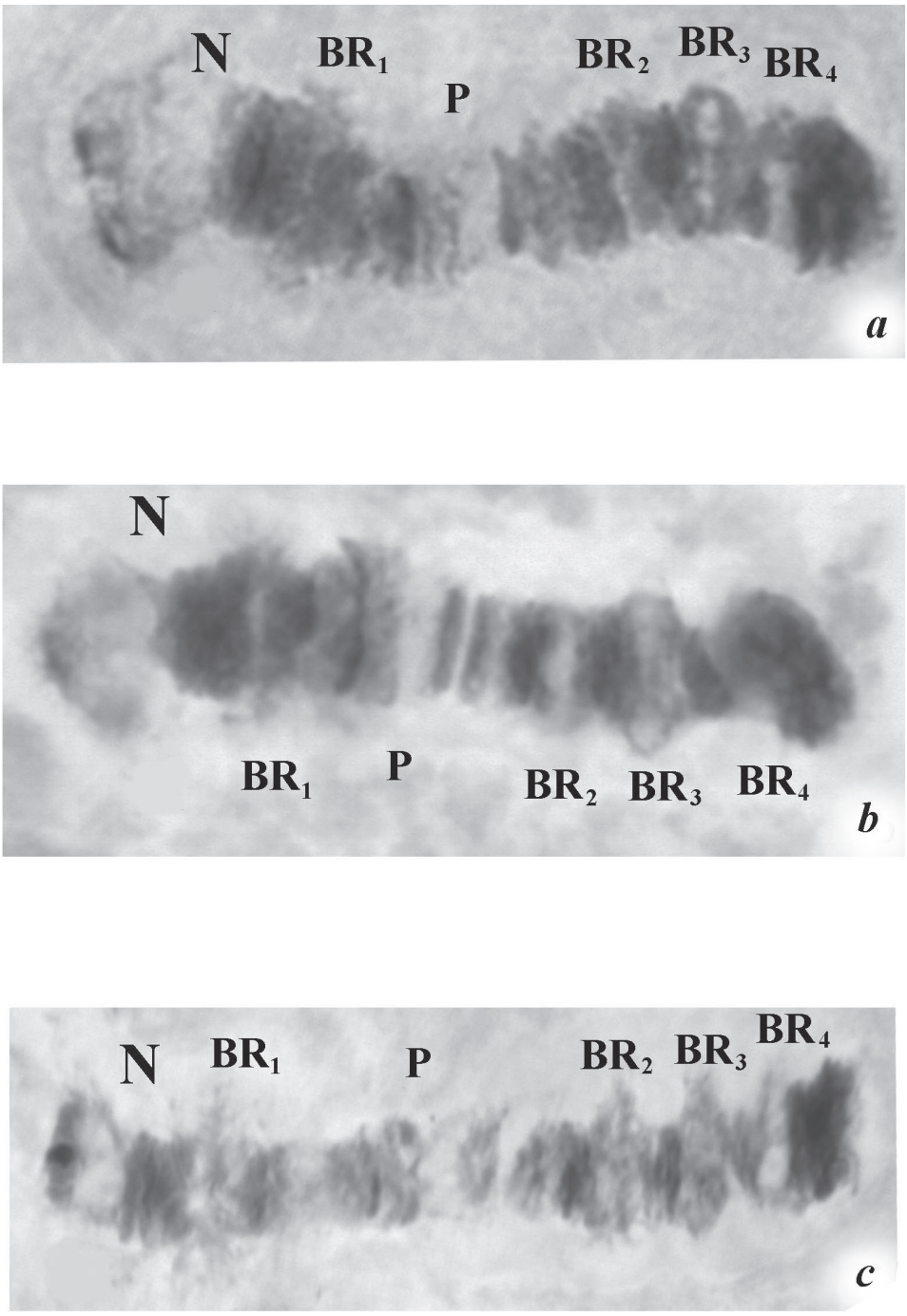

Figure 5. Chromosome IV patterns from different larvae of the Malawian population. Active regions show different levels of condensation. **a** N appears active, BR_1, BR_2, BR_3, BR_4 are slightly active **b** N and BR_3 appear active, BR_1 and BR_2 are slightly active **c** N, BR_1, BR_2, BR_3 and BR_4 are active. N: nucleolus. BR_1, BR_2, BR_3, BR_4: Balbiani rings, P: puff.

N and BR_1 were constitutively active, while other BRs showed variable activity, with various combinations (Fig. 5).

Sometimes all BR were faintly active (Fig. 5a) and in other cases, BR_3 showed a maximal activity (Fig. 5b). In the last case, BR_2 and BR_3 were expressed distinctly, whereas BR_4 was only weakly expressed (Fig. 5c).

Chromosomal polymorphism: For the majority of the studied individuals, we observed mispairing of the homologues. Uncoupled chromosome portions, as a result of torsion, were forming various structures. For example in the AB chromosome (Fig. 6a, b), homologous sections near the centromere were often situated nearby each other, due to asynapsis, and thus the area near the centromere appeared as a thickening.

Sometimes the regions of non-pairing (np) due to heterozygous inversions in the chromosome I (AB) were restricted to some bands only (Fig. 6a). Thus, the band of the centromere in one homologue was normally condensed. When the mispaired chromosome region was more extended, the band corresponding to the putative centromere was almost indistinguishable (Fig. 6b). The accurate band sequence in the arm A, close to the telomere on sec. 17–20 was broken too, due to non-pairing of the homologues and torsion of chromosomes. However, the non-pairing was sometimes observed throughout a large portion in the middle of the chromosome (Fig. 6b). In the chromosome II (arms CD), regions of non-pairing and torsions appeared as large heterochromatic knots (Fig. 6c, double lines). In this chromosome, extended non-pairing near the centromere was also observed quite often (Fig. 6d). Inversion polymorphism was not observed in this population.

Karyotypical distinctions between the two populations

Karyotypical comparison between the two populations of *P. vanderplanki* showed considerable inter-population differences. The diploid number of chromosomes was identical (2n=8), but the band sequences on the chromosomes and the organization of active regions were substantially different. Whereas there was only one N in the Malawian population, the Nigerian population showed two major N. No similarity in the arrangement of the marker groups of bands was found in the long chromosomes. The differences in the morphology of the chromosomes IV were especially noticeable: in the Nigerian population, this chromosome showed one N_1 and one BR, whereas one N and four BR were active in the Malawian population. In both populations, the fact that nucleoli and Balbiani Rings were active or not, should be related to the physiological status of the larva before fixation. Six days after rehydration, the influence of anhydrobiosis was probably negligible, but some larvae may have been engaged in the processes of metamorphosis, which could influence greatly the aspect of chromosomes by activating different transcriptional regions of the genome. However, the relative positions of nucleoli and Balbiani Rings on the chromosomes were completely different between the two populations and this may thus result most probably from populational variation, rather than from differences in the physiological or developmental status.

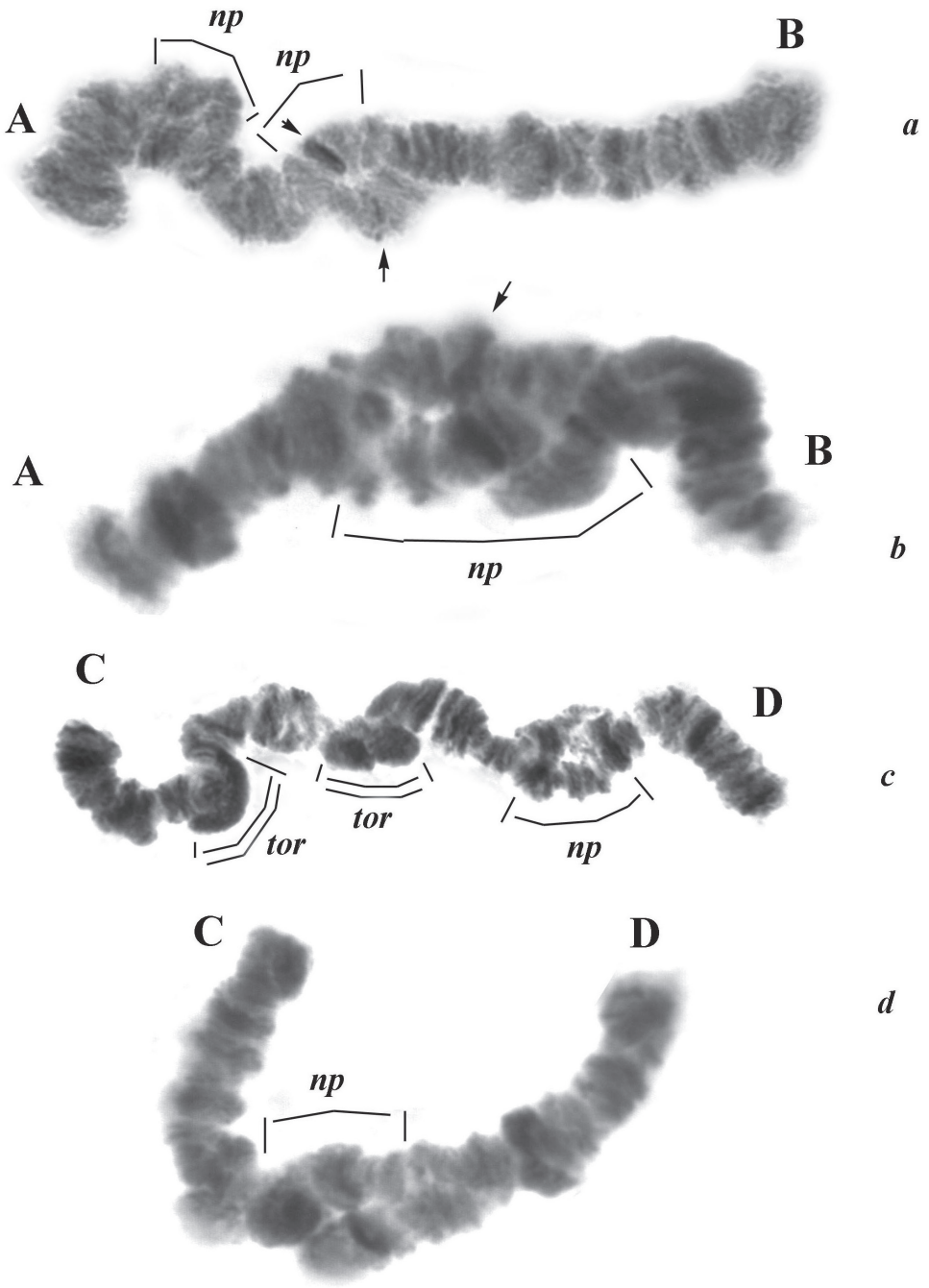

Figure 6. Different patterns of polymorphism for the chromosomes I and II in the Malawian population. **a** and **b** chromosome I. **c** and **d** chromosome II. Chromosome arms are labeled **A–B** and **C–D**. Arrows: putative centromeres, *np*: regions of non-pairing, *tor*: heterochromatic knots due to chromosome torsion.

The major unique feature in the Nigerian *P. vanderplanki* karyotype was the low degree of polytenization of chromosome IV: it was only half as thick as the other chromosomes of the karyotype. Finally, the large heterozygous inversion present in the arm F of chromosome III was observed with a frequency of 100% in the Nigerian population, whereas the Malawian population did not show this chromosome rearrangement in the arm F.

It should be noted that the Nigerian population of *P. vanderplanki* is a highly inbred strain and this could explain the stability of its chromosomal rearrangement. In comparison, the Malawian population was a natural one, not inbred in the laboratory. Strong heterozygosity in this population could induce the mispairings, nonpairings and torsions, which were observed, especially on chromosomes I and II. In addition, desiccation-rehydration cycles are known to induce severe lesions to DNA and subsequent repair (Gusev et al. 2010). When larvae experience anhydrobiosis, chromosome morphology is thus likely to be affected. However, the karyotype pattern in the Nigerian population of *P. vanderplanki* was very stable and constant, even after rehydration. The detailed effect of anhydrobiosis on chromosome morphology is thus an issue that remains to be addressed. In addition to these important differences in bands pattern and general organization of active regions between both Nigerian and Malawian populations, similarities in the organization of the chromosomes were hardly observed in comparison to other published karyotypes in the genus *Polypedilum* (Porter and Martin 1977, Kerkis et al. 1996). The genus *Polypedilum* is actually very diverse with 8 subgenera described (Saether et al. 2010) and this could explain the poor similarity observed in the karyotypes between species spread over these subgenera.

To conclude, the karyotype and precise chromosome map of *P. vanderplanki* were determined for the first time and these data will be useful for future physical mapping of the genome data on the chromosomes. Besides, analysis of the karyotypes of Nigerian and Malawian samples showed important differences between both populations. Whereas chromosomal numbers were identical (2n=8), the morphology of chromosomes was totally divergent. Such important differences between populations exceed physiological variation and intraspecific polymorphism and to our point of view, these *Polypedilum* populations from Nigeria and Malawi should be considered as distinct species. The Nigerian population was originally collected in the Northern part of Nigeria, close to the locality where the type specimen used for the description of *P. vanderplanki* by Hinton (1951) was discovered. In addition, DNA sequence data showed that our laboratory Nigerian strain and Hinton's samples were identical. Consequently, the karyotype presented here for the Nigerian population should be considered as the reference karyotype for *P. vanderplanki*. As a consequence, the Malawian population probably constitutes a new species of anhydrobiotic *Polypedilum*. Since examples of karyological studies on African chironomids are still scarce, accurate morphological description and physiological characterization of this new species of anhydrobiotic *Polypedilum* will be needed in the future.

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RESEARCH ARTICLE

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Genomic *in situ* hybridization identifies parental chromosomes in hybrid scallop (Bivalvia, Pectinoida, Pectinidae) between female *Chlamys farreri* and male *Argopecten irradians irradians*

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Abstract

Interspecific crossing was artificially carried out between *Chlamys farreri* (Jones & Preston, 1904) ♀ and *Argopecten irradians irradians* (Lamarck, 1819) ♂, two of the dominant cultivated scallop species in China. Genomic *in situ* hybridization (GISH) was used to examine the chromosome constitution and variation in hybrids at early embryonic stage. The number of chromosomes in 66.38% of the metaphases was 2n = 35 and the karyotype was 2n = 3 m + 5 sm + 16 st + 11 t. After GISH, two parental genomes were clearly distinguished in hybrids, most of which comprised 19 chromosomes derived from their female parent (*C. farreri*) and 16 chromosomes from their male parent (*A. i. irradians*). Some chromosome elimination and fragmentation was also observed in the hybrids.

Keywords

Scallop, interspecific hybridization, GISH, karyotype, chromosome aberration

Introduction

Utilization of heterosis has become one of the most important strategies for increasing productivity of commercial plants and animals (Hua et al. 2003). As a major approach for this attempt, crossbreeding programs have been extensively employed in agriculture (Vyn and Tollenaar 1998, Kumar 2002, Laurila et al. 2001, Xu et al. 2003) and stockbreeding production (Kahi et al. 2000, Carrapiso et al. 2003). In aquaculture, crossbreeding has been widely developed at both research and application aspects, particularly with some teleost fish species (Rahman et al. 1995, Gross et al. 1996, Kari et al. 1997, Gharrett et al. 1999). With respect to the breeding of marine shellfish, thus far, crossbreeding methods in oyster (Stiles 1978, Menzel 1987, Scarpa and Allen 1992) and abalone (Leighton and Lewis 1982, Yan et al. 1999, Wan et al. 2001, Cai et al. 2010) have been tentatively attempted or even commercially established for aquaculture. Scallop production comprises a pillar component of Chinese shellfish aquaculture in both value and weight. Interspecific hybridization of different pairs of species have been tentatively conducted for investigating their survival potential, growth and fertility for aquaculture purpose (Bower et al. 1997, Yang et al. 2004, Bi et al. 2005, Liu and Chang 2006, Lü et al. 2006a, Lü et al. 2006b, Huang et al. 2011, Wang et al. 2011, Hu et al. 2013). However, so far, only two successful cases of scallop crossbreeding were reported. One is hybrid *Chlamys farreri* (Jones & Preston, 1904) ♀ × *Patinopecten yessoensis* (Jay, 1857) ♂, whose offspring has a high production trait as well as strong disease resistance ability (Yang et al. 2002). The other is reciprocal hybrid between *Argopecten irradians irradians* (Lamarck, 1819) and *A. purpuratus* (Lamarck, 1819), and the hybrids exhibited a great increase in production traits as well as some interesting new characteristics (Wang et al. 2011).

To understand the genetic basis of heterosis, sequences of some nuclear gene and mitochondrial DNA and GISH were used to analyze the genomic constitution of scallop hybrids. Lü et al. (2006a and 2006b) reported that the chromosome number in most of the scallop hybrid between *C. farreri* and *P. yessoensis* was 38, which was accordant to that of their parents. But some abnormal chromosome constitutions were found including haploid, triploid, aneuploid and some gynogenesis-like individuals. The analysis of chromosome components in scallop hybrids between *Mimachlamys nobilis* (Reeve, 1852) and *C. farreri* by Huang et al. (2011) indicated that most of reciprocal hybrids contained 35 chromosomes, corresponding to the theoretical expectation of hybrids between the two species, and a few gynogenetic individuals, as well as chromosome fragmentations, aneuploids and allopolyploids were also detected in some F1 individuals. In the scallop hybrid between *A. purpuratus* and *A. i. irradians* (Hu et al. 2013), GISH verified a combination of haploid genomes of duplex parents in the hybrids. The sequence of the ribosomal DNA internal transcribed spacer region (ITS) showed that the hybrid offspring not only harbored alleles from their parents but also produced some recombinant variants,

which revealed some alterations in the nuclear gene of the hybrids. The mitochondrial 16S rDNA indicated a matrilineal inheritance in scallops. These progresses of genomic analysis in interspecific hybrids showed us some interesting phenomena of genomic structure in scallop hybrids.

The Zhikong Scallop, *C. farreri* is a native species of Northern China. It is an important cultivated scallop species and has accounted for over 60% of the total scallop production in China. The Bay Scallop, *A. i. irradians*, was introduced from North America to Qingdao in 1982 (Zhang et al. 1986). Bay scallops grow quickly and can reach market size (50–60 mm) within a year, which is much faster than Zhikong scallops which usually take 1.5–2.0 years to reach market size. Because of the short grow-out time, bay scallops became an important marine cultured species in China. The production of bay scallops increased considerably due to severe summer mortalities of Zhikong scallops since 1997. These two species have different cytogenetic features. *C. farreri* has a diploid number of 38 with a karyotype of 6m + 10cm + 22st (Wang et al. 1990), but the karyotype of *A. i. irradians* is 2n = 32 = 10st + 22t (Wang and Guo 2004). In addition, in *C. farreri*, the major and minor rRNA genes had one locus each and were mapped to the same chromosome. While in *A. i. irradians*, the major rRNA genes had two loci, the minor rRNA gene had one locus, and all of these three loci were on different chromosomes (Wang and Guo 2004). With these apparent ecological and genetic differences, *C. farreri* and *A. i. irradians* may be potentially useful for crossbreeding to obtain desirable scallop breeds.

We artificially carried out interspecific crossing between *C. farreri* and *A. i. irradians* as an initial step of the ongoing crossbreeding project. In the present study, we reported experimental results of using GISH to verify the hybrid identity of the larvae, and documented a number of interesting patterns of karyotypic abnormalities in some hybrids.

Material and methods

Scallop materials

Sexually mature scallop *C. farreri* ♀ and *A. i. irradians* ♂ (two years old) were obtained from Changfei Scallop Hatchery in Shandong Province, China. Artificial hybridization was carried out in the lab. The main procedures are as followed. Mature parents were induced to spawn by exposure to air for 30 min followed with a temperature shock in 20 °C seawater. Because *A. i. irradians* is hermaphroditic, sperm was filtered by a 25 μm mesh screen in order to avoid introducing eggs of *A. i. irradians*. After collection of the gametes, eggs from *C. farreri* were mixed with sperms from *A. i. irradians* to produce hybrids. Hybrid larvae were reared at 20 °C and sampled at the swimming trochophore stage (approximate 20 h after fertilization) and used for chromosome preparation.

Chromosome preparations

Following colchicine (0.01%) treatment for 2 h at room temperature, the larvae were exposed to 0.075 M KCl for about 30 min. After fixation in Carnoy's fixative (methanol: glacial acetic acid=3:1 v/v) for 3 times (each 15 min), samples were stored at -20 °C. The fixed larvae were dissociated into fine pieces by pippetting in 50% acetic acid. The cell suspension was dropped on hot-wet glass slides and air-dried. For FISH analysis, the chromosome preparations were air-dried and preserved in a moist chamber at -20 °C until use.

Genomic DNA extraction and labeling

Total genomic DNA was extracted from adductor muscle using traditional phenol/chloroform method described by Sambrook et al. (1989). Genomic DNA from one parent was labeled with biotin-11-dUTP by nick translation following the manufacturer's protocol (ROCHE). The length range of probe fragments was approximately 100~600 bp. Labeled probe was purified, ethanol-precipitated and then resolved at a concentration of 5 ng/μl in a hybridization solution of 2×SSC, 50% deinoized formamide, 10% dextran sulphate and 100 μg/μl salmon testis DNA, pH 7.0. A 10-fold unlabeled blocking DNA from the other parental scallop species, which was sonicated to generate fragments of approximately 100~300 bp in length, was added into probe solution in order to block the DNA of the corresponding species.

Genomic *in situ* hybridization

Genomic *in situ* hybridization and probe detection were performed as described by Bi and Bogart (2006) with minor modifications. Before hybridization, slides were incubated at 50 °C for about 3 h, treated with 100 μg/ml RNase A in 2×SSC at 37 °C for 30 min, washed with 2×SSC at room temperature for 15 min, and denatured in a mixture contains 75% formamide and 2×SSC for 2~3 min at 72 °C , dehydrated through a ice-cold ethanol series including 70%, 90% and 100%, 5 min each, and air-dried. Genomic DNA probe mixture was denatured for 5 min at 80 °C , followed by immediately putting on ice for at least 10 min. Probe was pre-annealed by incubating for at 32 °C 5 min prior to hybridization. The probe hybridization mix was applied to the slide and DNA-DNA in situ hybridization was carried out in a dark humid container at 37 °C for 16~18 h. Following hybridization the slides were washed twice in 2×SSC, and 50% formamide at 42 °C for 10 min, 1×SSC at 42 °C for 10 min and finally in 2×SSC at room temperature for 10 min. Biotinylated probes were detected with fluorescein isothiocyanate (FITC) conjugated avidin DCS (Cell Sorting Grade VECTOR) for 1 h at 37 °C . Chromosomes were counterstained with propidium iodide (VECTOR) for 40 min at 37 °C . Hybridization signals were detected by using Nikon epifluorescence microscope E-600 equipped with the appropriate filter sets for FITC and PI. More than 50 metaphase plates were examined by GISH.

Image processing

Digital images were recorded using a CCD camera (COHU) and analyzed with software of Lucia - FISH Image System. The karyotype was determined from more than 10 good metaphase plates and classified according to the criteria defined by Levan et al. (1964).

Results and discussion

The chromosome number of hybrids was determined by observing more than one hundred metaphase plates. The statistic results showed that 66.38% of 116 metaphase plates present a diploid component of 2n = 35 in the hybrids. Ten metaphase plates of hybrids were selected to measure arm length and calculate arm ratio and relative length of chromosomes. The karyotype of hybrids is 2n = 35 = 3m + 5sm + 16st + 11t. Typical mitotic spread of the hybrids was shown in Figure 1. The karyotype of *C. farreri* is 2n = 38 = 6m + 10sm + 22st (Wang et al. 1990), while that of *A. i. irradians* is 2n = 32 = 10st + 22t (Wang and Guo 2004). Most of the hybrid metaphase plates had a diploid chromosome number of 35, as expected from the parental haploid complements. According to the chromosome configuration, all 3 metacentric chromosomes belonged to *C. farreri*, but not *A. i. irradians*. And all 11 telocentric chromosomes, on the contrary, belonged to *A. i. irradians* but not *C. farreri*. These chromosome morphological characteristics can be used for chromosome identification in the hybrid metaphases.

By using blocking DNA and pre-annealing to block homoeologous sequences, labeled genomic DNA probes from one parent could not hybridize to chromosomes from the other one. GISH effectively distinguished all chromosomes of *C. farreri* and *A. i. irradians* in their hybrids, respectively. Examples of GISH results with detection of respective parental genomic DNA probes in hybrids were shown in Figure 1. FITC-labeled genomic DNA of *A. i. irradians* blocked with unlabeled *C. farreri* genomic DNA was hybridized *in situ* to mitotic metaphase chromosome of the hybrids (Fig. 1A, B). At the same time, FITC-labeled genomic DNA of *C. farreri* blocked with unlabeled *A. i. irradians* genomic DNA was hybridized *in situ* to mitotic metaphase chromosome of the hybrids (Fig. 1C, D). On metaphase plates, though much genome cross-hybridization existed, strong contrast could be detected between fluorescein and PI staining. The karyotype of hybrids was 2n = 3m + 5sm + 16st + 11t, which credibly proved to be a combination of haploid genomes of two parents. Of a complement of 35 chromosomes, 19 chromosomes originated from *C. farreri*, whereas the remaining 16 were of *A. i. irradians* origin. Chromosome investigation is an effective method for hybrid genomic analysis. GISH is an efficient cytogenetic technique which allows chromosomes from different parents or ancestors to be distinguished. Labeled total genomic DNA from one parental species was used as a probe, and has often been found to be specific enough to mark the chromosomes from the other parent. Using this technique in hybrids, it is possible to determine the genome origin of paired and unpaired chromosomes in metaphase. GISH has been successfully used in analysis of genome origin

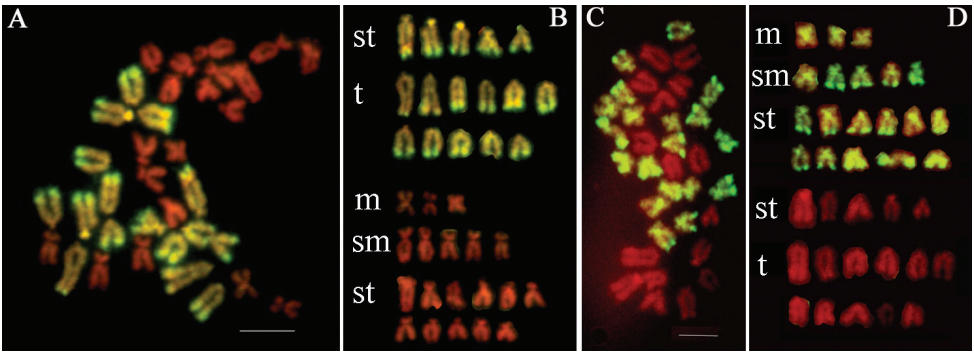

Figure 1. Representative metaphase chromosomes and karyotypes of F1 hybrids of *C. farreri* ♀ × *A. i. irradians* ♂ examined by GISH. Chromosomes were labeled by FITC (green) and counterstained by PI (red). In (**A**, **B**), chromosomes originated from *A. i. irradians* were painted green using the labeled genomic DNA probes from *A. i. irradians*. In (**C**, **D**), chromosomes from *C. farreri* were painted green using the labeled genomic DNA probes from *C. farreri*. m: metacentric, sm: submetacentric, st: subtelocentric, t: telocentric. Bars = 5 μm.

and organization of the hybrid plant (Brysting et al. 2000, Gavrilenko et al. 2001, Falistocco et al. 2002), fish (Fujiwara et al. 1997; Sakai et al. 2007; Ráb et al. 2008) and shellfish (Cai et al. 2010; Lü et al. 2006b; Huang et al. 2011; Hu et al. 2013).

In most metaphases, hybridization signals were not uniform along chromosomes. Some strong signals are located on telomeric region of long arms and centromeric regions in *C. farreri* (Fig. 1A), and on the telomeric region of almost all long arms and two short arms in *A. i. irradians* (Fig. 1C). These uneven signals along chromosomes indicated that some repetitive sequences were located on these regions, which was revealed by FISH using species-specific satellite probes in *C. farreri* (Zhang et al. 2008, Hu et al. 2011, Huang et al. 2011). In *A. i. irradians*, these strong signal locations were accordance with the heterochromatic regions on chromosomes revealed by C-bands, DAPI-bands and FISH using C_0t-1 DNA probes (Huang et al. 2007, Hu et al. 2011). The heterochromtic regions were found mainly in telomeric and centromeric regions by some banding methods in mollusk including scallops (Insua et al. 1998, López-Piñón et al. 2005, Huang et al. 2007), mussels (Martínez-Expósito et al. 1997, Torreiro et al. 1999, Pérez-García et al. 2011) and oysters (Li and Havenhand 1997, Wang et al. 2001, Cross et al. 2005). In addition, the nonuniform distribution of the signals reflected genomic repetitive DNAs to the chromosomes by self-GISH, which were observed in fishes (Targino et al. 2009), plants (She et al. 2007), insects (Pita et al. 2014) and mammals (Suarez-Villota et al. 2012). In *A. i. irradians*, the short arm of two subtelocentric chromosomes showed strong signals after GISH (Fig. 1A). The morphology of these two chromosomes was similar with those two pairs of chromosomes with NORs verified by silver staining and FISH using 18S-28S rDNA probes (Huang et al 2007). The strong signals in NORs were also found in heterochromatic region by self-GISH in plants (She et al. 2007). So we speculated the strong signal regions on short arm of two chromosomes in *A. i. irradians* were the nucleolus organizer regions (NORs).

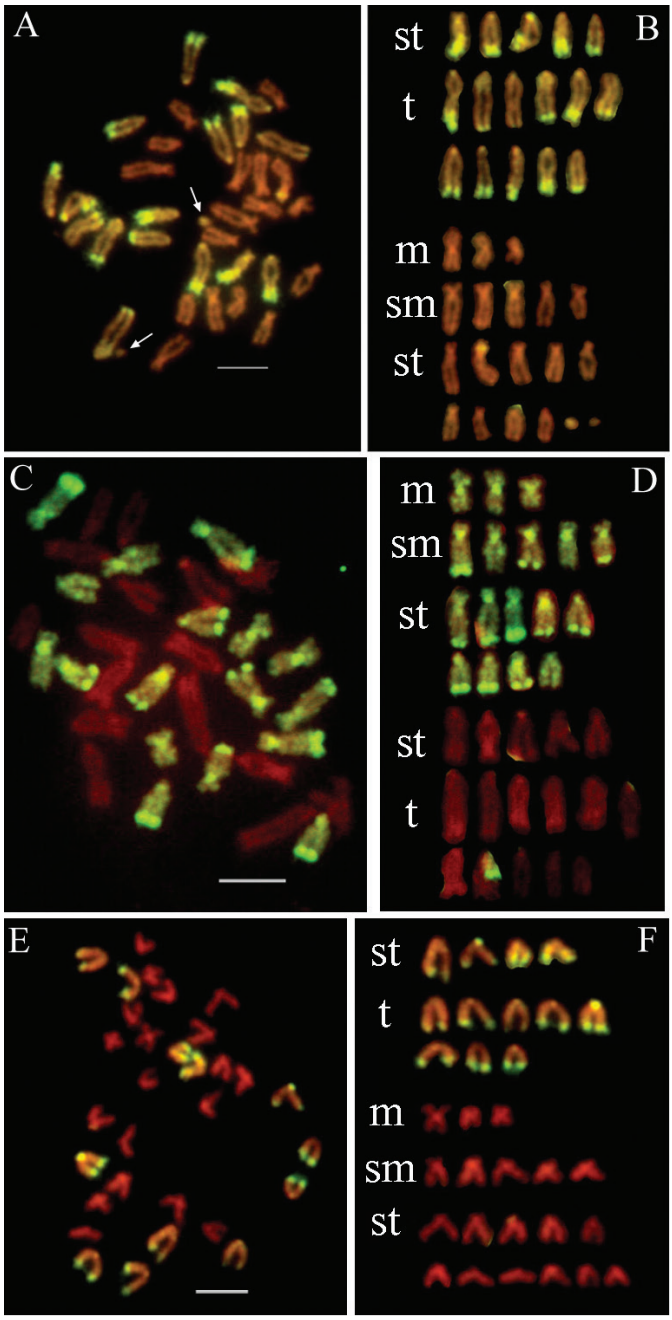

Figure 2. Examples of chromosome fragments (**A, B**) and chromosome eliminations (**C, D, E, F**) in the F1 hybrids. In (**A, B**), chromosome fragments originated from *C. farreri* were marked with arrows. In (**C, D**), some chromosomes from *C. farreri* eliminated in the metaphase spread. In (**E, F**), some chromosomes from *A. i. irradians* eliminated in the metaphase spread. In (**A, B, E, F**), chromosomes were labeled by GISH using *A. i. irradians* genomic DNA probes (green). In (**C, D**), chromosomes were labeled by GISH using *C. farreri* genomic DNA probes (green). Bars = 5 μm.

Table 1. Chromosome number of hybrids (*C. farreri* ♀ × *A. i. irradians* ♂).

	Chromosome number									
	≤30	31	32	33	34	35	36	37	≥38	Total
Number of analyzed metaphases	2	3	9	10	7	77	7	1	0	116
Frequency (%)	1.72	2.59	7.76	8.62	6.03	66.38	6.04	0.86	0	100

During the examination, we also found some metaphases containing chromosome fragments and chromosome elimination. Chromosome fragments were found to originate from *C. farreri* (Fig. 2A, B) in only two metaphases. This phenomenon of chromosome fragments was not reported in other scallop interspecific hybridization. In addition, we found chromosomes derived from *C. farreri* were eliminated in 17.24% metaphases (Fig. 2C, D), which was apparently higher than those from *A. i. irradians* in 9.32% metaphases (Fig. 2E, F). In Table 1, totally 33.62% metaphases were aneuploid, much higher than the intraspecific cross groups 15.6% for *C. farreri*, indicating the instability of the hybrid genome (Huang et al. 2011). Chromosome abnormality is known to be one of the causes for hybrid inviability in some salmonid interspecific hybrids, which is induced by a possible incompatibility between paternal genome and maternal cytoplasm (Fujiwara et al. 1997). Chromosome elimination is observed in natural hybrids of insects such as *Nasonia* (Ashmead, 1904) (Breeuwer and Werren 1990, Reed and Werren 1995). We speculated that the observed chromosome elimination in scallop hybrids was influenced by the ratio or property of parental nuclear genomes and cytoplasms, where chromosomes from one parent were always eliminated by their asynchronous behaviors during mitosis.

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Chromosomal organization of the ribosomal RNA genes in the genus *Chironomus* (Diptera, Chironomidae)

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Abstract

Chromosomal localization of ribosomal RNA coding genes has been studied by using FISH (fluorescence *in situ* hybridization) in 21 species from the genus *Chironomus* Meigen, 1803. Analysis of the data has shown intra- and interspecific variation in number and location of 5.8S rDNA hybridization sites in 17 species from the subgenus *Chironomus* and 4 species from the subgenus *Camptochironomus* Kieffer, 1914. In the majority of studied species the location of rDNA sites coincided with the sites where active NORs (nucleolus organizer regions) were found. The number of hybridization sites in karyotypes of studied chironomids varied from 1 to 6. More than half of the species possessed only one NOR (12 out of 21). Two rDNA hybridization sites were found in karyotypes of five species, three – in two species, and five and six sites – in one species each. NORs were found in all chromosomal arms of species from the subgenus *Chironomus* with one of them always located on arm G. On the other hand, no hybridization sites were found on arm G in four studied species from the subgenus *Camptochironomus*. Two species from the subgenus *Chironomus* – *Ch. balatonicus* Devai, Wuelker & Scholl, 1983 and *Ch.* "annularius" sensu Strenzke, 1959 – showed intraspecific variability in the number of hybridization signals. Possible mechanisms of origin of variability in number and location of rRNA genes in the karyotypes of species from the genus *Chironomus* are discussed.

Keywords

Chironomus, 5.8S rDNA, ribosomal gene localization, polytene chromosomes, NOR, gene mapping, FISH

Introduction

The ribosomal RNA genes in eukaryotic genomes are multiply repeated and form the family of ribosomal genes. They are arranged in clusters comprising hundreds of tandemly repeated units, each consisting of three genes – 18S, 5.8S, and 28S rRNA – separated by transcribed and untranscribed intergenic spacers (Long and David 1980). The clusters of ribosomal RNA genes in chromosomes are located to the nucleolus organizer regions (NORs). Two methods are currently used to detect these regions, namely, FISH (fluorescence *in situ* hybridization) with rDNA probes, allowing for localization of rRNA genes, and silver nitrate staining, allowing for detecting their activity. Studies involving numerous animal and plant groups have demonstrated that the number of NORs and their location on chromosomes may differ not only in distant species, but also in closely related ones. Research into NOR variation in karyotypes has clarified the patterns in chromosome evolution of many insect groups (Cabrero and Camacho 2008, Cabral-de-Mello et al. 2010, 2011, Grzywacz et al. 2011, Oliveira et al. 2011, Neto et al. 2013).

The chromosome evolution of the species belonging to the genus *Chironomus* Meigen, 1803 has been studied in much more detail as compared with the other insect groups owing to the presence of polytene chromosomes with a distinct species-specific banding pattern in the nuclei of their salivary glands (Keyl 1962, Martin 1979, Wuelker 1980, Shobanov 2002, Kiknadze et al. 2008). Seven arms of the *Chironomus* haploid chromosome set comprise over 1000 robustly identifiable bands (chromosome markers), and the homology of banding sequences in chromosomes of different species can be detected using the mapping system devised for this genus (Keyl 1962). Correspondingly, comparison of the banding sequences reliably detects the changes in the linear structure emerging in chromosomes (inversions, deletions, duplications, and translocations). A high density of the known markers – chromosome bands – in chironomid chromosomes makes it possible to find even small chromosome rearrangements involving only one or two bands (Kiknadze et al. 2004a, b). Studies of intraspecific and interspecific polymorphism in banding sequences of the chironomid polytene chromosomes have provided the insight into emergence and spreading patterns of chromosome polymorphism in the distribution ranges of individual species. They also allowed to better understand the phylogenetic relationships between species as well as to reconstruct cytogenetic evolution of the genus *Chironomus* (Keyl 1962, Martin 1979, Shobanov 2002, Kiknadze et al. 2004a, b, 2008).

On the other hand, the information about the number and location of NORs in the chromosomes of *Chironomus* species is mainly based on a phase contrast analyses of acetorcein-stained chromosomes (Beermann 1960). Silver nitrate staining (Lentzios and Stocker 1979) and *in situ* hybridization (Hollenberg 1976, Eigenbrod 1978, Razmakhnin et al. 1982) have been used to study NORs of only a few *Chironomus* species. The absence of these data prevents clarification of the patterns for chromosome evolution of the rRNA gene family in the genus *Chironomus*.

The goal of this work was to study the chromosomal localization of the rRNA locus in the genus *Chironomus* species by means of FISH. The DNA sequences of chironomid species from the rRNA locus carrying 5.8S rRNA gene (5.8S rDNA) and the internal transcribed spacer (ITS-1) separating 18S and 5.8S rRNA genes were selected as the probes.

Material and methods

The IV instar larvae of 21 *Chironomus* species belonging to the subgenera *Chironomus* and *Camptochironomus* Kieffer, 1914 sampled in aquatic bodies of the Novosibirsk region, Russia, were examined. The larvae of North-American species *C. dilutus* Shobanov, Kiknadze & Butler, 1999 were obtained from the laboratory culture maintained at the Institute of Biology of Inland Waters, Russian Academy of Sciences (Borok, Yaroslavl region, Russia). Seven examined species of the subgenus *Chironomus* belong to the group of *Ch. plumosus* sibling species, namely, *Ch. agilis* Schobanov & Djomin, 1988, *Ch. balatonicus* Devai, Wuelker & Scholl, 1983, *Ch. borokensis* Kerkis, Filippova, Shobanov, Gunderina & Kiknadze, 1988, *Ch. entis* Schobanov, 1989, *Ch. muratensis* Ryser, Scholl & Wuelker, 1983, *Ch. nudiventris* Ryser, Scholl & Wuelker, 1983, and *Ch. plumosus* (Linnaeus), 1758. These species as well as *Ch.* "annularius" sensu Strenzke, 1959, *Ch. riparius* Meigen, 1804, *Ch. cingulatus* Meigen, 1830, *Ch. nuditarsis* Keyl, 1961, and *Ch. sororius* Wuelker, 1973 belong to the «thummi» cytocomplex, characteristic of which is the arm combination AB CD EF G in the chromosomes of their karyotype. *Ch. dorsalis* Meigen, 1818, *Ch. luridus* Strenzke, 1959, *Ch. melanescens* Keyl, 1961, and *Ch. pseudothummi* Strenzke, 1959 (arm combination, AE BF CD G) belong to the pseudothummi cytocomplex and *Ch. lacunarius* Wuelker, 1973 (arm combination, AD BC EF G), to the lacunarius cytocomplex. The four species of the *Camptochironomus* subgenus – *C. dilutus*, *C. pallidivittatus* sensu Beermann, 1955, *C. setivalva* Shilova, 1957, and *C. tentans* Fabricius, 1805 – belong to the camptochironomus cytocomplex (arm combination, AB CF ED G).

The larvae were fixed with 96% ethanol (for further DNA extraction) or 3 : 1 *v/v* of 96% ethanol and glacial acetic acid (for making preparations of salivary gland polytene chromosomes for FISH hybridization) and stored at –20 °C. Species were identified according to morphological characteristics of larvae and by cytogenetic analysis of banding patterns of polytene chromosomes from salivary glands (Kiknadze et al. 1991).

Genomic DNA was isolated from individual larvae using a DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's protocol. DNA probes were produced by polymerase chain reaction (PCR) with the primers 5'-GTAACAAGGTTTCCGTAGG-3' (chir5F) and 5'-CGACACTCAACCATATGTACC-3' (chir5R) (Gunderina and Katokhin 2011, Gunderina 2014). Either genomic DNA or isolated, purified, and characterized DNA fragments with a length of ~480 bp from the 18S–5.8S rDNA region of the chironomid species listed in Table 1 were used as a template. The rDNA probes were labeled with biotin-11-dUTP or digoxigenin-11-dUTP (Roche,

Germany). The DNA probes were precipitated according to a standard technique with fragmented salmon DNA as a DNA carrier. The ITS-1 and 5.8S rDNA sequences used as a DNA probes were aligned to characterize the interspecific differences using the MUSCLE program (Edgar 2004) (http://www.ebi.ac.uk). Molecular genetic analysis of these sequences was conducted using the MEGA 6 software package (Tamura et al. 2013). For NJ-tree construction sequences ITS1 and 5.8S rDNA of *Drosophila melanogaster* M21017 from GenBank database were used as an outgroup.

For FISH, the polytene chromosomes were prepared from the larvae fixed with 3 : 1 *v/v* of 96% ethanol and glacial acetic acid according to the following procedure. A larva was placed into 70% ethanol to extract its salivary glands and transfer them onto a glass slide into a drop of 45% acetic acid. The cells were separated from secretion by removing it from the glass, gently covered with a cover glass, and squashed, removing excess acid with filter paper. The ready preparation was placed for 10–15 min onto a metal table cooled with liquid nitrogen to remove the cover glass; the slide was then kept for 5 min at a room temperature, 5 min in 70% ethanol, and air-dried for 1 week.

FISH was conducted according to the following protocol. The preparations were air-dried for one week. They were then incubated with RNase A (100 mg/ml in 2× SSC) for 1 h at 37 °C, washed at a room temperature for 5 min with 2× SSC, dehydrated with alcohols (70, 90, and 96% ethanol, 5 min in each), and air-dried for 10 min. Then the slide was incubated with 0.02% pepsin in 10 mM HCl for 6 min at 37 °C, washed with a series of phosphate buffers (5 min in PBS, 5 min in PBS with 50 mM $MgCl_2$, 10 min in PBS with 50 mM $MgCl_2$ and 1% formaldehyde, and again in PBS and PBS with 50 mM $MgCl_2$, 5 min each) at a room temperature, and dehydrated in alcohols as described above. DNA probe (dissolved in 20 µl of 2× SSC with 50% deionized formamide for 1 h at 37 °C in a thermoshaker at 800 rpm) was applied to dry slide, covered with a cover glass, and incubated for 12–15 h at 37 °C in a humid chamber. The slides were then washed in a shaker (100 rpm, 37 °C) two times for 10 min in 2× SSC with 50% deionized formamide and 0.1% NP40, two times for 5 min in 2× SSC, two times for 5 min in 0.2× SSC, and one time in 4× SSC with 3% BSA; then antibody solution (20 µl) was added, the slide was covered with a cover glass and incubated in a humid chamber at 37 °C for 40 min. The DNA probes labeled with biotin or digoxigenin were detected using the antibodies labeled with the fluorochromes avidin-Alexa fluor®488 or Cy3, respectively. The antibodies were diluted with 4× SSC containing 3% BSA (1–2 µl antibodies per 100 µl reaction mixture) and dissolved for 1 h in a thermoshaker (800 rpm, 37 °C) in parallel with washings after the hybridization with DNA probes. On completion of the incubation with antibodies, the slides were washed in a shaker (110 rpm, 37 °C) three times, 5 min each, in 4× SSC with 0.1% NP40; dehydrated with alcohols; air-dried for 15 min; mounted in a DAPI-containing antifade; and covered with a cover glass. Homologous DNA probes (the karyotype and DNA probe belongs to the same species) and heterologous DNA probes (the karyotype and DNA probe belongs to different species) were used for FISH.

The slides were examined using the equipment of the Joint Access Center for Microscopy of Biological Objects with the Siberian Branch of the Russian Academy of

Sciences, namely, AxioPlan2 Imaging microscope and Axio Cam HRc CCD camera with the help of Isis 4 software package (Zeiss, Germany).

Mapping of polytene chromosomes in arms A, C, D, E and F was done according to Keyl–Devai system (Keyl 1962, Devai et al. 1989). Arm B was mapped according to Keyl–Devai system (Keyl 1962, Devai et al. 1989) in *Ch. riparius*, according to Maximova–Shobanov system (Maximova 1976, Shobanov 1994) in species of *Ch. plumosus* group of sibling species, and was not mapped in other species studied in this paper. Arm G was mapped according to Keyl–Hägele system (Keyl 1957, Hägele 1970) in *Ch. riparius* and according to Maximova–Shobanov system (Maximova 1976, Shobanov 1994) in *Ch. plumosus*, *Ch. borokensis* and *Ch. balatonicus*. Mapping of polytene chromosomes of species from the subgenus *Camptochironomus* was done according to Beermann system (Beermann 1955).

Results

Karyotypes of most *Chironomus* species studied in this work have four polytene chromosomes, which corresponds to the haploid chromosome set n = 4 (Figs 1–4). The only exception is *Ch. nudiventris* that have three chromosomes in its haploid set. The chromosome number in the karyotype of this species reduced via fusion of arms G (chromosome IV) and E (chromosome III, EF) to form the joint chromosome GEF (Fig. 3d, Table 3).

DNA-probe used for FISH analysis consists of two main components: gene coding 5,8S rRNA and internal transcribed spacer (ITS1). The percent of identity between ITS-1 sequences of the *Chironomus* species used for producing DNA probes is considerably lower as compared with the percent of identity between their 5.8S rDNA sequences (Table 2). However, FISH has demonstrated that despite considerable interspecific differences in ITS-1, if the probe contains conserved 5.8S rDNA sequences, the number and location of hybridization sites for homologous and heterologous marker DNAs in the karyotypes of examined chironomid species completely coincide (Fig. 1). It should be also noted that the hybridization sites of rDNA probes in most of the studied species coincide with the positions of NORs on chromosomes. This suggests that hybridization of the DNA probes to the chromosomes is mainly determined by the 5.8S rDNA nucleotide sequences and that the regions where rDNA probes hybridize to chromosomes are NORs.

The number of NORs in the studied *Chironomus* species is different (Figs 1–4, Table 3). Only one chromosome site of rDNA probe hybridization is observed in 12 chironomid species; two sites, in five species; and three, five, or six sites, in the remaining four species (Table 3).

The species belonging to the subgenera *Chironomus* and *Camptochironomus* are similar in the number of rDNA loci in their karyotypes (one or two NORs) but differ considerably in their chromosomal positions. In species from the subgenus *Chironomus* NORs have been found in all chromosomal arms, whereas in species from the

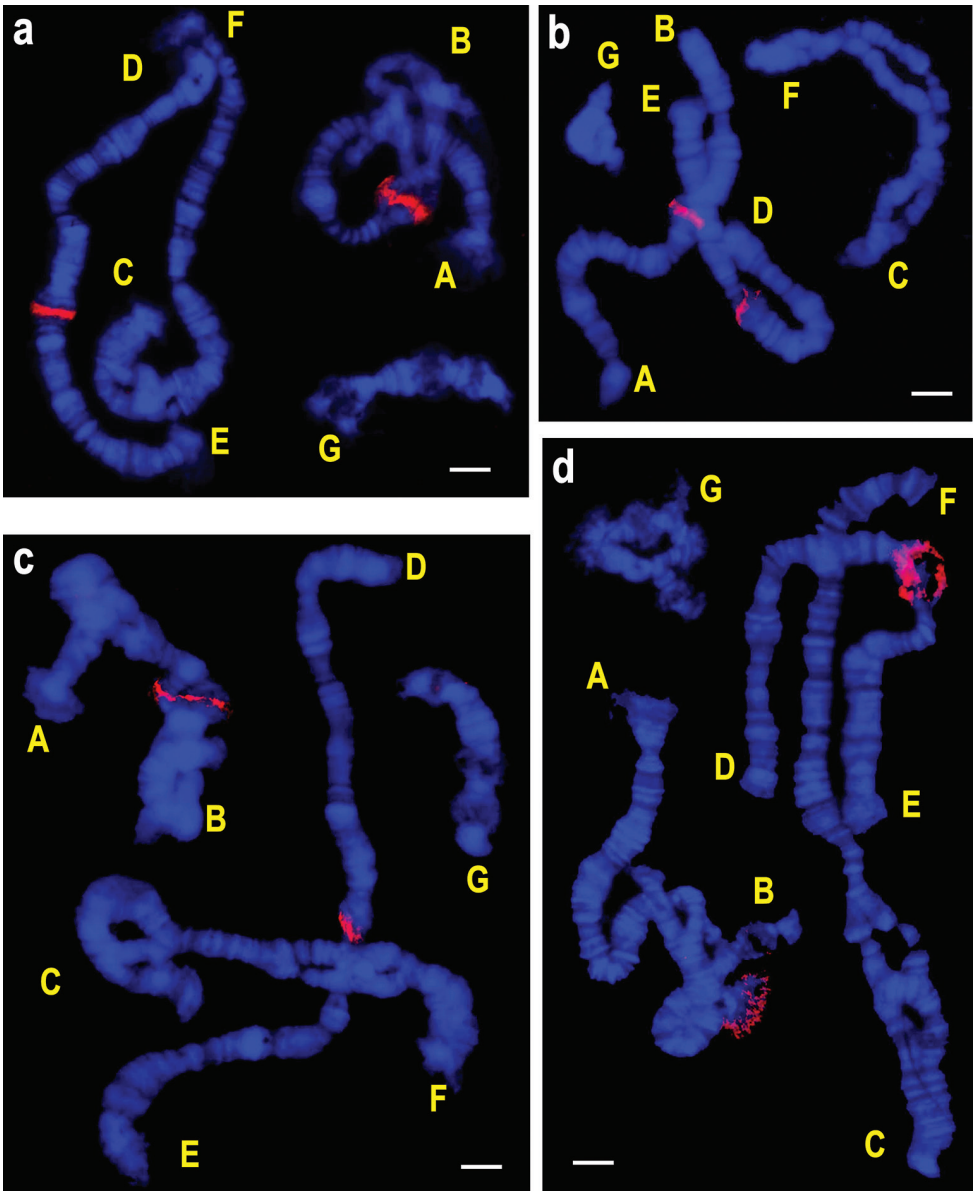

Figure 1. FISH of homologous (**a**) and heterologous (**b–d**) rDNA probes on the polytene chromosomes of *C. tentans.* **a** *ITS-1 + 5.8S_ten* (Cy3) **b** *ITS-1 + 5.8S_pal* (Cy3) **c** *ITS-1 + 5.8S_dil* (Cy3) **d** *ITS-1 + 5.8S_set* (Cy3). Letters designate chromosomal arms. Bar = 10 μm.

subgenus *Camptochironomus* NORs have been detected in arms A, B, C and D only. Unlike species belonging to the subgenus *Chironomus* with obligatory presence of one of the NORs in arm G (Figs 2–3), no NOR in this arm has been detected in all four

Table 1. The DNA probes used in the work.

	Species	DNA probe	GenBank accession number
1	*Ch. agilis*	*ITS-1 + 5.8S_agi*	GU053584
2	*Ch.* "annularius"	*ITS-1 + 5.8S_ann*	HQ656600
3	*Ch. balatonicus*	*ITS-1 + 5.8S_bal*	GU053586
4	*C. dilutus*	*ITS-1 + 5.8S_dil*	KP985232
5	*Ch. dorsalis*	*ITS-1 + 5.8S_dor*	GU053590
6	*Ch. muratensis*	*ITS-1 + 5.8S_mur*	GU053605
7	*C. pallidivittatus*	*ITS-1 + 5.8S_pal*	KP985231
8	*Ch. plumosus*	*ITS-1 + 5.8S_plu*	GU053597
9	*Ch. riparius*	*ITS-1 + 5.8S_rip*	GU053603
10	*C. setivalva*	*ITS-1 + 5.8S_set*	-
11	*C. tentans*	*ITS-1 + 5.8S_ten*	KP985230

Table 2. The percent of identity between 5.8S rDNA nucleotide sequences (above) and ITS-1 (below) in *Chironomus* species.

	Ch. agilis	*Ch. balatonicus*	*Ch. muratensis*	*Ch. plumosus*	*Ch.* "annularius"	*Ch. riparius*	*Ch. dorsalis*	*C. dilutus*	*C. pallidivittatus*	*C. tentans*
Ch. agilis		**100**	**100**	**100**	**99**	**99**	**100**	**100**	**100**	**100**
Ch. balatonicus	92		**100**	**100**	**99**	**99**	**100**	**100**	**100**	**100**
Ch. muratensis	94	95		**100**	**99**	**99**	**100**	**100**	**100**	**100**
Ch. plumosus	91	94	96		**99**	**99**	**100**	**100**	**100**	**100**
Ch. "annularius"	87	90	87	87		**98**	**100**	**100**	**100**	**100**
Ch. riparius	75	76	76	77	76		**100**	**100**	**100**	**100**
Ch. dorsalis	74	78	76	76	73	80		**100**	**100**	**100**
C. dilutus	76	79	77	78	79	73	73		**100**	**100**
C. pallidivittatus	77	79	78	78	79	73	73	96		**100**
C. tentans	77	80	79	78	78	74	73	95	98	

Camptochironomus species (Fig. 4). In *Camptochironomus* NOR is most frequently found in arm B, being observed in three of the four examined species, namely, *C. setivalva*, *C. dilutus*, and *C. tentans*. In the *C. setivalva* karyotype it is the only one NOR found, while *C. dilutus* and *C. tentans* carried one additional NOR in arms C and D, respectively. Only one NOR has been found in arm A of *C. pallidivittatus* (Fig. 4).

Seven species from the subgenus *Chironomus* carried rDNA hybridization sites in other chromosomal arms besides the NOR in arm G. These species can be divided into three groups according to the hybridization pattern of DNA probes.

Two NORs are always observed in the karyotypes of the first group (*Ch. agilis* and *Ch. cingulatus*) and the hybridization sites of DNA probes are similar in the intensity of hybridization and completely coincide with the localized NORs. Both NORs of *Ch. agilis* are located in arm G (one in the centromeric and the other in the telomeric regions); as for the *Ch. cingulatus* NORs, they are located on arms B and G (Fig. 3a, b, Table 3).

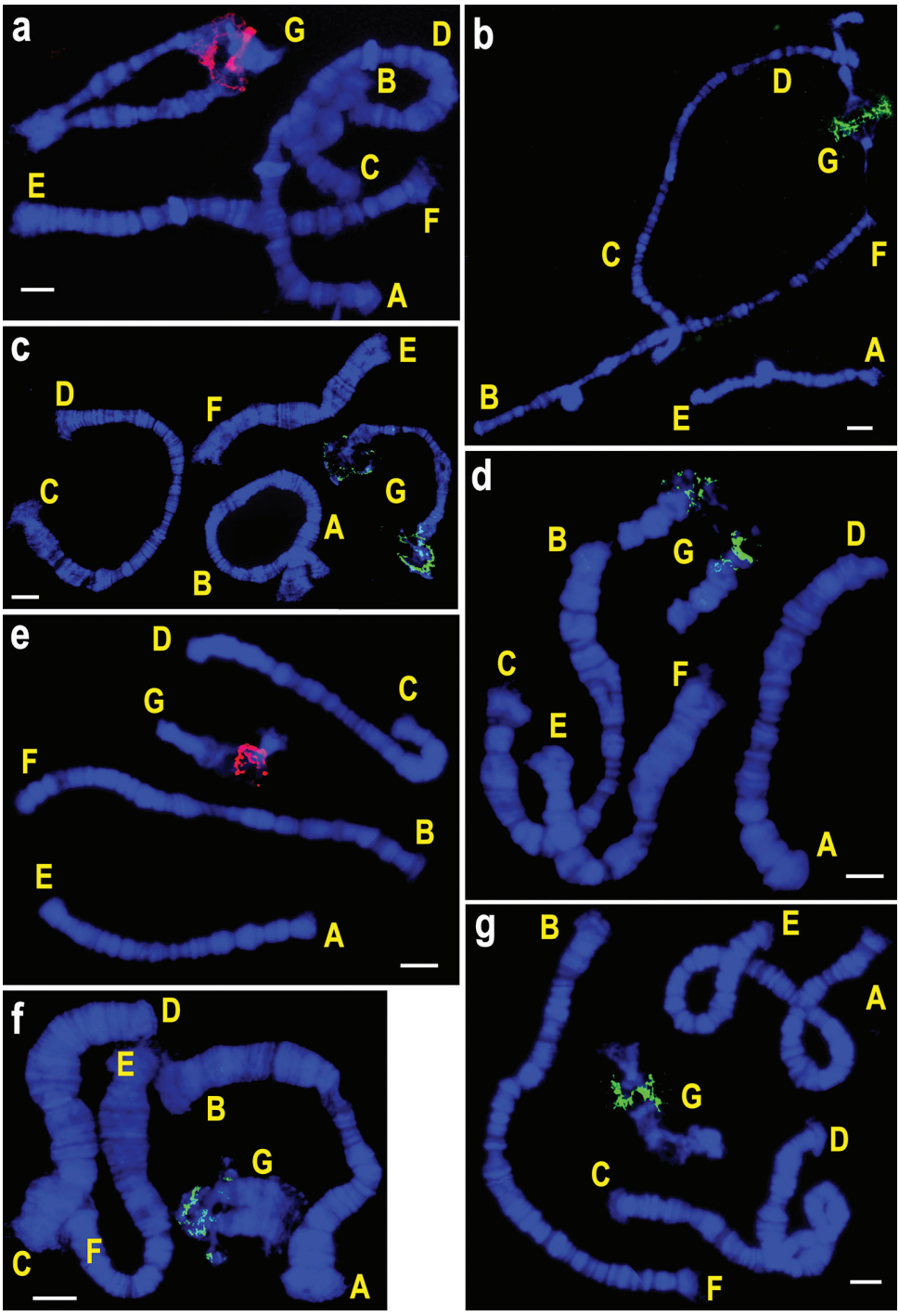

Figure 2. FISH of rDNA probes on polytene chromosomes of species from the subgenus *Chironomus* with one NOR in karyotype. **a** *Ch. borokensis* **b** *Ch. dorsalis* **c** *Ch. entis* **d** *Ch. lacunarius* **e** *Ch. luridus* **f** *Ch. nuditarsis* **g** *Ch. melanescens*.

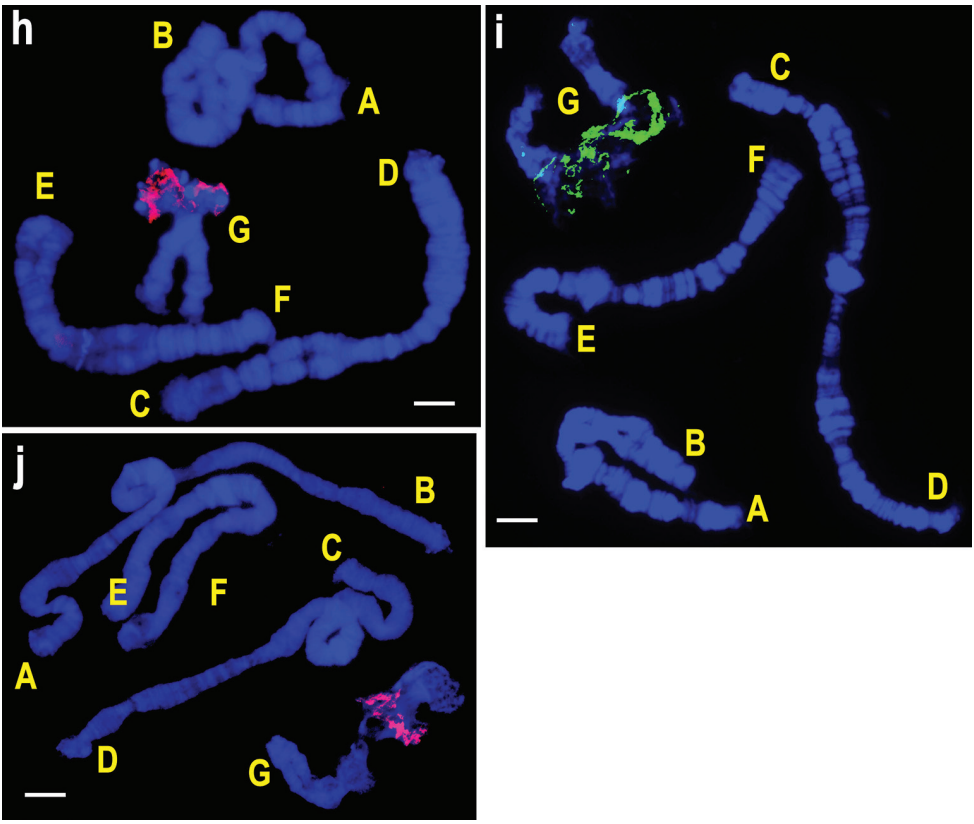

Figure 2. Continued. FISH of rDNA probes on polytene chromosomes of species from the subgenus *Chironomus* with one NOR in karyotype. **h** *Ch. plumosus* **i** *Ch. sororius* **j** *Ch. riparius*. Letters designate chromosomal arms. Bar = 10 μm.

The second group includes species with the number of hybridization sites for DNA probes exceeding the number of cytologically identifiable NORs and with the intensity of hybridization varying between hybridization sites (*Ch. muratensis*, *Ch. nudiventris*, and *Ch. pseudothummi*). In the karyotype of *Ch. muratensis*, two strong rDNA hybridization signals are always detected in regions developing NORs – one in arm G and the other in arm C, and in addition, weak hybridization signals varying in their intensity and number are detected in arms B, C, D, and F in the regions, where a developed nucleolus has never been observed (Fig. 3c, Table 3). Three rDNA hybridization signals are detected in the karyotypes of *Ch. nudiventris* and *Ch. pseudothummi*; the strongest signal is located in arm G and coincides with the active NOR in both species, while two weaker signals are located in arm D region 2h–d in *Ch. nudiventris* and arms G and C in *Ch. pseudothummi* (Fig. 3d, e, Table 3). It is necessary to note that active NORs were never been detected in regions with weak hybridization signals in karyotypes of these three species.

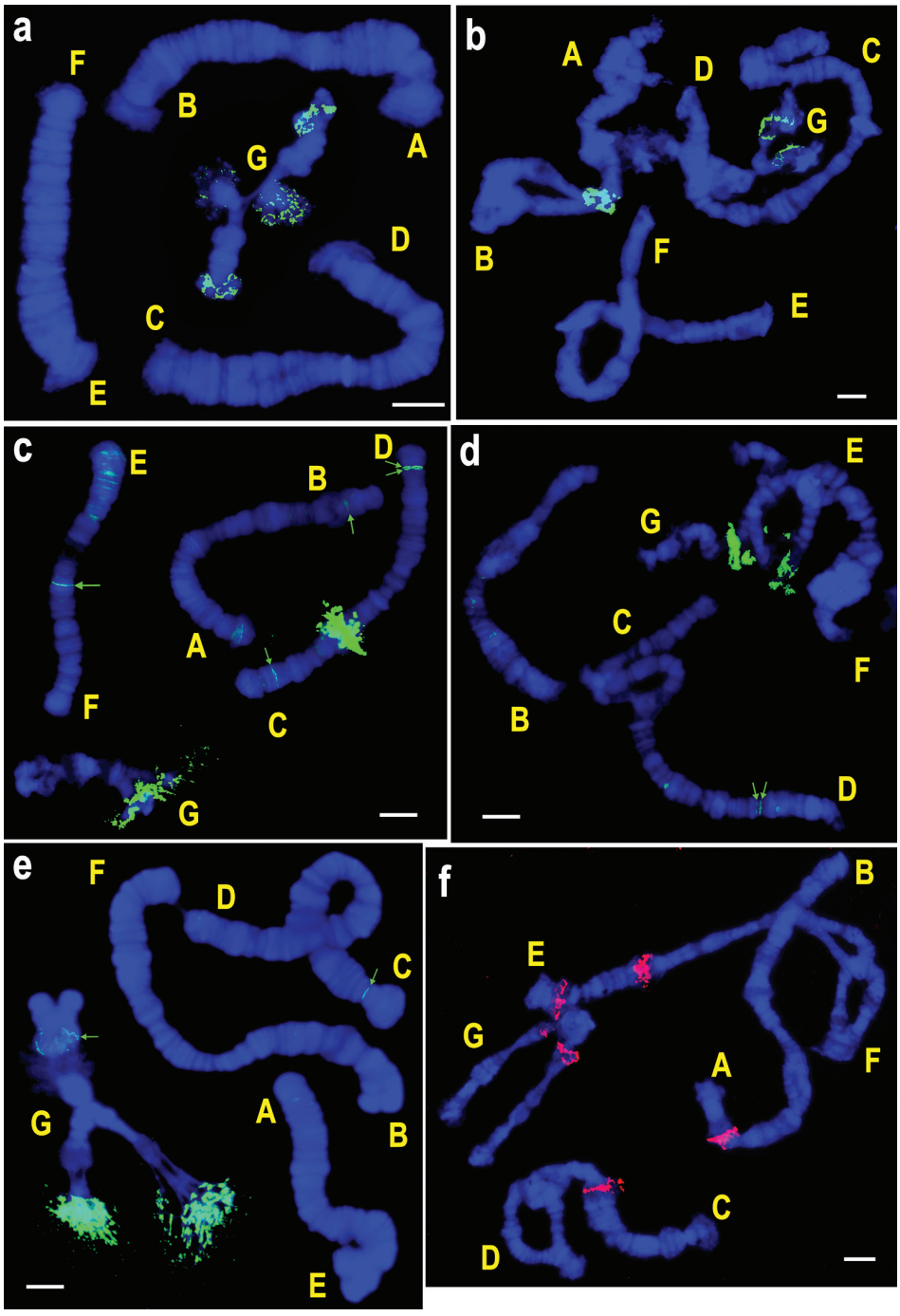

Figure 3. FISH of rDNA probes on polytene chromosomes of species from the subgenus *Chironomus* with multiple localization of hybridization sites. **a** *Ch. agilis* **b** *Ch. cingulatus* **c** *Ch. muratensis* **d** *Ch. nudiventris* **e** *Ch. pseudothummi* **f** *Ch.* "annularius". Letters designate chromosomal arms. Green arrows show sites of weak hybridizations signals. Bar = 10 μm.

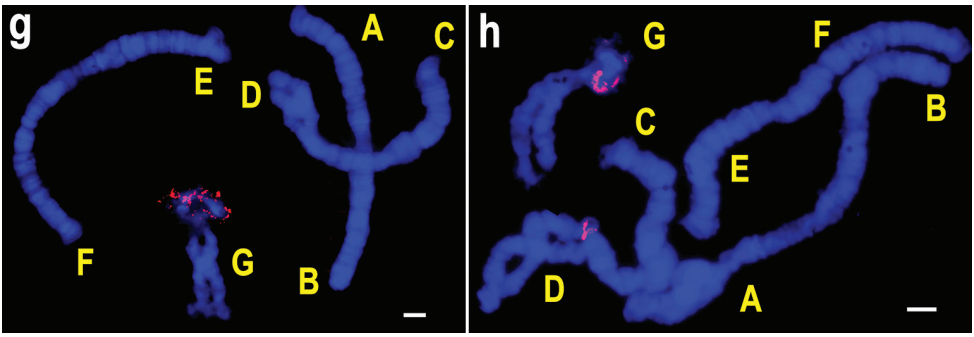

Figure 3. Continued. FISH of rDNA probes on polytene chromosomes of species from the subgenus *Chironomus* with multiple localization of hybridization sites. **g** *Ch. balatonicus* with one NOR **h** *Ch. balatonicus* with additional NOR in arm D. Letters designate chromosomal arms. Green arrows show sites of weak hybridizations signals. Bar = 10 μm.

The number of NORs in the karyotypes of the third group of species (*Ch. balatonicus* and *Ch.* "annularius") may vary, however the hybridization sites of DNA probes always coincide with the active NORs (Fig. 3f–h, Table 3). The karyotype of *Ch. balatonicus* may have one or two NORs: one constantly present in arm G and the other, polymorphic, in arm D (Fig. 3g, h). However, rDNA hybridization signals were detected in arm D only if the arm carried one of the banding sequences balD3, balD17, or balD23. No hybridization signals or developed nucleoli were observed in other arm D banding sequences of *Ch. balatonicus* (Fig. 3g).

The karyotype of *Ch.* "annularius" has either four or five NORs. Four NORs are found in all studied specimens (two NORs in arm E and one in each of arms C, and G). An intraspecific NOR polymorphism has been observed in *Ch.* "annularius" arm A, region 3g (Fig. 3f). NOR localized to this region is present in either homozygous or heterozygous state in approximately 70% of the larvae (Kiknadze et al. 2012). The rDNA hybridization signals in arm A have not been detected in one-third of the examined larvae, which coincide with the absence of active NOR in this region.

Discussion

The genus *Chironomus* comprises over 150 species (Shobanov et al. 1996). The karyotypes of these species are usually studied using the salivary gland polytene chromosomes rather than mitotic or meiotic chromosomes as in the majority of other insect species. The fact is that the mitotic and meiotic chromosomes of chironomids are very tiny, 1–5 μm, which prevents from distinguishing secondary constrictions and other chromosome markers, while karyotypes of species are very similar. Polytene chromosomes of chironomids are considerably longer. The average lengths of *Ch. riparius* metacentric and submetacentric polytene chromosomes (I–III) are 110, 100, and 85 μm, respectively, and the shortest acrocentric chromosome (IV) reaches 30 μm (Kiknadze and

Table 3. The number of chromosome pairs in karyotype, combinations of arms in chromosomes, and number and locations of nucleolus organizer regions (NORs) in *Chironomus* species.

Species	Number of chromosome pairs in karyotype	Arm combination in chromosomes	Number of NORs	NOR location	
				Chromosome arm	Chromosome region
Subgenus *Chironomus*					
1. *Ch. agilis*	4	AB CD EF G	2	G	1a, 1bc†
2. *Ch. balatonicus*	4	–//–	2	G D	1† 18fg‡
3. *Ch. borokensis*	4	–//–	1	G	1†
4. *Ch. entis*	4	–//–	1	G	1†
5. *Ch. muratensis*	4	–//–	6	B C D F G	24i-j† 16‡ 2h, 2d‡ 10c‡ 1†
6. *Ch. plumosus*	4	–//–	1	G	1†
7. *Ch. nudiventris*	3	AB CD GEF	3	G D	1† 2h, 2d‡
8. *Ch.* "annularius"	4	AB CD EF G	5	A C E G	3g‡ 15c–17b‡ 3a–4h, 9–10b‡ not mapped
9. *Ch. riparius*	4	–//–	1	G	3§
10. *Ch. cingulatus*	4	–//–	2	B G	not mapped not mapped
11. *Ch. nuditarsis*	4	–//–	1	G	not mapped
12. *Ch. sororius*	4	–//–	1	G	not mapped
13. *Ch. lacunarius*	4	AD BC EF G	1	G	not mapped
14. *Ch. dorsalis*	4	AE BF CD G	1	G	not mapped
15. *Ch. luridus*	4	–//–	1	G	not mapped
16. *Ch. melanescens*	4	–//–	1	G	not mapped
17. *Ch. pseudothummi*	4	–//–	3	G C G	not mapped 4d‡ not mapped
Subgenus *Camptochironomus*					
18. *C. dilutus*	4	AB CF ED G	2	B C	9| 10|
19. *C. pallidivittatus*	4	–//–	1	A	12|
20. *C. setivalva*	4	–//–	1	B	9|
21. *C. tentans*	4	–//–	2	B D	9a–b| 9b|

† mapping according to Maximova-Shobanov system (Maximova 1976, Shobanov 1994)

‡ mapping according to Keyl-Devai system (Keyl 1962, Devai et al. 1989)

§ mapping according to Keyl-Hagele system (Keyl 1957, Hagele 1970)

| mapping according to Beermann system (Beermann 1955)

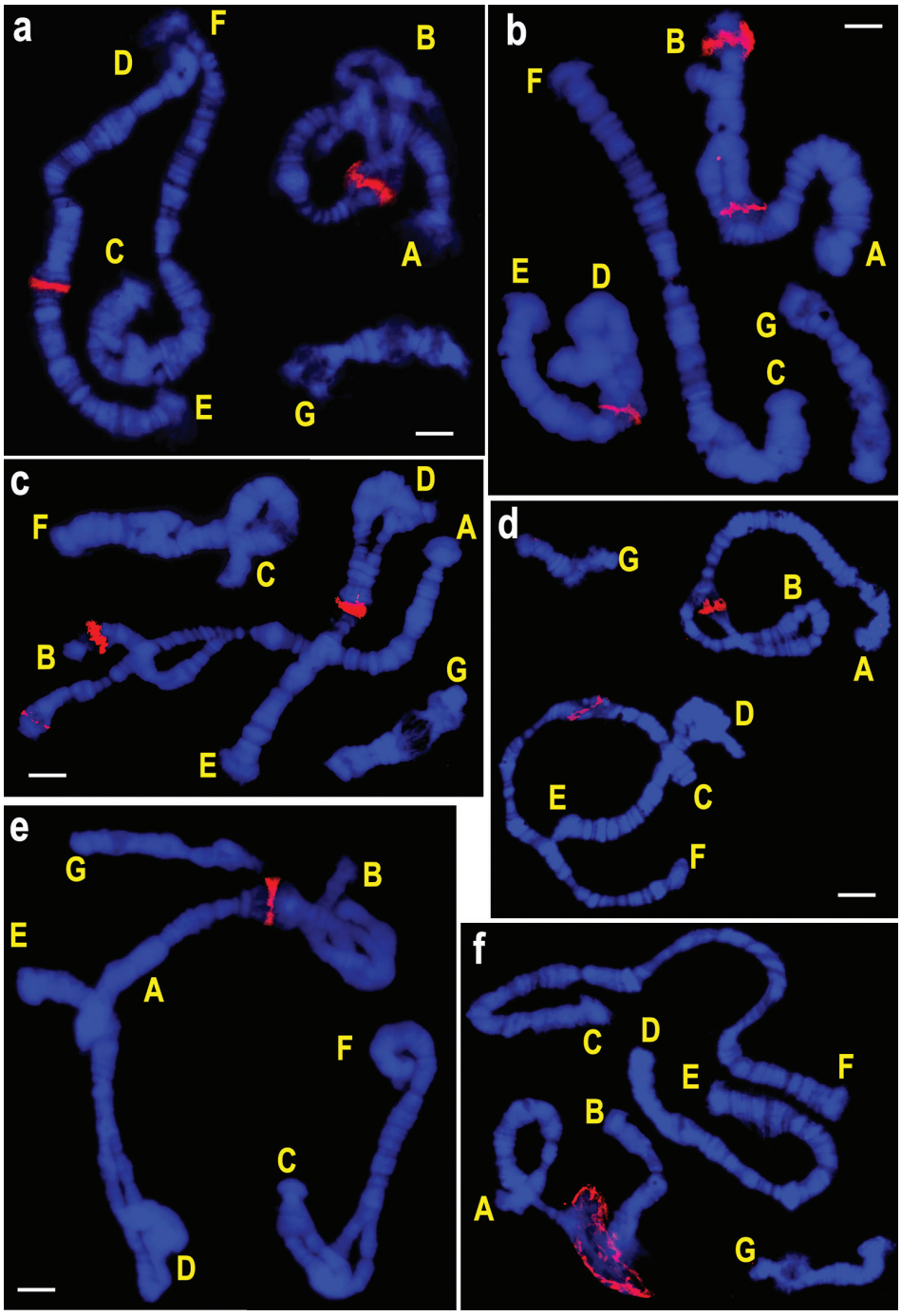

Figure 4. FISH of rDNA probes on polytene chromosomes of species from the subgenus *Camptochironomus.* **a–c** *C. tentans*, where **b** and **c** specimens with heterozygous inversions in arm B that change the position of NOR in one of the homologues **d** *C. dilutus;* **e** *C. pallidivittatus* **f** *C. setivalva*. Letters designate chromosomal arms. Bar = 10 μm.

Gruzdev 1970). Four chromosomes of chironomid haploid karyotype comprise over 1000 precisely mapped bands (Kiknadze et al. 2004a, b). Thus, the density of markers (bands and interbands) is sufficient to robustly identify individual species, detect chromosome rearrangements, and study chromosome evolution of the genus *Chironomus*.

NORs are additional markers in polytene chromosomes. Nucleoli are actively transcribed regions of chromosomes, visible on chromosomes as giant puffs. Phase contrast microscopy of orcein-stained chromosomes allows them to be distinguished from any other functionally active chromosome regions (Beermann 1960). However, the activity of nucleoli significantly varies during chironomid development (Kiknadze et al. 1981, Razmakhnin et al. 1982), creating certain problems with their precise identification. Most of these problems can be resolved by the use of *in situ* hybridization, $AgNO_3$ staining, and FISH (Hollenberg 1976, Eigenbrod 1978, Lentzios and Stocker 1979, Razmakhnin et al. 1982). The data on NORs detected using silver staining in 11 Australian chironomid species (Lentzios and Stocker 1979) and FISH in 21 Palearctic chironomid species (this work) demonstrate that the number and locations of the NORs detected in polytene chromosomes of the examined species mainly coincide with their number and locations determined by phase contrast microscopy, although there were several exceptions from these rule.

In some cases the number of NORs detected by FISH or $AgNO_3$ staining does not match to number of NORs detected by phase contrast analysis. In particular, staining with $AgNO_3$ detected six NORs in the polytene chromosomes of *Ch. duplex* Walker, 1856 salivary gland, but only one NOR in interphase ganglion cells and none in the meiotic late prophase and metaphase I as well as mitotic chromosomes. The authors assume that the observed differences are determined by tissue-specific features in the NOR function, namely, fusion of nucleoli in ganglion cells and a decrease in the NOR transcription activity after the pachytene in meiosis (Lentzios and Stocker 1979). This phenomenon is characteristic not only of chironomids, but also of other insect groups (Cabrero and Camacho 2008, Cabral-de-Mello et al. 2010, Grzywacz et al. 2011).

One of the possible reasons underlying the variation in NOR activity in chironomids is a change in the number of transcriptionally active copies of ribosomal genes. A special study into the chromatin structure of *Ch. riparius* ribosomal genes has shown that not all these copies are equally active in transcribing rRNA. Along with transcriptionally active copies of ribosomal genes, free of nucleosomes, populations of these genes also contain transcriptionally inactive copies displaying nucleosome organization. The share of transcriptionally active copies in the population of ribosomal genes is tissue-specific, amounting to 80% in the fat body cells, to 50% in the salivary glands, and only 20% in the Malpighian tube cells (Sanz et al. 2007). An analogous ratio is observed in the *C. tentans* salivary gland cells, where 40% of the ribosomal genes are in a transcriptionally active state (Madalena et al. 2012). Since silver staining predominantly detects active NORs, the variation in NOR number observed in chironomids using this technique may be actually determined by the variation in the transcriptional activity of their ribosomal genes. However, this factor does not influence the NOR detection by FISH.

Variability in activity of NORs might be also determined by such characteristics of this locus as multiple copies of rRNA genes and a presence of transposable elements (TE) (Long and Dawid 1980, Jakubczak et al. 1991). The presence of multiple gene copies allows part of them to be separated by crossing-over, while mobile elements enhance their transfer to new genomic regions. If these events do not involve regulatory sites for ribosomal genes, then localization and activity of the initial NOR are retained and additional new NORs appear; the activity of the latter depends on the rDNA copy number in the transferred fragment (Eickbush and Eickbush 2007). The number of ribosomal gene copies in these fragments may be different, as demonstrated by the length variation of extrachromosomal circular DNA (eccDNA) formed by multimers of tandemly repeated rDNA genes (Cohen et al. 2003, 2010) as a result of recombination between adjacent gene clasters and intergene spacers.

If this mechanism is involved, the variability in intensity of hybridization signals of rDNA on chromosomes of *Ch. muratensis*, *Ch. nudiventris* and *Ch. pseudothummi* might be determined by the difference in the number of gene copies presented in each NOR. Thus, intense hybridization signals were detected in regions with active NORs while weak signals occurred in regions with no visible NOR activity. A similar pattern has been observed in wheat (Dubcovsky and Dvořák 1995) and many other species (Cabrero and Camacho 2008).

The analysis involving FISH and silver staining has shown a considerable diversity in the NOR number and locations in the chromosomes constituting karyotypes of 32 Palearctic and Australian *Chironomus* species (Lentzios and Stocker 1979; our data, Table 3). The number of NORs in these species may vary from one to eight. The variant with a single NOR is prevalent in the chironomid karyotypes, being observed in 17 of the 32 examined species; two NORs are found in eight species; and three NORs in three species. Four species contain considerably larger number of NORs, namely, five (*Ch.* "annularius"), six (*Ch. duplex* and *Ch. muratensis*), and eight (*Ch. nepeanensis*).

NORs can be located on all seven chromosome arms of the chironomid karyotypes; however, none of the NORs have been detected on the same chromosome arm in all 32 species of the genus *Chironomus*. Most frequently, NOR is located on arm G (in 25 species out of 32), but none of the species belonging to the subgenus *Camptochironomus* had NOR on this arm. The locations of NORs is also different in species from the subgenus *Chironomus* inhabiting remote geographic regions: species from Western Siberia may carry NOR in all chromosome arms (Table 3), while the Australian species lack NOR in arm E (Lentzios and Stocker 1979). The interspecific differences in the NOR number and location are also observed in closely related chironomid species.

Along with the interspecific variation in the NOR number and location, chironomids also display intraspecific variation in these characteristics. Three species (*Ch. balatonicus*, *Ch.* "annularius", and *Ch. tepperi*) may carry different numbers of NORs in individual karyotypes. *In situ* hybridization of *Ch. tepperi* chromosomes with 28S rRNA (Eigenbrod 1978) and FISH of *Ch. balatonicus* and *Ch.* "annularius" chromosomes with 5.8S rDNA (this work) have demonstrated that the additional NORs develop only in the individuals that carry ribosomal genes in the corresponding chromosome regions.

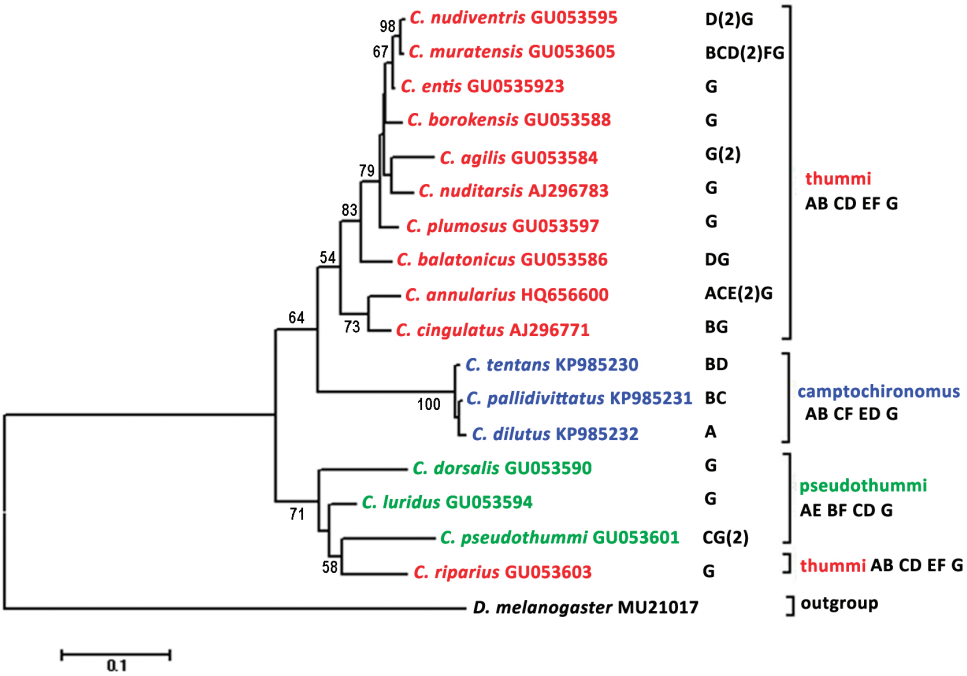

Figure 5. NJ tree based on maximum likelihood distances for ITS1 and 5,8S rDNA sequences from the genus *Chironomus* species. *Drosophila melanogaster* is used as an outgroup species. Maximum likelihood bootstrap values (1000 replicates) (> 50%) are shown next to the nodes. NORs chromosomal arms location, arm combinations and name of cytocomplexes are listed at the right.

Variety in number and locations of NOR on chromosomes in karyotypes of species from the genus *Chironomus* can occur due to several reasons: as a result of chromosomal rearrangemens, mainly inversions and translocations that are widespread in chironomids (Kiknadze et al. 2008), in consequence of transpositions of chromosomal fragments containing NORs into other regions of homologous and non-homologous chromosomes due to sister chromatid exchanges, homologous recombination, crossing-over or other mechanisms. All of this can result in an emergence of NORs in regions where they did not occur before or in a loss of NORs from regions of their traditional occurrence.

Transposable elements (TE) can also cause considerable changes in organization of NORs in karyotypes of species from the genus *Chironomus*. They can change activity of NORs or cause their complete inactivation. Several types of TE were found in the genus *Chironomus*. Common features for all of them are the presence in genome of multiple copies of each element, multiple location sites, species-specific but demonstrate intraspecific, intra- and interpopulations variability (Papusheva et al. 2004, Zampicinini et al. 2011). All of this allow to consider TE as a source that can provide a possibility for transpositions and changes in number of NORs on chromosomes of different species of the genus *Chironomus*.

The obtained results allowed us to characterize chromosomal organization and evolution of rRNA genes family in the genus *Chironomus*. The tree of phylogenetic relationships between species from the genus *Chironomus* constructed on the basis of comparison sequences of ITS1 and 5,8S rDNA shows that species groups into tree distinct clusters that coincide with cytocomplexes that differ from each other by arm combinations in chromosomes (Fig. 5). The phylogenetic tree demonstrates mostly monophyletic evolution of rRNA genes in these species. The only exception is *Ch. riparius*, which belong to "thummi" cytocomplex on the basis of chromosomal arm combination but is clustered in the "pseudothummi" cytocomplex on the tree. It should be noted that the same picture can be observed on phylogenetic trees constructed on the basis of other markers, such as isozymes (Scholl et al. 1980), genes from nuclear and mitochondrial genomes (Guryev et al. 2001) or banding sequences (Shobanov 2002, Kiknadze et al. 2004b, 2008, Gunderina et al. 2005). According to hypothesis of Keyl (1962) the reason for such behavior is that originally *Ch. riparius* belonged to the "pseudothummi" cytocomplex but undergone the change in chromosome arm combination due to reciprocal translocation between chromosomes AE and BF, which resulted in its transfer into "thummi" cytocomplex. But if such an event occurred relatively recently in this species evolution its genome has not accumulate enough changes to differ it from other species from the "pseudothummi" cytocomplex.

Addition of data on the number and chromosomal positions of NORs to the phylogenetic tree of studied chironomid species shows that there is no correlation between evolution of nucleotide sequences of ribosomal genes and chromosomal organization of NORs in the karyotypes of species (Fig. 5). The analysis had shown that number and location of NORs in karyotypes had changed many times during evolution of the genus *Chironomus* while evolution of ribosomal genes was monophyletic.

At the same time the combined data allow us to suggest a hypothesis about location of NOR in the karyotype of an ancestor species of the genus *Chironomus*. As all species from both "thummi" and "pseudothummi" cytocomplexes always have one NOR in arm G it is possible to suppose that an ancestor chironomid species had a NOR in this arm. And the absence of NOR in the arm G of species from the "camptochironomus" cytocomplex is probably caused by its loss in the ancestor species of this cytocomplex after its separation from "thummi" cytocomplex.

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SHORT COMMUNICATIONS

COMPARATIVE Cytogenetics
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Chromosomes of *Belonocnema treatae* Mayr, 1881 (Hymenoptera, Cynipidae)

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Abstract

Chromosomes of the asexual and sexual generation of the gall wasp *Belonocnema treatae* Mayr, 1881 (Cynipidae) were analyzed. Females of both generations have 2n = 20, whereas males of the sexual generation have n = 10. Cyclical deuterotoky is therefore confirmed in this species. All chromosomes are acrocentric and form a continuous gradation in size. This karyotype structure is probably ancestral for many gall wasps and perhaps for the family Cynipidae in general. Chromosome no. 7 carries a characteristic achromatic gap that appears to represent a nucleolus organizing region.

Keywords

Hymenoptera, Cynipidae, *Belonocnema treatae*, gall wasps, chromosomes, karyotype

Introduction

Parasitic Hymenoptera are one of the largest, taxonomically complicated and economically important insect groups (Rasnitsyn 1980, Heraty et al. 2011). The overwhelming majority of this group attacks insects and some other arthropods; however, certain taxa of the 'parasitic' Hymenoptera are in fact secondarily phytophagous (Quicke 1997). Among these taxa, gall wasps of the family Cynipidae are the most diverse, with their

world fauna exceeding 1300 species (Ronquist 1999, Abe et al. 2007, Liljeblad et al. 2011). Many gall wasps exhibit cyclical parthenogenesis, i.e. they have heterogonous life cycles with temporally segregated sexual and asexual generations (Crozier 1975, Stone et al. 2002). The cynipid, *Belonocnema treatae* Mayr, 1881 induces galls on live oaks (*Quercus* spp.) in the series *Virentes* (Muller 1961, Melika and Abrahamson 2002). In the Edwards Plateau region of central Texas, USA, both generations are host specific to *Quercus fusiformis* Small (Lund et al. 1998). The asexual generation of *B. treatae* develops within single-chambered, spherical galls on the undersides of leaves during the summer and fall and emerges in the fall and winter, whereas the sexual generation develops within multi-chambered galls on the roots, and males and females emerge during the spring (Lund et al. 1998).

Chromosomes of more than twenty species of the family Cynipidae have now been studied (see Gokhman 2009 for review). Karyotypes of many cynipid gall wasps exhibit a relatively high degree of similarity. Indeed, most genera and species have the same chromosome number, n = 10 (Sanderson 1988). Nevertheless, all four studied members of the genus *Diplolepis* Fourcroy, 1785 show another n value, i.e. n = 9. Moreover, chromosome sets with deviating karyotype structure have been detected within the genus *Andricus* Hartig, 1840 (Abe 1998, 2007). In this genus, the majority of species also have n = 10, although a few closely related taxa have chromosome sets with n = 6 and 5. Furthermore, the latter karyotypes belong to a particular species complex where cryptic species were discovered (Abe 1998). Interestingly, similar chromosome numbers, n = 10 and 9, are characteristic of five studied species of another cynipoid family, Figitidae, in which two other species with n = 11 and 5 were also found (Gokhman 2009, Gokhman et al. 2011).

Recent observations reported by Hjelmen et al. (2013) suggest that observed values for the genome size of male and asexual female *B. treatae* differ from values expected from haplo-diploidy. We have undertaken the present study to investigate chromosomes of this species and to determine whether variation in karyotype structure is present within and/or between the asexual and sexual generations of *B. treatae* within a single population.

Material and methods

Samples of the asexual and sexual generations of *B. treatae* developing within galls on *Quercus fusiformis* from central Texas, USA, were collected near San Marcos (Texas) and husbanded in the lab during September 2013 and March 2014 respectively. Prepupae and early pupae of *B. treatae* were extracted from the dissected galls. Chromosomal preparations were obtained from developing ovaries and, in case of males, prepupal cerebral ganglia following the protocol provided by Imai et al. (1988) with some modifications. Mitotic divisions were studied and photographed using an optic microscope Zeiss Axioskop 40 FL fitted with a digital camera AxioCam MRc. To obtain karyograms, the resulting images were processed with image analysis programs

Zeiss AxioVision version 3.1 and Adobe Photoshop version 8.0. Mitotic chromosomes were measured on thirty haploid metaphase plates using Adobe Photoshop and then classified according to the guidelines provided by Levan et al. (1964).

Results

Mitotic metaphase plates from eleven females of the asexual generation as well as six females and five males of the sexual generation of *B. treatae* were analyzed. Females of both the asexual and sexual generations have identical karyotypes with 2n = 20 (Fig. 1a, b), whereas males of the sexual generation have n = 10 (Fig. 1c). All chromosomes form a continuous gradation in size (perhaps except for the smallest chromosome; Table 1) and are clearly acrocentric, although shorter arms are visible in many elements. No aneuploid specimens or individuals with other unusual karyotypic features were found. Chromosome no. 7 carries a characteristic achromatic gap in the longer arm near the centromere (Fig. 1a–c). This gap appears to represent a nucleolus organizing region (NOR) and is best visible in the male karyotype, possibly because of the stronger spiralization of the chromosomes.

Discussion

Our results show that *B. treatae* exhibits cyclical deuterotoky, similarly to many other members of the family Cynipidae studied in this respect (reviewed in Crozier 1975 and Stone et al. 2002). The chromosome number found in *B. treatae*, i.e. n = 10 (2n = 20), is the most common in the family. Moreover, all chromosomes of this species appeared to be acrocentric. Despite karyotypes of most members of the Cynipidae containing at least some biarmed chromosomes (see e.g. Sanderson 1988), only acrocentrics were found in the chromosome set of another species, i.e. *Dryocosmus kuriphilus* Yasumatsu, 1951

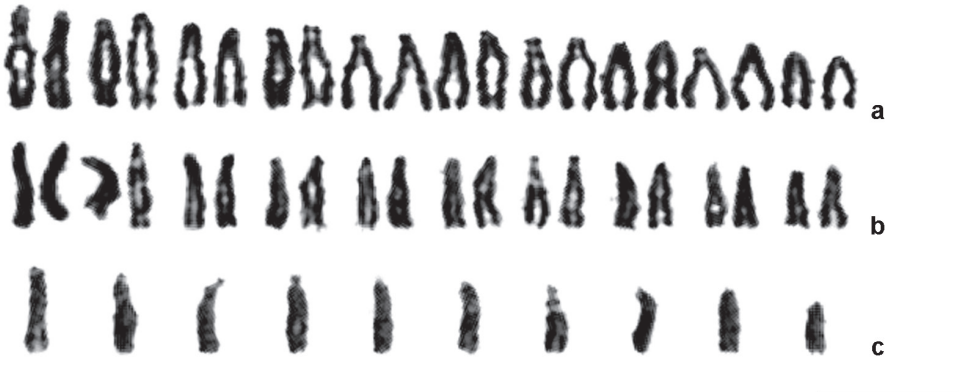

Figure 1. Karyograms of *Belonocnema treatae*. **a** asexual female **b** sexual female **c** male. Bar = 10 μm.

Table 1. Relative lengths (RL) of *Belonocnema treatae* chromosomes from haploid metaphase plates (mean ± SD).

Chromosome no.	RL
1	11.91 ± 0.52
2	11.34 ± 0.37
3	10.89 ± 0.36
4	10.44 ± 0.28
5	10.19 ± 0.21
6	9.88 ± 0.30
7	9.65 ± 0.28
8	9.35 ± 0.30
9	8.90 ± 0.41
10	7.45 ± 0.62

(Abe 1994). Interestingly, both *Dryocosmus* Giraud, 1859 and *Belonocnema* Mayr, 1881 represent the least advanced lineages within their clades, i.e. within the *Neuroterus*-group and *Cynips*-group respectively (see Tree 7 in Liljeblad et al. 2008), and therefore this karyotype structure is likely to be ancestral for members of their common clade within the tribe Cynipini, and perhaps for the family Cynipidae in general. However, biarmed chromosomes apparently predominate in the karyotype of *Callirhytis quercuspomiformis* (Bassett, 1881) with n = 10 (Goodpasture 1975). Since this species is the only studied member of *Callirhytis* Förster, 1869 (in turn, the least advanced examined genus of Cynipini), we cannot exclude the presence of metacentrics and/or submetacentrics within the ancestral karyotype of the above-mentioned tribe/family.

Although certain communications claimed that *B. treatae* possessed a special sex determination mechanism, these reports were mainly based on putative differences in the genome size between various populations and generations of this species (see e.g. Hjelmen et al. 2013). However, recent studies suggest that these results could be affected by tannins coming from the galls (Hjelmen et al. 2014).

The present study has also revealed a single achromatic gap (presumably NOR) in the haploid karyotype of *B. treatae*. Among other Cynipidae, similar results were obtained in the only species studied in this respect, *Diplolepis rosae* (Linnaeus, 1758) using FISH with 18S rDNA probe (Gokhman et al. 2014).

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SHORT COMMUNICATIONS

COMPARATIVE Cytogenetics
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Cytogenetic analysis of *Scinax auratus* and *Scinax eurydice* (Anura, Hylidae) with emphasis on cytotaxonomy

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Abstract

Scinax Wagler, 1830 is a species-rich genus of amphibians with relatively few detailed chromosomal reports. In this work, cytogenetic analyses of *Scinax auratus* (Wied-Neuwied, 1821) and *Scinax eurydice* (Bokermann, 1968) were carried out based on conventional (Giemsa staining, Ag-NOR and C-banding) and cytomolecular (base-specific fluorochrome staining and fluorescence *in situ* hybridization – FISH of ribosomal probes) techniques. Both species shared the same karyotype, location of active nucleolar organizer regions on pair 11 and GC-rich heterochromatin, as reported for most species in *S. ruber* clade. Interpopulation chromosomal variation was observed in *S. eurydice*, indicating the occurrence of cryptic species. The mapping of 18S ribosomal genes by FISH is reported for the first time in both species.

Keywords

Amphibians, chromosomes, FISH, heterochromatin

Introduction

Classic and cytomolecular chromosomal studies have been efficient to infer intra and interspecific relationships in anurans, besides supporting the validation of new and cryptic species (Siqueira et al. 2004; Medeiros et al. 2006; Bruschi et al. 2012; Gruber et al. 2012).

The genus *Scinax* Wagler, 1830 encompasses 114 species (Frost 2014), but only 39 of them have been karyotyped (Cardozo et al. 2011) while chromosomal mapping of particular DNA sequences is available solely for *Scinax fuscovarius* (Lutz, 1925) (Kasahara et al. 2003). A review of cytogenetic reports in this genus indicated that all *Scinax* species present a diploid number (2n) of 24 and fundamental number of chromosomal arms (FN) equal to 48. In *S. catharinae* clade, the pairs 1 and 2 are submetacentric and nucleolus organizer regions (NORs) in most species are located on pair 6. This pattern differs from *S. ruber* clade in which the pairs 1 and 2 pairs are metacentric and the NOR-bearing chromosomes correspond to pair 11 in most species (Cardozo et al. 2011).

S. auratus (Wied-Neuwied, 1821) inhabits rocky areas in Atlantic forest and forest borders in northeastern Brazil (Alves et al. 2004). This species belongs to *S. ruber* clade and, according to biological and anatomical studies would be related to the following species: *Scinax alter* (Lutz, 1973), *S. cretatus* (Nunes & Pombal, 2011), *S. crospedospilus* (Lutz, 1925), *S. cuspidatus* (Lutz, 1925), *S. imbegue* Nunes, Kwet & Pombal, 2012, *S. juncae* Nunes & Pombal, 2010 and *S. tymbamirim* Nunes, Kwet & Pombal, 2012 (Pombal et al. 1995, Alves et al. 2004, Nunes and Pombal 2010, 2011, Nunes et al. 2012, Mercês and Juncá 2012) . Cardozo et al. (2011) showed that the karyotype of *S. alter* in unique in *S. ruber* clade because of a distinctive NOR-bearing pair (3q).

S. euridyce (Bokermann, 1968) is also widespread in Brazil with records in five states of northeastern and southeastern Brazil (Bahia, Espírito Santo, Minas Gerais, Rio de Janeiro and São Paulo) (Pombal et al. 1995, Hartmann 2002, Canelas and Bertolucci 2007, Araújo et al. 2009, Magrini et al. 2011). Cytogenetic analyses in samples from southeastern Brazil have shown polymorphic NORs since two specimens presented terminal marks on 11q while a single female presented interstitial Ag-NORs (Cardozo et al. 2011).

In the present work, we provide new chromosomal data for both *S. auratus* and *S. eurydice* in order to respond the following questions: (1) Are the NORs observed in 3q of *S. alter* also present in *S. auratus*? (2) Is the polymorphism of NORs previously reported in *S. eurydice* from southeastern Brazil shared by populations from Bahia? (3) Are there chromosomal differences among geographically distant populations? (4) Can the mapping of 18S rDNA by FISH reveal additional non-active NORs previously undetected by silver nitrate staining?

Material and methods

Five specimens of *S. auratus* and *S. euridyce* were collected for cytogenetic analyses in Jequié, state of Bahia, northeastern Brazil (13°51'4"S, 40°4'52"W) (Table 1). Voucher

Table 1. Analyzed species, number of individuals (N), sex (J = juveniles of undentified sex) and collection site.

Species	Voucher	N	Locality
S. auratus	MZUESC11051 (♀), MZUESC11052 (♀), MZUESC11053 (♀), MZUESC11054 (♀), MZUESC11055 (♀)	5	Jequié - BA
S. eurydice	MZUESC11047 (♂), MZUESC11049 (J), MZUESC11005 (J), MZUESC11006 (♂), MZUESC11007 (♂)	5	Jequié - BA

specimens were deposited in the herpetological collection at Universidade Estadual de Santa Cruz – UESC. Mitotic chromosomes were obtained from epithelial cells of intestine as reported by Schmid (1978).

The slides were stained with Giemsa at 10% in phosphate buffer (pH 6.8) for about 10 minutes and air dried. For karyotyping, the chromosomes were classified according to centromere position into: m (metacentric), sm (submetacentric) and st (subtelocentric) following the nomenclature suggested by Green and Session (1991). Active nucleolar organizer regions (Ag-NORs) were detected by silver nitrate staining (Howell and Black 1980) and heterochromatin was visualized by C-banding (Sumner 1972), with slight modifications according to Siqueira et al. (2008). Base-specific fluorochrome with chromomycin A_3 (CMA_3), distamycin (DA) and 4,6-diamidino-2-fenilindole (DAPI) was performed to reveal GC- and/or AT-rich sites (Schmid 1980).

Fluorescence *in situ* hybridization using 18S rDNA probes was carried out according to Pinkel et al. (1986), under stringency conditions of 77%. The ribosomal probes were obtained via PCR of genomic DNA of both species (White et al. 1990, Hatanaka and Galetti 2004). In the case of *S. eurydice*, the probe was labeled with cyanine 3 (Cy3) by nick translation using Bionick Labeling System kit (Invitrogen) according to manufacturer's instructions. In *S. auratus*, the 18S rDNA probe was labeled using fluorescein-12-dUTP (Roche). The chromosomes were counterstained with DAPI and slides were mounted in Vectashield medium (Vector).

The best metaphase spreads were photographed using an Olympus BX51 epifluorescence microscope equipped with digital image capture system (ImagePro Plus – Media Cybernetics) and processed in the software Adobe Photoshop CS 8.0.1.

Results

S. auratus and *S. eurydice* presented 2n = 24 and FN = 48 besides sharing the same chromosomal formula: 16 metacentric (pairs 1, 2, 7, 8, 9, 10, 11 and 12) and eight submetacentric (pairs 3, 4, 5 and 6) chromosomes (Table 2; Fig. 1).

Silver nitrate staining revealed active nucleolus organizer regions (Ag-NORs) at interstitial region of 11q (Fig. 1a–b, box). However, a single homologous presented silver nitrate marks in *S. eurydice*, being coincident with secondary constrictions in all metaphases (Fig. 1b).

Table 2. Chromosomal measurements of studied species: relative length (RL), centromeric index (CI) and classification (CT) according to Green and Sessions (1991).

Species		Chromosomal Pairs											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
S. auratus	RL	16.35±0.09	13.66±0.07	11.40±0.03	10.34±0.55	9.22±0.04	8.73±0.01	6.76±0.26	6.30± 0.01	5.48±0.50	5.30±0.01	5.21±0.01	5.11±0.01
	CI	0.49±0.01	0.42±0.01	0.34±0.01	0.36±0.01	0.34±0.01	0.31±0.01	0.45±0.01	0.48±0.01	0.41±0.01	0.38±0.01	0.48±0.01	0.47±0.01
	CP	M	M	SM	SM	SM	SM	M	M	M	M	M	M
S. eurydice	RL	14.35±0.09	11.52±0.15	10.82±0.43	9.56±0.55	9.01±0.37	7.81±0.21	6.61±0.21	6.45± 0.09	5.79±0.60	5.71±0.09	5.56±0.96	4.53±0.15
	CI	0.48±0.01	0.42±0.01	0.27±0.01	0.32±0.01	0.32±0.01	0.3±0.01	0.41±0.01	0.37±0.01	0.43±0.01	0.48±0.01	0.45±0.02	0.47±0.01
	CP	M	M	SM	SM	SM	SM	M	M	M	M	M	M

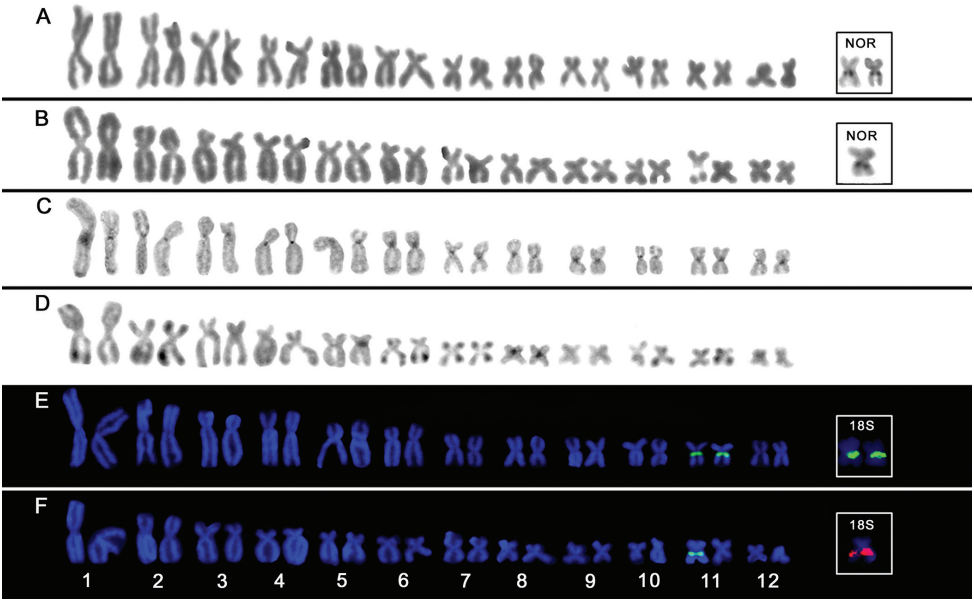

Figure 1. Karyotypes of *S. auratus* (**a, c, e**) and *S. eurydice* (**b, d, f**) after Giemsa-staining (**a, b**), C-banding (**c, d**) and base-specific fluorochrome staining (**e, f**). The NOR-bearing chromosomes after silver nitrate staining and FISH with 18S rDNA probes of each species are shown in boxes. Bar = 10 µm.

Heterochromatin was distributed over centromeric regions of all chromosomes in *S. auratus* while telomeric C-bands were observed in most chromosomes of *S. eurydice* along with telomeric heterochromatic blocks at centromeric regions of pairs 5 and 8 (Fig. 1c–d). In some metaphases, C-bands were also observed interspersed to NORs at interstitial position of pair 11. After base-specific fluorochrome staining, CMA_3^+ signals were detected at NORs in both species, indicating the presence of GC-rich heterochromatin segments (Fig. 1e–f).

FISH with 18S rDNA probes confirmed the single NOR-bearing pair visualized by silver nitrate staining in the analyzed species (Fig. 1e–f , box).

Discussion

The karyotypes of *S. auratus* and *S. eurydice* followed the pattern proposed for *Scinax* (2n = 24 and FN = 48). Similarly, the karyotype formulae agree with those reported for species within *S. ruber* clade (Faivovich 2002, Kasahara et al. 2003, Cardozo et al. 2011).

Based on morphological traits and vocalization, *S. auratus* seems to be closely related to *Scinax alter*, *S. cretatus*, *S. crospedospilus*, *S. cuspidatus*, *S. imbegue*, *S. juncae* and *S. tymbamirim* (Pombal et al. 1995, Alves et al. 2004, Nunes and Pombal 2010, 2011, Nunes et al. 2012, Mercês and Juncá 2012) . Karyotypic studies in this group of species are available only for *S. alter*, a distinctive species in *S. ruber* clade by the presence of terminal Ag-NORs on long arms of pair 3 (Cardozo et al. 2011). Even though *S.*

auratus and *S. alter* shared the same karyotype formulae, the Ag-NORs in the former was identified on pair 11, a plesiomorphic condition reported in most species within *S. ruber* clade. Therefore, cytogenetic studies based on mapping of 18S rDNA in closely related species such as *S. cretatus*, *S. crospedospilus*, *S. cuspidatus*, *S. imbegue*, *S. juncae* and *S. tymbamirim* are encouraged to evaluate whether the presence of NORs among the largest pairs is an autopomorphic condition or a synapomorphy of this subclade.

The NORs were associated with CMA_3^+ signals in both analyzed species, indicating the presence of GC-rich repetitive DNA interspersed with ribosomal genes, as commonly observed in anurans (Ananias et al. 2007, Campos et al. 2008). In spite of this correlation between base-specific fluorochrome and rDNA, the mapping of 18S rDNA by FISH is necessary to validate the precise location and number of NORs. In the present study, the FISH results confirmed the presence of a single NOR-bearing pair (11q) in analyzed species (Fig. 1e–f). This pattern has been reported in other species submitted to FISH analyses, with exception of *S. fuscovarius* whose 18S rDNA signals were mapped onto pair 12 (Kasahara et al. 2003). Nonetheless, Cardozo et al. (2011) stated that the NOR-bearing pair in *S. fuscovarius* actually corresponds to the 11th pair, once the smallest chromosomal pairs in *Scinax* are hardly distinguished.

The specimens of *S. eurydice* from the state of São Paulo, southeastern Brazil (Cardozo et al. 2011) and those analyzed in the present study had the same karyotype formulae, but different patterns in heterochromatin distribution. While the population from São Paulo presented C-bands at centromeric position in all chromosomes (Cardozo et al. 2011), the population of *S. eurydice* from northeastern Brazil showed heterochromatin at terminal regions of most chromosomes and centromeric regions of pairs 5 and 6 only (Fig. 1d). Telomeric C-bands were also reported in other hylids (Kasahara et al. 2003; Busin et al. 2006; Gruber et al. 2012). Similarly, NORs were also differentiated between both populations of *S. eurydice* once they were located at interstitial region of a single homologous in pair 11 whereas specimens from São Paulo presented terminal NORs at 11q besides interstitial cistrons in the same chromosome in one female (Cardozo et al. 2011). The physical mapping of 18S rDNA confirmed the location of NORs, even though a single chromosome was marked by FISH.

Other cases of NOR polymorphism have been previously reported in anurans such as *Hyla nana* (Boulenger, 1889) (Medeiros et al. 2006), *Hyla chrysocelis* Cope 1880, *Hyla versicolor* LeConte, 1825 (Willey et al. 1989), *Engystomops petersi* Jiménez de la Espada, 1872 (Lourenço et al. 1998), *Paratelmatobius poecilogaster* Giaretta & Castanho, 1990 (Lourenço et al. 2001), *S. alter* and *S. hiemalis* (Haddad & Pombal, 1987) (Cardozo et al. 2011). According to some models of evolution of ribosomal genes in eukaryotes as well as experimental evidence in yeasts, the rDNA are tandemly arranged in chromosomes being particularly susceptible to unequal exchanges between sister chromatids (Eickbush and Eickbush 2007). This phenomenon could account for the presence of a larger (and active) cluster of 18S rDNA in one homologue of pair 11in *S. eurydice*. Nonetheless, other events such as errors during DNA replication could also lead to this polymorphic NOR state (Amaro-Ghilardi et al. 2008). Apparently, specimens bearing larger amounts of ribosomal DNA have been

fixed in the analyzed population either by natural selection (if this NOR phenotype is somewhat adaptive) or by genetic drift.

The presence of heterozygous NORs (Ag^+/Ag^-) in *S. eurydice* might be related to sex, since this heteromorphic pattern was observed only in males. For instance, females and males of *Gastrotheca riobambae* (Fowler, 1913) were characterized by two and single NOR marks, respectively, mapped on X chromosomes (Schmid et al. 1983). If sex-related NORs are also valid for *S. eurydice*, the sex chromosomes in this species would be morphologically homogeneous and further analyses should be carried out to identify putative mechanisms of sex chromosomal determination by other cytogenetic techniques.

Nonetheless, experimental evidence has shown that individuals of salamanders *Plethodon cinereus* (Green, 1818) and *Xenopus laevis* (Daudin, 1802) bearing heterozygous NORs (Ag^+/Ag^-), independently on sex, are viable but their fertility is reduced since crosses between heterozygous specimens will produce unviable tadpoles bearing homozygous NORs (Schmid 1982). Therefore, it is possible that fertility of *S. eurydice* is also affected by this unusual pattern of NORs what remains to be investigated by inheritance studies in both natural and controlled conditions.

The interpopulation variation of NOR and C-banding pattern among populations of *S. eurydice*, associated with slight differences in vocalization between samples from northeastern and southeastern Brazil (Magrini et al. 2011), reinforces the necessity of a taxonomic review of this species.

In conclusion, the detailed cytogenetic characterization of *S. auratus* and *S. eurydice* showed that *S. auratus* shares some chromosomal traits with most of species in *S. ruber* clade, but diverges from the putatively closely related *S. alter*. The results in *S. eurydice* from Bahia revealed differences in chromosomal banding when compared to populations of southeastern Brazil, indicating the presence of cryptic species that should be systematically revised. Therefore, the chromosomal analyses in *Scinax* are potentially useful to both taxonomy and systematics of this group of anurans.

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RESEARCH ARTICLE

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Comparative analysis of karyotypes of *Chironomus solitus* Linevich & Erbaeva, 1971 and *Chironomus anthracinus* Zetterstedt, 1860 (Diptera, Chironomidae) from East Siberia

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Abstract

A comparative chromosome banding analysis of *Chironomus solitus* Linevich & Erbaeva, 1971 and *Chironomus anthracinus* Zetterstedt, 1860 from East Siberia (Lakes Baikal, Gusinoe, Arakhley and Irkutsk Reservoir) showed close similarity of banding sequences. *Ch. solitus* differs from *Ch. anthracinus* in one species-specific sequence of arm B. Arms C (43%) and D (30%) had inversion banding sequences previously reported in *Ch. anthracinus* The similarity of karyotypic features of *Ch. solitus* and *Ch. anthracinus* in combination with morphological features of larvae provide evidence in favour of including *Ch. solitus* in the *C. anthracinus* group of sibling species long with *Ch. reservatus* Shobanov, 1997.

Keywords

Karyotype, banding sequences, inversion, *Chironomus solitus*, *Chironomus anthracinus*

Introduction

Chironomus solitus Linevich & Erbaeva, 1971 and *Chironomus anthracinus* Zetterstedt, 1860 are abundant chironomid species (Diptera: Chironomidae), inhabiting the silty bottoms of various water bodies in Pribaikalye and Zabaikalye. *Ch. solitus* was first

registered in the Irkutsk Reservoir as well as in the Angara River, Bratsk, Ust-Ilimsk water reservoirs (Linevich and Erbaeva 1971; Linevich 1981; Proviz et al. 1991; Erbaeva and Safronov 2009), in lakes and rivers of the Barguzin River basin (Buyantuev 1999) and recently encountered in the near-shore zone of Lake Baikal. *Ch. anthracinus* is a widespread Holarctic species known from the Angara River and its tributaries, Irkutsk Reservoir, lakes of Western Zabaikalye (Linevich and Erbaeva 1971; Linevich 1981; Kiknadze et al. 2005) and the basin of the Barguzin River (Buyantuev 1999).

Ch. solitus and *Ch. anthracinus* live in the single type water environments (lakes, water reservoirs), and are characterized by similar larval morphology in the features used in the distinction of *Chironomus* species, which makes their differentiation complicated. Thus, accurate identification of these species requires analysis of their karyotypes, rather than only external larval morphology. Until recently, the *Ch. solitus* karyotype had only been examined in one population from the Irkutsk Reservoir. The first data were reported by Bukhteeva (1979); later, banding chromosome patterns and polymorphisms were described (Proviz 2009). Karyological analysis was made of *Ch. anthracinus* from many Palearctic and Nearctic regions (Belyanina 1983; Kiknadze et al. 1991, 1996; Shobanov 1996; Petrova and Rakisheva 2003; Kiknadze et al. 2005). In East Siberia, karyotypes of larvae from Lake Shchuchie (Buryatia) were briefly reported by Bukhteeva (1979, 1980). Later, Kiknadze and co-authors (Kiknadze et al. 1991, 1996) described the karyotypic of *Ch. anthracinus* from the Vilyuy Reservoir (Yakutia).

The present work is aimed at comparative analysis of *Ch. solitus* and *Ch. anthracinus* karyotypes from the largest lakes of East Siberia, Baikal, Gusinoe, Arakhley and Irkutsk Reservoir, and determination of cytogenetic features for their identification.

Material and methods

Fourth instar larvae of *Ch. solitus* were collected in January 1992 in the Irkutsk Reservoir (depth 3 m, 52 larvae), and in June 2008 in Lake Baikal opposite the Bolshye Koty Settlement (depth 6 m, 12 larvae). *Ch. anthracinus* were collected in May 2013 in Lake Gusinoe (10–22 m, 65 larvae), and in March 2014 in Lake Arakhley (10–17 m, 78 larvae). Larvae were fixed in a 3:1 mixture of 96% ethanol and glacial acetic acid. Karyological preparations were made using the ethyl-orcein method (Demin and Shobanov 1990). In 1992 and 2008, chromosomes were photographed by a micro-camera unit MCU-1 with 90× zoom magnification; in 2013–2014, this was performed using an Axiostar plus (Zeiss) microscope (Centre for Microscopic Analysis LIN SB RAS) with AxioVision Rel. 4.7.1 software. Mapping of arms A, C, D, E, and F of *Ch. anthracinus* chromosomes was performed according to Kiknadze et al. (2005) based on piger-standard (Keyl 1962, Devai et al. 1989), while standard map of *Ch. plumosus* suggested by Shobanov (1994, 1996) was used for mapping of arm B. Symbols designating banding sequences are as follow: distribution areas marked by p' for Palearctic,

n' for Nearctic, and h' for Holarctic zoogeographical regions (Kiknadze et al. 2005) and followed by abbreviated species name (sol), arm designation (A) and banding sequence number–p'solx1 (in homozygote–p'solA1.1).

Results

Larval morphology

Both species have a light yellow (from the dorsal part) cephalic capsule, including the frontal sclerite. Abdominal segment VIII bears two pairs of long ventral appendages; lateral appendages on segment VII are absent (bathophilus type after: Lenz 1926). Premandible with two uneven teeth. Fourth lateral cusp of mentum is smaller than fifth cusp. Third antennal segment is shorter than the fourth. The colour of the fourth lower mandibular tooth varies; that of *Ch. solitus* is dark yellow, while the remaining teeth are dark brown. The results of our examination of the population from Lake Arakhley showed that *Ch. anthracinus* tooth was either dark yellow or of the same colour as the rest of the teeth.

Karyotype characteristics

Karyotypes of *Ch. solitus* (Fig. 1) and *Ch. anthracinus* (Fig. 2) have common morphological features: 2n=8. A combination of chromosome arms is typical for species from "thummi" cytocomplex. Chromosomes AB and CD are metacentric, EF, submetacentric, and G, telocentric. The species differ in the size of cenromeric heterochromatin. The centromeric areas of *Ch. solitus* are well defined, and the centromeres of *Ch. anthracinus* look like thin disks. Arm G homologues are unconjugated and carry a Balbiani Ring (BR) and a nucleolus (N). In *Ch. anthracinus* there is a second nucleolus in the arm F.

Banding sequences

Arms A of *Ch. anthracinus* and *Ch. solitus* are monomorphic with a single identical banding sequence h'antA1=h'solA1 (Fig. 3, a, b): h'antA1= h'solA1 1a-2c 10a-12a 13ba 4a-c 2g-d 9e-4d 2h-3i 12cb 13c-19f C

Arms B of *Ch. anthracinus* and *Ch. solitus* are monomorphic with banding sequences h'antB1 (Fig. 3, c) and p'solB1 (Fig. 3, d) differ by a simple inversion:

h'antB1 25s-24i 18c-16b 22b-21a 23l-24h 18d-20n 23k-d 15m-16a 22c-23c 15l-12v C
p'solB1 25s-24i 18c-16b 18d 24h-23l 21a-22b 19a-20n 23k-d 15m-16a 22c-23c 15l-12v C

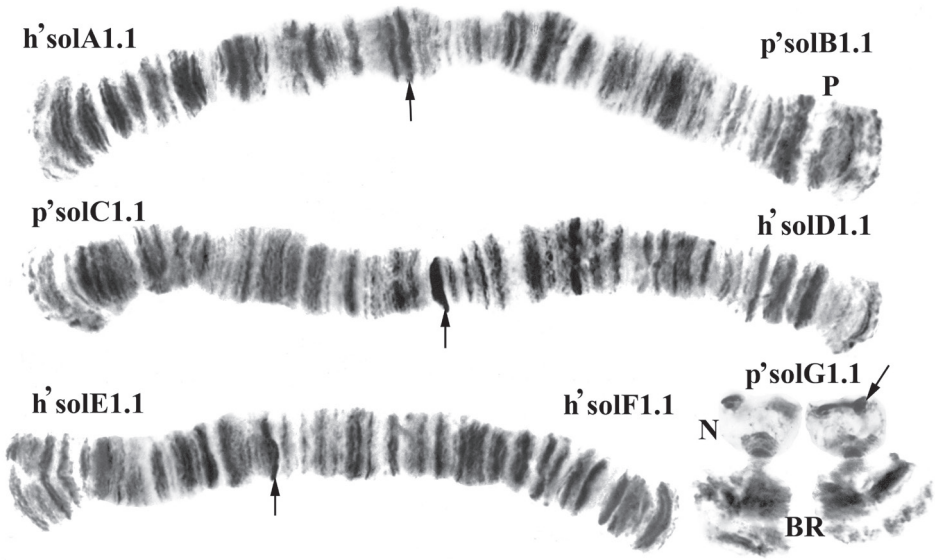

Figure 1. Karyotype of *Chironomus solitus*. h'solA1.1, p'solB1.1 ets.–genotypic combinations of banding sequences in chromosomal arms; N – nucleolus; BR – Balbiani Ring, p – puff, arrows show centromeric bands.

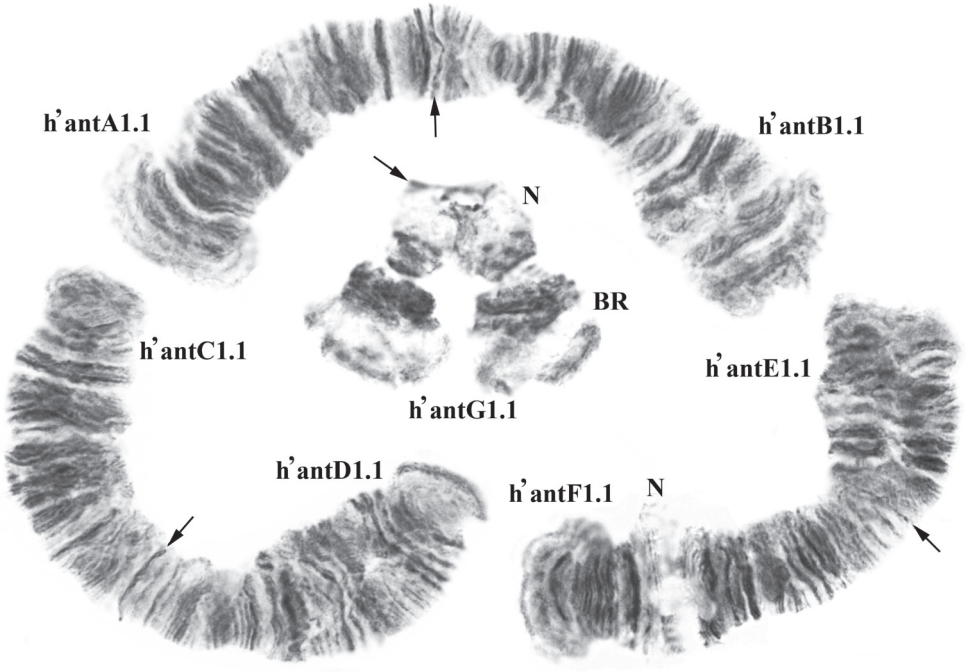

Figure 2. Karyotype of *Chironomus anthracinus*. The designations are the same as in Fig. 1.

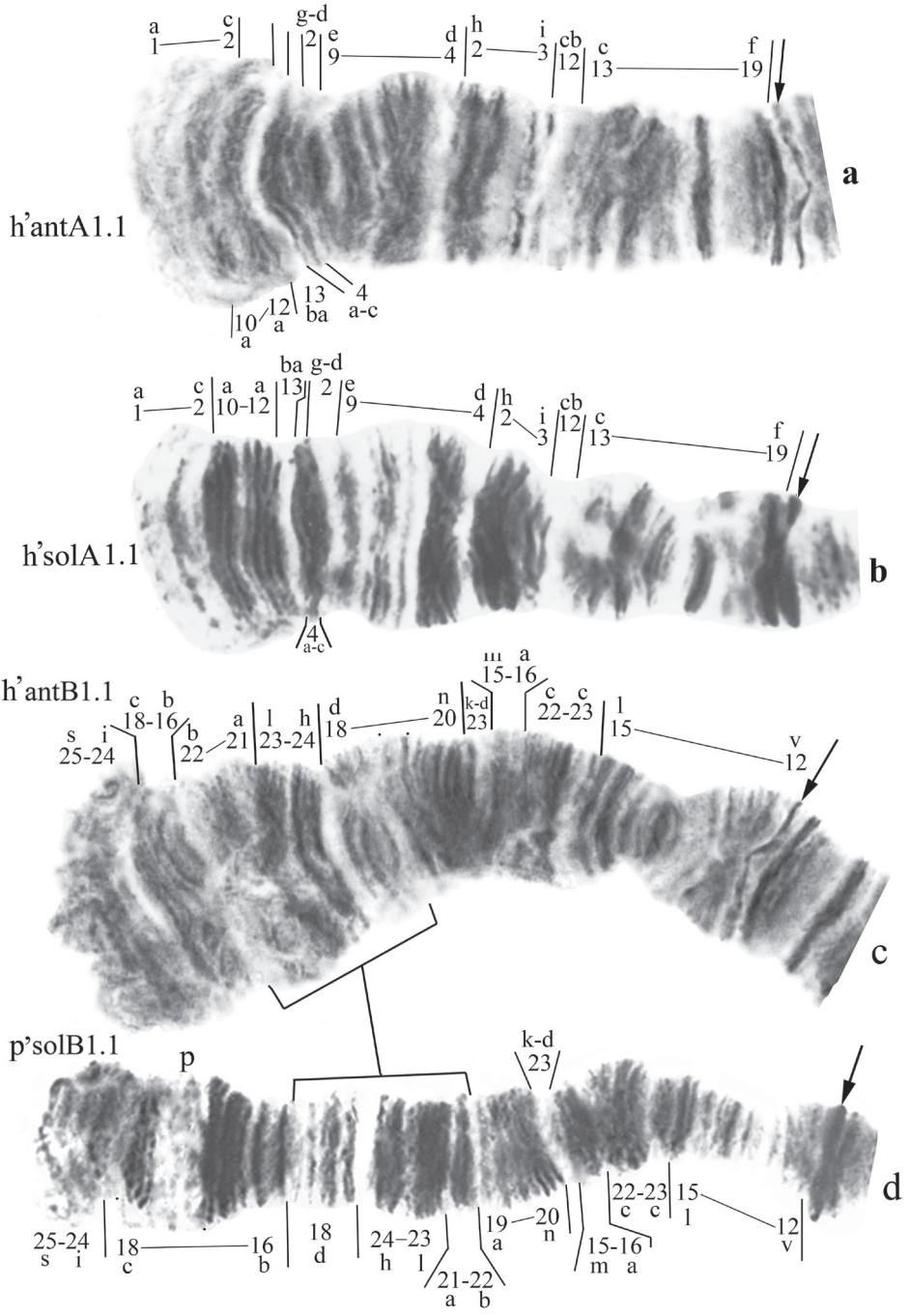

Figure 3. Homozygous banding sequences in the arms A and B of *Chironomus anthracinus* and *Chironomus solitus.* **a** h'antA1.1 **b** h'solA1.1 **c** h'antB1.1 **d** p'solB1.1. Numbers and small letters under chromosome arm correspond to banding sequences, brackets near chromosome arms show inversions.

In addition to the inversion, *Ch. solitus* differs from *Ch. anthracinus* by the presence of a puff in the region 17. The banding sequence h'antB2 (Kiknadze et al. 2005) were found in Palearctic and Nearctic *Ch. anthracinus* populations. The borders of this inversion located close to the borders of inversion that differ banding sequence p'solB1 from h'antB1. Standard mapping of *Ch. plumosus* (Shobanov, 1994) allows it to be represented as follows:

h'antB2 25s-24i 18c-17a 23l 21a-22b 16b 24h 18d 19a-20n 23k-d 15m-16a 22c-23c 15l-12v C

Arm C of *Ch. anthracinus* is monomorphic, with a single banding sequence h'antC1 (Fig. 4, a). Arm C of *Ch. solitus* is polymorphic and has two banding sequences–p'solC1 (Fig. 4, b) and h'solC2–differing by one simple inversion (Fig. 4, c). Inversion heterozygotes p'solC1.h'solC2 made up 25% and 43% of Baikal and Irkutsk Reservoir populations, respectively. The same banding sequences, h'antC1 (=h'solC2) and p'antC2 (=p'solC1), were also registered in *Ch. anthracinus* populations from other localities within this area, although in somewhat different proportions:

h'antC1= h'solC2 1a-2c 2d-6b 11c-8a 15ed 15c-11d 6gh 17a-16a 7d-a 6f-c 17b-22g C
p'solC1=p'antC2 1a-2C 15de 8a-11c 6b-2d 15c-11d 6gh 17a-16a 7d-a 6f-c 17b-22g C

h'antC1 sequence dominated in all of the populations studied, while p'antC2 was less common and occurred in both homo- and heterozygous states (Kiknadze et al. 2005).

Arm D of *Ch. anthracinus* is monomorphic, with one h'antD1 banding sequence (Fig. 5, a). Arm D of *Ch. solitus* is polymorphic and has two banding sequences–h'solD1 (Fig. 5, b, c), identical to h'antD1, and p'solD2 (Fig. 6), which differs by a simple inversion. Inversion heterozygotes h'solD1.p'solD2 were found in 17% of specimens from the Baikal population, and in 30% from the Irkutsk Reservoir. *Ch. anthracinus* from western parts of Palearctics also had a p'antD2 banding sequence identical to that of p'solD2 and was found in homo- and heterozygous states (Kiknadze et al. 2005):

h'antD1=h'solD1 1a-3g 14g-16e 8c-7g 5d-7f 18d-17a 8d-10a 13a-11a 14f-13b 10b-e 4a-5c 18e-24g C
p'antD2=p'solD2 1a-3g 14g-16e 5c-4a 10e-b 13b-14f 11a-13a 10a-8d 17a-18d 7f-5d 7g-8c 18e-24g C

Arms E of *Ch. anthracinus* (Fig. 7, a) and *Ch. solitus* (Fig. 7, b) are monomorphic and have an identical banding sequence:

h'antE1= h'solE1 1a-3e 5a-10b 4h-3f 10c-13g C

Arms F of *Ch. anthracinus* (Fig. 7, c) and *Ch. solitus* (Fig. 7, d) also have an identical banding sequence that is only found in a homozygous state in East Siberia:

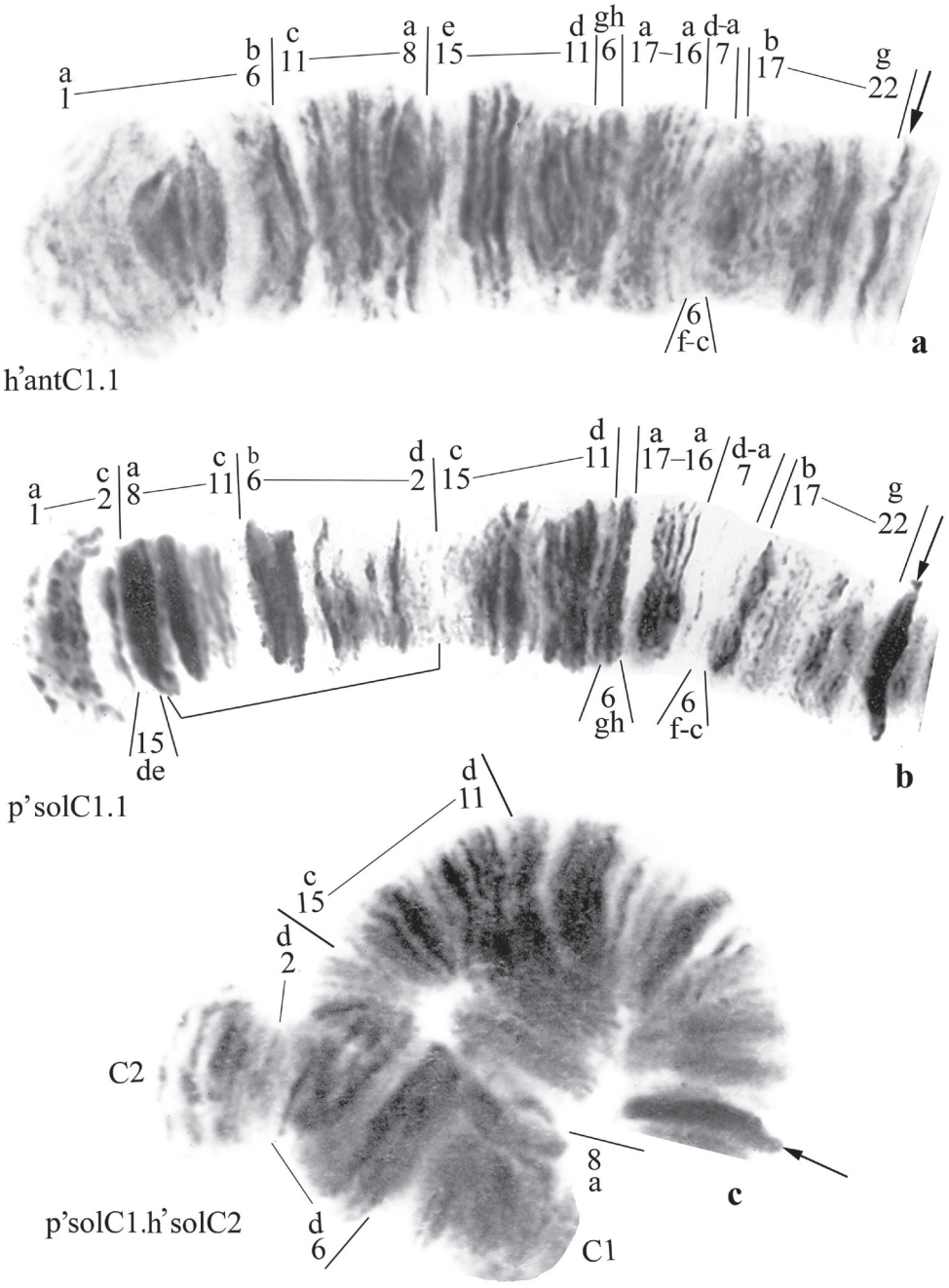

Figure 4. Banding sequences in the arm C of *Chironomus anthracinus* and *Chironomus solitus*. **a** homozygotes h'antC1.1. **b** homozygotes p'solC1.1 **c** heterozygous inversions p'solC1.h'solC2. Designations as in Fig. 3.

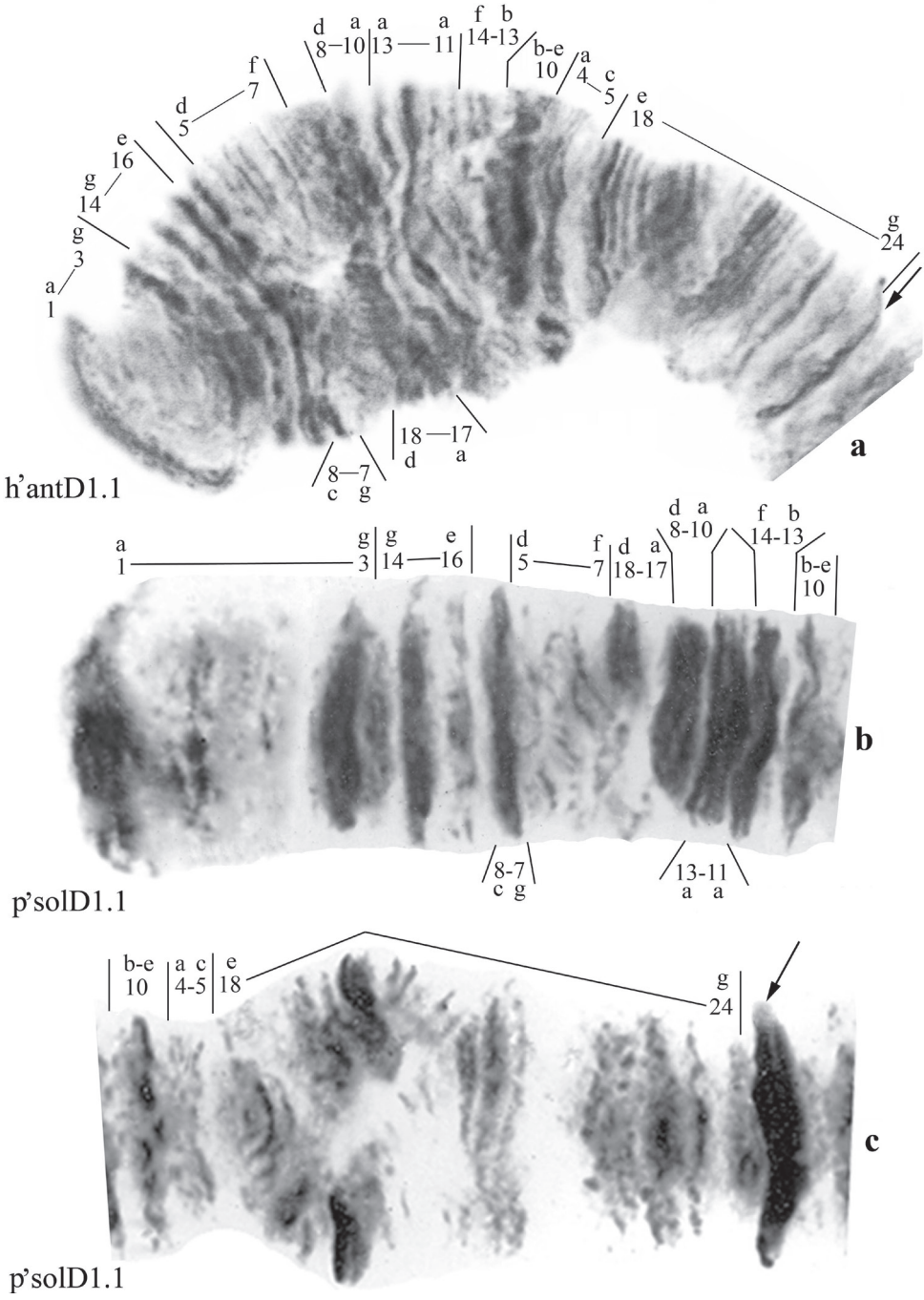

Figure 5. Homozygous banding sequences in the arm D of *Chironomus anthracinus* and *Chironomus solitus*. **a** h'antD1.1 **b** and **c** h'solD1.1. Designations as in Fig. 3.

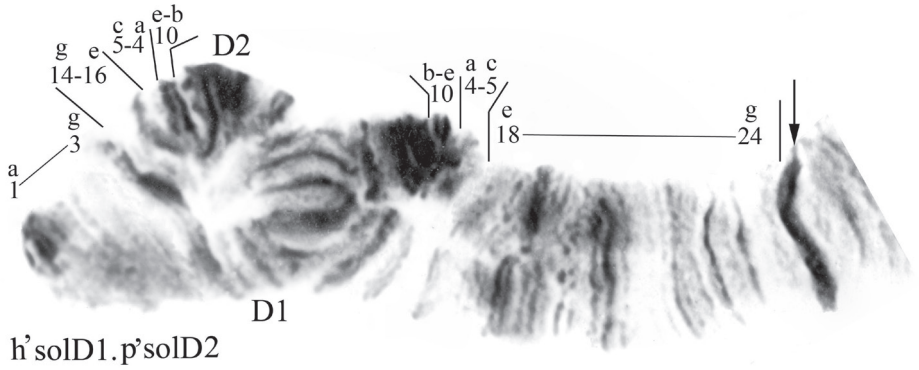

Figure 6. Inversion heterozygote h'solD1.p'solD2 in the arm D *Chironomus solitus*. Designations as in Fig. 3.

h'antF1= h'solF1 1a-8e 9c-17d 18a-23f C

A second nucleolus in Arm F of *Ch. anthracinus* is a species-specific feature of *Ch. anthracinus* that makes it different from *Ch. solitus* with a single nucleolus in arm G.

Arms G of *Ch. anthracinus* and *Ch. solitus* (Figs 1, 2) have similar morphology: unconjugated homologues with a constriction, unclear banding pattern, similar location of Balbiani Ring and nucleolus. In general, homologues have ectopic contacts in active loci.

Discussion

As a result of comparative analysis of banding patterns of *Ch. solitus* and *Ch. anthracinus* from East Siberia, the similarity of these species in morphological features of larvae as well as karyotypes was revealed. Most of the chromosomal arms, A, D, E and F, have identical banding sequences, and a similar structure of arm G. The principal distinctive features of *Ch. solitus* karyotype are the species-specific p'solB1 sequence and the absence of a nucleolus in arm F. Previous investigators (Belyanina 1979, Kiknadze et al. 1991, 1996, Shobanov 1996, Petrova and Rakisheva 2003, Kiknadze et al. 2005) reported a low level of chromosome polymorphisms in *Ch. anthracinus*. Analysis of the populations with standard banding sequences from Lakes Gusinoe and Arakhley also confirmed these observations. The overall banding sequence pool of *Ch. anthracinus* from other regions includes h'antC2, h'antC1 and p'antD2 sequences, which are identical to p'solC1, h'solC2 and p'solD2 from East Siberia; this is suggestive of karyological similarity of *Ch. solitus* and *Ch. anthracinus*.

There is one more species of the genus *Chironomus* – *Ch. reservatus* Shobanov, 1997, which has close similarity of karyotypic and morphological features at all developmental instars of *Ch. anthracinus* (Shobanov, 1997). Based on these results, the author included the two species in the *C. anthracinus* group. Banding sequence p'resB1,

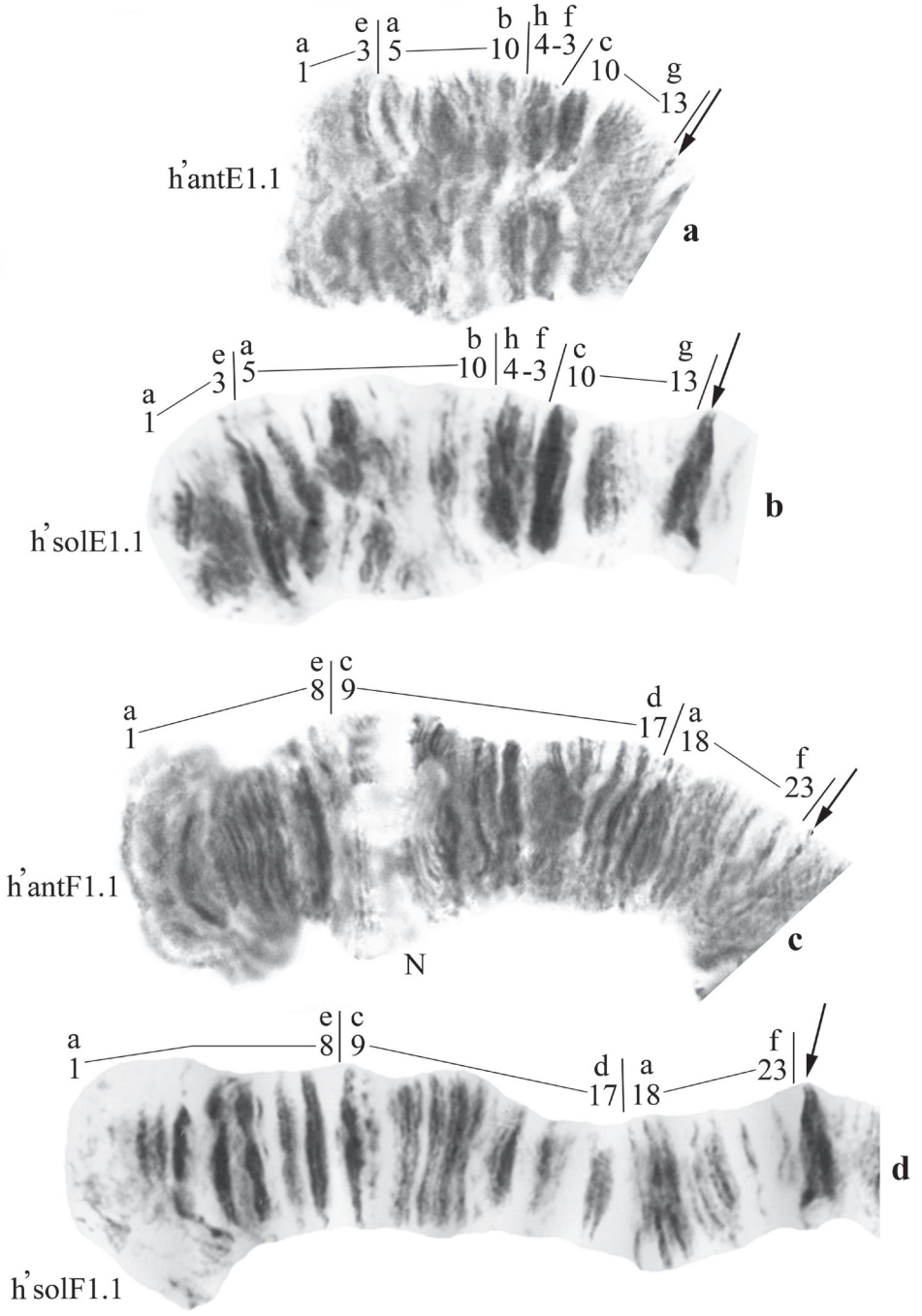

Figure 7. Homozygous banding sequences in the arms E and F of *Chironomus anthracinus* and *Chironomus solitus*. **a** h'ant E1.1 **b** h'solE1.1 **c** h'antF1.1 **d** h'solF1.1.

alongside h'antB2, localised close to p'solB1, and is regarded one of the species-specific markers:

p'resB1 25s-24i 18c-16b 22b-18d 24h-23d 15m-16a 22c-23c 15l-12v C

The morphology of *Ch. anthracinus* and *Ch. solitus* imagines from East Siberia is insufficiently studied (Linevich and Erbaeva 1971), therefore, it is possible to compare only several characteristics of these species. For instance, AR of *Ch. solitus* (3.8) is most closely related to *Ch. anthracinus* (4.14–4.43) from the European part (Shobanov 1996), and *Ch. anthracinus* from East Siberia (5.0) – to *Ch. reservatus* (4.8–5.6) (Shobanov 1997). Further research into metamorphosis of these species should be conducted to make reliable conclusions.

The results of our investigation, similarity of karyotypic features of *Ch. solitus* and *Ch. anthracinus* in combination with morphological features of larvae provide evidence in favour of their close similarity and enable us to include *Ch. solitus* as well as *Ch. reservatus* in the *C. anthracinus* group.

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SHORT COMMUNICATIONS

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Chromosomal and molecular evidence for presence of *Polyommatus* (*Agrodiaetus*) *poseidon* (Lepidoptera, Lycaenidae) in Caucasus region

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Abstract

We show how combination of chromosomal and molecular markers can be applied for proper species identification in *Agrodiaetus* Hübner, 1822 blue butterflies. Using this approach we provide first evidence for presence of *P.* (*A.*) *poseidon* (Herrich-Schäffer, [1851]) in Georgia.

Keywords

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

Introduction

The blue butterfly subgenus *Agrodiaetus* Hübner, 1822 belongs to the genus *Polyommatus* Latreille, 1804 (Talavera et al. 2013). In the last years, this group become a model system for study of speciation and chromosome evolution (Lukhtanov et al. 2015, Vershinina et al. 2015). Despite this, its taxonomy is still poorly elaborated and identification of individual species is difficult due to their morphological similarity. Species within the subgenus are mostly uniform and exhibit few differences in

characters traditionally used in classification, such as wing pattern and/or aspects of the male and female genitalia (Lukhtanov et al. 2006, Vila et al. 2010). The genus was estimated to have originated very recently (Kandul et al. 2004) and, thus, many *Agrodiaetus* species may have not had sufficient time to acquire extensive genetic differences. In particular, *COI* barcode gap is low or even absent between numerous closely related species of *Polyommatus* (*Agrodiaetus*) (Wiemers and Fiedler 2007). In opposite to majority of other butterflies and moths (Lukhtanov 2014), many *Agrodiaetus* species have evolved distinctive karyotypes. They show one of the highest interspecific karyotypic diversities known in the animal kingdom with haploid chromosome numbers ranging from n = 10 to n = 134 (Lukhtanov et al. 2005). Therefore, karyotypic features provide important identification characters for many described species that are virtually indistinguishable by their morphology. However, it should be noted that in few cases the chromosome number may be identical in different species (see Results and Discussion).

Here we show how combination of chromosomal and molecular markers can be applied for proper species identification in *Agrodiaetus*. Using this approach we provide first evidence for presence of *P.* (*A.*) *poseidon* (Herrich-Schäffer, [1851]) in Georgia.

Material and methods

The samples used for molecular and chromosomal analysis were collected in Georgia (Akhaltsikhe, 41.60N, 43.06E, 1000 m alt., 18 July 2014, V. Lukhtanov et V. Tikhonov leg., samples 2014VL56, 2014VL57, 2014VL58, 2014VL62, 2014VL63, 2014VL64, 2014VL65, 2014VL68, 2014VL69, 2014VL70). The methods of DNA sequencing, chromosomal analysis and phylogenetic inference were described previously (Lukhtanov and Dantchenko 2002a, Lukhtanov et al. 2008, 2014, Vershinina and Lukhtanov 2010, Przybyłowicz et al. 2014). Additional samples of *Polyommatus* belonging to *P.* (*A.*) *poseidon* species complex (Kandul et al. 2007) were used for comparison.

Results and discussion

The species *P.* (*A.*) *poseidon* (= *Lycaena poseidon* var. *mesopotamica* Staudinger, 1892, synonymized with *P. poseidon* by Schurian et. 1992) is known to be an endemic of the Middle East sporadically distributed from Kütahya in West Turkey to Artvin in North-East Turkey (Hesselbarth et al. 1995). Phenotypically similar, but chromosomally distinct species *P.* (*A.*) *putnami* (Lukhtanov & Dantchenko, 2002) was described from East Turkey (provinces Erzurum and Ağri) (Lukhtanov and Dantchenko 2002b). The last taxon is allopatric in distribution with *P.* (*A.*) *poseidon* and differs from *P. poseidon* by chromosome number and karyotype structure (Lukhtanov and Dantchenko 2002b). *P.* (*A.*) *poseidon* has relatively low haploid chromosome number (from n=19 on the south and east of the distributional range to n=21 in the north), all the chro-

mosomes form a gradient size row with no especially large or small chromosomes (de Lesse 1963, Kandul and Lukhtanov 1997). Chromosome numbers n=22 and n=23 were also found in the northern population as intraindividual occasional deviations from the basic n=21 (de Lesse 1963). *P.* (*A.*) *putnami* has higher chromosome numbers (from n=24 to n=27, with n=26 as a distinct mode). Its karyotype is asymmetrical and includes chromosomes of two distinct classes: class of large chromosomes and class of small chromosomes (Lukhtanov and Dantchenko 2002b). Currently *P.* (*A.*) *putnami* is treated as a distinct species (Lukhtanov and Dantchenko 2002b, Wiemers 2003, Wiemers and Fiedler 2007) or a subspecies of *P.* (*A.*) *poseidon* (Tshikolovets 2011).

The taxon *P.* (*A.*) *deebi* (Larsen, 1974) discovered in Lebanon and Syria is often considered as a subspecies of *P.* (*A.*) *poseidon* (e.g. Tshikolovets 2011), however, it differs in chromosome number (n=17, Larsen 1975) and may represent a different species (Eckweiler and Häuser 1997). The taxon *P.* (*A.*) *damocles krymaeus* (Sheljuzhko, 1928) was also considered as subspecies of *P.* (*A.*) *poseidon* (Hesselbarth et al. 1995), however, with respect to mitochondrial genes *COI* and *COII* it is very distant from *P.* (*A.*) *poseidon* and was shown to be a subspecies of *P.* (*A.*) *damocles* (Herrich-Schäffer, [1844]) (Lukhtanov et al. 2005, Kandul et al. 2007).

Males of *P.* (*A.*) *poseidon* have plesiomorphic (Kandul et al. 2004, Lukhtanov et al. 2005) blue colouration of the upper side of the wings with no specific morphological characters. Therefore their morphological discrimination from phenotypically similar *P.* (*A.*) *caeruleus* (Staudinger, 1871), *P.* (*A.*) *damocles* and *P.* (*A.*) *damonides* (Staudinger, 1899) is difficult. With respect to *COI* barcodes, *P.* (*A.*) *poseidon* is indistinguishable from *P.* (*A.*) *hopfferi* (Herrich-Schäffer, [1851]) and *P.* (*A.*) *putnami* (Wiemers & Fiedler, 2007). As it was stated above, the chromosome number varies within *P.* (*A.*) *poseidon* (de Lesse 1963, Kandul and Lukhtanov 1997, Lukhtanov and Dantchenko 2002b) and thus overlap with chromosome numbers found in *P.* (*A.*) *elbursicus* (Forster, 1956), *P.* (*A.*) *cyaneus* (Staudinger, 1899), *P.* (*A.*) *ectabanensis* (de Lessse, 1963), *P.* (*A.*) *hamadanensis* (de Lesse, 1959), *P.* (*A.*) *alcestis* (Zerny, 1932), *P.* (*A.*) *altivagans* (Forster, 1956), *P.* (*A.*) *mithridates* (Staudinger, 1878), *P.* (*A.*) *shirkuhensis* ten Hagen

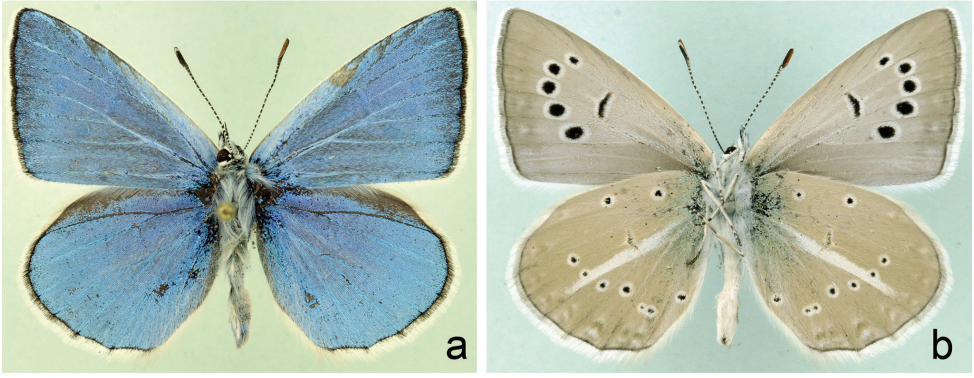

Figure 1. *Polyommatus* (*Agrodiaetus*) *poseidon* from Akhaltsikhe, Georgia. **a** male, upperside **b** male, underside.

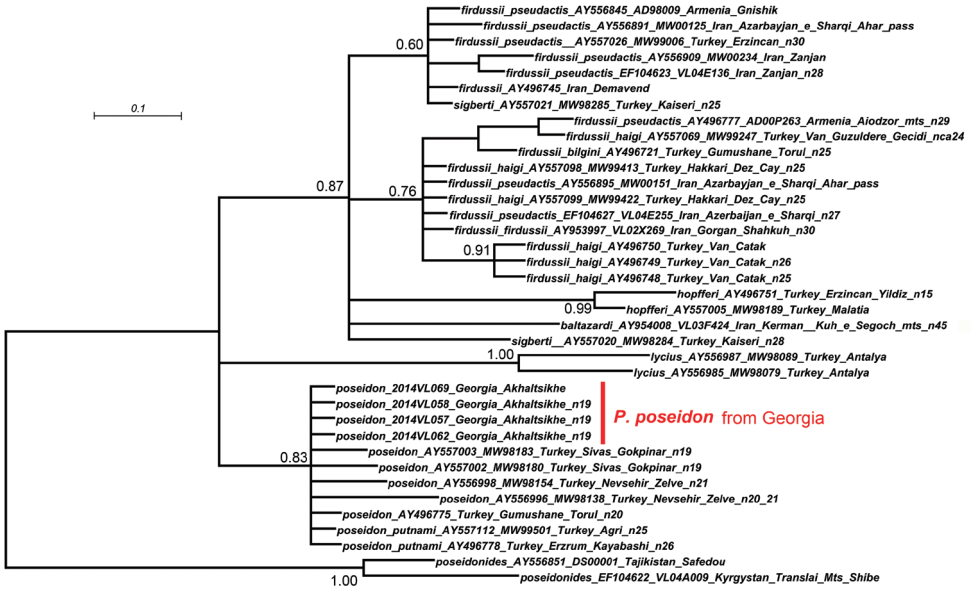

Figure 2. Bayesian tree of the species close to *Polyommatus* (*Agrodiaetus*) *poseidon* inferred from *COI* sequences. Posterior probability values >50% are shown.

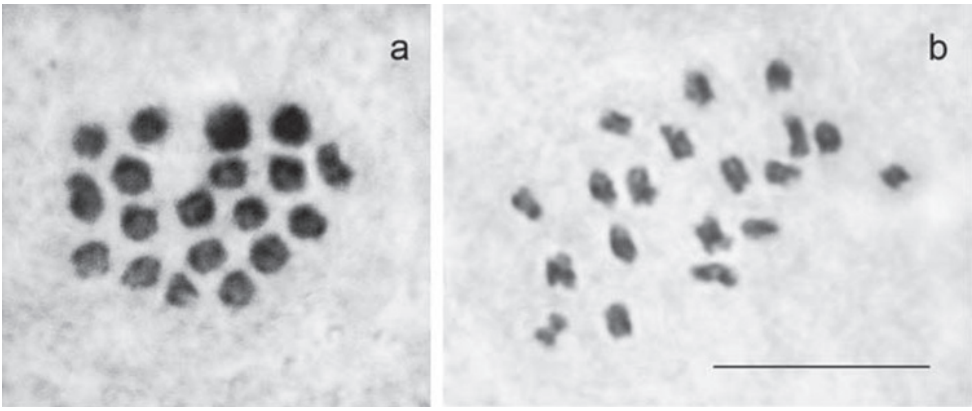

Figure 3. Male karyotype of *Polyommatus* (*Agrodiaetus*) *poseidon* from Georgia. **a** sample 2014VL57, metaphase I, n = 19 **b** sample 2014VL62, metaphase II, n = 19. Bar = 10 μm.

et Eckweiler, 2001 and *P.* (*A.*) *pierceae* (Lukhtanov & Dantchenko, 2002) (Kandul et al. 2007, Lukhtanov et al. 2014).

A population of blue butterflies which were morphologically similar to *P.* (*A.*) *poseidon* (Fig. 1) was discovered near Akhaltsikhe in Georgia in 2013 by V.Tikhonov and I. Kostyuk. In 2014 the locality was visited again in order to collect material available for molecular and chromosomal study. Molecular analysis of this material revealed

that *COI* barcodes were completely identical or nearly identical (barcode gap from 0 to 0.6%) in population from Akhaltsikhe and other populations of *P.* (*A.*) *poseidon* and *P.* (*A.*) *putnami* (Fig. 2).

The haploid chromosome number n=19 was found in MI and MII cells of three studied individuals (2014VL57, 2014VL58, 2014VL62) (Fig. 3). All chromosome elements formed a gradient size row. The karyotype contained no exceptionally large or small chromosomes. In this respect, the population from Akhaltstikhe is indistinguishable from populations of *P.* (*A.*) *poseidon* from Amasya (de Lesse 1963) and Artvin (Kandul and Lukhtanov 1997), but differs from *P.* (*A.*) *putnami* (n=26) (Lukhtanov and Dantchenko 2002b).

Thus, although in the studied case neither the DNA barcodes nor chromosomal numbers are species-specific characters, their combination clearly indicates that the population from Akhaltsikhe should be identified as *P.* (*A.*) *poseidon*. This is the first evidence of *P.* (*A.*) *poseidon* for Georgia and for Caucasus region at whole.

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Phylogenetic relationships of some species of the family Echinostomatidae Odner, 1910 (Trematoda), inferred from nuclear rDNA sequences and karyological analysis

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Abstract

The family Echinostomatidae Looss, 1899 exhibits a substantial taxonomic diversity, morphological criteria adopted by different authors have resulted in its subdivision into an impressive number of subfamilies. The status of the subfamily Echinochasminae Odhner, 1910 was changed in various classifications. Genetic characteristics and phylogenetic analysis of four Echinostomatidae species – *Echinochasmus* sp., *Echinochasmus coaxatus* Dietz, 1909, *Stephanoprora pseudoechinata* (Olsson, 1876) and *Echinoparyphium mordwilkoi* Skrjabin, 1915 were obtained to understand well enough the homogeneity of the Echinochasminae and phylogenetic relationships within the Echinostomatidae. Chromosome set and nuclear rDNA (ITS2 and 28S) sequences of parthenites of *Echinochasmus* sp. were studied. The karyotype of this species (2n=20, one pair of large bi-armed chromosomes and others are smaller-sized, mainly one-armed, chromosomes) differed from that previously described for two other representatives of the Echinochasminae, *E. beleocephalus* (von Linstow, 1893), 2n=14, and *Episthmium bursicola* (Creplin, 1937), 2n=18. In phylogenetic trees based on ITS2 and 28S datasets, a well-supported subclade with *Echinochasmus* sp. and *Stephanoprora pseudoechinata* clustered with one well-supported clade together with *Echinochasmus japonicus* Tanabe, 1926 (data only for 28S) and *E. coaxatus*. These results supported close phylogenetic relationships between *Echinochasmus* Dietz, 1909 and *Stephanoprora* Odhner, 1902. Phylogenetic analysis revealed a clear separation of related species of Echinostomatoidea restricted to prosobranch snails as first intermediate hosts, from other species of Echinostomatidae and Psilostomidae, developing in Lymnaeoidea snails as first intermediate hosts. According to the data based on rDNA phylogeny, it was supposed

that evolution of parasitic flukes linked with first intermediate hosts. Digeneans parasitizing prosobranch snails showed higher dynamic of karyotype evolution provided by different chromosomal rearrangements including Robertsonian translocations and pericentric inversions than more stable karyotype of digenean worms parasitizing lymnaeoid pulmonate snails.

Keywords
Echinochasmus, *Stephanoprora*, Echinostomatidae, karyotype evolution, intermediate host, rDNA, ITS2, 28S

Introduction

The family Echinostomatidae Looss, 1899 is a heterogeneous group of cosmopolitan, hermaphroditic digeneans. Adult echinostomatids are predominantly found in birds, and also parasitize mammals including man, and occasionally reptiles and fishes (Huffman and Fried 1990, Kostadinova and Gibson 2000, Kostadinova 2005a). Morphological diversity of this group and/or the diversity of the criteria adopted by different authors have resulted in its subdivision into an impressive number of subfamilies (Kostadinova and Gibson 2000). The Echinostomatidae has been viewed as a monophyletic taxon, with some exceptions, but some authors suggested that the family Echinostomatidae is polyphyletic and elevated the Echinochasminae Odhner, 1910 to full family rank (Odening 1963, Sudarikov and Karmanova 1977). Kostadinova (2005a) accomplished the last revision of the Echinostomatidae accepting 11 subfamilies and 44 genera after the vast comparative morphological study based on the examination of type and freshly collected material, and a critical evaluation of published data. Afterward, she retained the subfamilial status of the Echinochasminae with similar composition to that proposed in 1971 by Yamaguti.

The karyotypes of more than 20 species of the subfamily Echinostomatinae Looss, 1899 belonging to the genera *Echinostoma* Rudolphi, 1809, *Echinopharyphium* Dietz, 1909, *Hypoderaeum* Dietz, 1909, *Neoacanthoparyphium* Yamaguti, 1958, *Moliniella* Hübner, 1939, and *Isthmiophora* Lühe, 1909 have been described; most species had 2n=20 or 2n=22, except some species (for review, see Baršienė 1993). The karyotypes of two species of the subfamily Echinochasminae, namely *Echinochasmus beleocephalus* (von Linstow, 1893), 2n=14, and *Episthmium bursicola* (Creplin, 1937), 2n=18, have been reported by Baršienė and Kiselienė (1990).

The use of molecular approaches to determine phylogenetic relationships of digeneans has grown very rapidly since 1990s and molecular-based studies on echinostomes have been carried out to date (Morgan and Blair 1995, 1998a, 1998b, 2000, Petrie et al. 1996, Grabda-Kazubska et al. 1998, Kostadinova et al. 2003, Saijuntha et al. 2011, Georgieva et al. 2013, 2014, Noikong et al. 2014, Selbach et al. 2014, Kudlai et al. 2015). The genus *Echinochasmus* Dietz, 1909 (as well as *Echinostoma* and *Echinopharyphium*) is one of the most species–rich genera in Echinostomatidae (Kostadinova and Gibson 2000); however, no one species of this genus was involved in molecular phylogenetic studies of the Digenea (Cribb et al. 2001, Olson et al. 2003, Olson and Tkach 2005).

The present study is mainly focused on comparative analysis of species belonging to the subfamily Echinochasminae. Two regions of rDNA, ITS2 and partial 28S, and karyotype of cercaria of *Echinochasmus* sp., parasite of the gravel snail *Lithoglyphus naticoides* (C. Pfeiffer, 1828) are presented there as well as DNA sequences of adult specimen of type-species of *Echinochasmus*, *Echinochasmus coaxatus* Dietz, 1909 from the final host *Podiceps nigricollis* C. L. Brehm, 1831. Morphology of the *Echinochasmus* sp. cercaria from the same population of *L. naticoides* was previously described by Stanevičiūtė et al. (2008).

Materials and methods

The digeneans for this study were obtained from naturally infected hosts. Seven specimens of gravel snail *Lithoglyphus naticoides* infected with parthenites of *Echinochasmus* sp. were collected at water reservoir of the dammed up River Nemunas near Kaunas in Lithuania (54°51.38'N, 24°09.08 E'). The specimens of snail *Valvata piscinalis* (Müller, 1774) infected with parthenites of *Echinoparyphium mordwilkoi* Skrjabin, 1915 were collected from the River Ūla, Lithuania (54°7.76'N, 24°27.76'E). The ethanol fixed adult specimen of *Echinochasmus coaxatus* recovered from *Podiceps nigricollis* in Kherson region (Ukraine) was received from collection of Department of Parasitology, I.I. Schmalhausen Institute of Zoology of NAS of Ukraine. Adult trematodes from *Larus melanocephalus* (Temminck, 1820) and cercariae from *Hydrobia acuta* (Draparnaud, 1805) were described as *Stephanoprora pseudoechinata* (Olsson, 1876) by Kudlai and Stunžėnas (2013); rDNA sequences of these specimens were used for comparative analysis in this study.

Living *L. naticoides* snails were incubated in 0.01% colchicine in well water for 12–14 h at room temperature and afterward, dissected. The infected tissues from crushed snails were transferred to distilled water for 40–50 min and fixed in a freshly prepared Carnoy's solution I (Farmer's solution) composed of 3 parts of 95% ethanol and 1 part glacial acetic acid. Chromosome slides were prepared using air-dried method and analysed after conventional Giemsa staining (Petkevičiūtė and Stanevičiūtė 1999). The karyotypes were constructed by arranging the chromosome pairs in order of decreasing size. Chromosomes of 11 high quality metaphase plates were measured using Image-Pro Plus v3 software. Chromosome measurements included length of individual chromosomes, relative length, and centromeric index. These parameters were used for description of chromosome morphotype according to standard nomenclature of Levan et al. (1964). Data were analyzed using the Student's *t* test. Results were considered significant when $P<0.05$. The same nomenclature was applied to the karyotype of the other seven species used for comparison: *Episthmium bursicola*, *Echinochasmus beleocephalus*, *Echinopharyphium aconiatum* Dietz, 1909, *Istmiophora melis* (Schrank, 1788) Lühe, 1909, *Hypoderaeum conoideum* (Bloch, 1782), *Sphaeridiotrema globulus* (Rudolphi, 1814), and *Echinostoma revolutum* (Fröelich, 1802) Looss, 1899. Karyotypic data of these taxa were obtained from Baršienė and Kiselienė (1990), Baršienė (1993) and Mutafova (2001).

The DNA extraction (without proteinase or lysis buffer treatment) was performed in sterile Tris-borate-EDTA (TBE) buffer. In previous study this method allowed us to extract high quality DNA from tissue of molluscs (Stunžėnas et al. 2011) and trematodes (Petkevičiūtė et al. 2014). An entire nuclear 5.8S-ITS2-28S DNA sequence of ribosomal DNA (~460bps: 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence) was amplified using primers: 3S (5'- CGG TGG ATC ACT CGG CTC GTG -3'), forward direction; 28S (5'- CCT GGT TAG TTT CTT TTC CTC CGC -3'), reverse direction (Bowles et al. 1995). The 5' end of the 28S rRNA gene sequence (~1,200 bps), not overlapping with the previous sequence, was amplified using two primers: Digl2 (5'- AAG CAT ATC ACT AAG CGG -3') forward direction; L0 (5'- GCT ATC CTG AG(AG) GAA ACT TCG-3') reverse (Tkach et al. 1999). DNA fragments were amplified via a standard Polymerase Chain Reaction (PCR) according to Petkevičiūtė et al. (2014).

DNA sequences of representative species of the superfamily Echinostomatoidea and outgroup taxa were downloaded from GenBank and included in the phylogenetic analysis and/or pairwise sequence comparisons together with our data. For phylogenetic analyses the sequences were aligned with ClustalW (Thompson et al. 1994) with an open gap penalty of 15, and a gap extension penalty of 6.66. For data sets we estimated the best-fit model of sequence evolution using jModeltest v. 0.1.1 software (Posada 2008). Neighbour-joining (NJ) (Saitou and Nei 1987), maximum parsimony (MP) (Nei and Kumar 2000) and maximum likelihood (ML) phylogenetic trees were obtained and analysed using MEGA 5 (Tamura et al. 2011). Supports to internal branches for the trees were estimated by bootstrap analyses with 1000 replicates. The genetic distances of neighbour joining tree were calculated by Tamura-Nei (Tamura and Nei 1993) for 28S gene and 5.8S-ITS2-28S rDNA region datasets. Maximum likelihood trees were obtained using general time reversible model with a gamma distribution of rates and a proportion of invariant sites (GTR+G+I) for the both datasets. Gamma shape and number of invariant sites were estimated from the data. Parsimony analysis based on subtree pruning and regrafting (SPR) was used with default parsimony settings.

Results

Karyotype of *Echinochasmus* sp.

Chromosomes of 113 mitotic metaphase spreads from three molluscs revealed that karyotype of *Echinochasmus* sp. is 2n=20; it consists of one pair of large chromosomes and nine pairs of smaller-size chromosomes. Also, the percentage of aneuploid cells (2n=18–19) was 10.62%. Twelve spreads displaying values lower than modal, represent aneuploidies or (more likely) loss of chromosomes during processing, a technical artefact commonly encountered with the slide preparation method used. The measure-

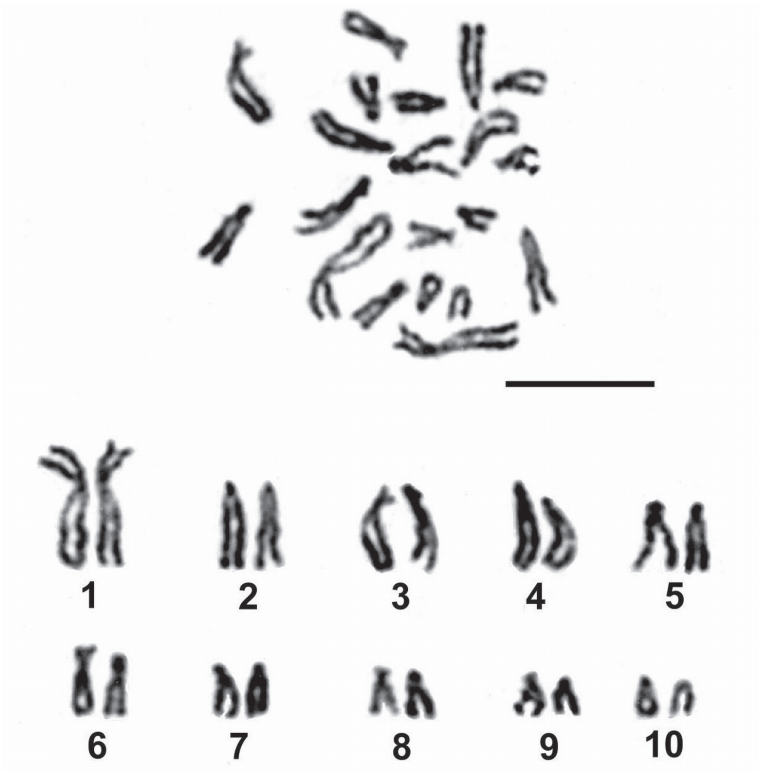

Figure 1. Mitotic metaphase and karyotype of *Echinochasmus* sp. Bar = 10 μm.

Table 1. Morphometric analysis of chromosomes of *Echinochasmus* sp. Stanevičiūtė, Petkevičiūtė & Kiselienė, 2008.

Chromosome number	Absolute length (mm)	Relative length (%)	Centromeric index	Classification
1	7.64*±1.69	18.97±1.61	37.45±1.64	sm-m
2	4.99±0.79	12.51±0.68	10.44± 2.66	a-st
3	4.72±0.98	11.73±0.66	23.64±2.25	st-sm
4	4.46±0.88	11.09±0.58	14.18±3.62	st-a
5	3.98±0.78	9.89±0.60	13.95±4.13	st-a
6	3.69±0.63	9.23±0.64	30.39±5.27	sm
7	3.16±0.53	7.89±0.41	20.71±2.82	st
8	2.81±0.40	7.05±0.44	19.41±2.93	st
9	2.51±0.28	6.33±0.46	22.92±5.25	st
10	2.11±0.38	5.29±0.71	19.17±4.32	st

* - mean±SD; m - metacentric; sm - submetacentric, st - subtelocentric; a - acrocentric chromosomes

ments of mitotic chromosomes showed ten chromosome pairs ranging in size from 2.11 to 7.64 μm (Fig. 1, Table 1). The mean total length of the haploid complement is 40.07 μm. The homologues of the 1st pair are significantly large than the remaining chromosomes and comprise about 19% of the total chromosome complement length.

According to the centomeric index value they are of submeta-or metacentrics. The remaining chromosomes decrease in size fairly gradually. Three pairs (2nd, 4th and 5th) fall into an intermediate position between acrocentric and subtelocentric; pair 3rd is subtelocentric - submetacentric; pair 6th is submetacentric and four last chromosome pairs (7th – 10th) are subtelocentric.

Molecular analysis

New sequences from two different regions of nuclear ribosomal DNA were obtained: the 5.8S-ITS2-28S and the 5' end of the 28S gene, which does not overlap with the previous sequence. Complete nucleotide sequences are available in GenBank (Figs 2, 3). Pairwise comparisons of newly obtained sequences demonstrated that *Echinochasmus* sp. was closest to *Stephanoprora pseudoechinata*. These sequences of *Echinochasmus* sp. differed from sequences of *S. pseudoechinata* by 12 out of 653 base pairs (1.84%) in the 5.8S-ITS2-28S region and by 15 out of 1070 base pairs (1.4%) in the sequenced portion of the 28S gene. All other differences among the new sequences were more significant, sequence divergence ranged from 13.59 to 23.15% in the 5.8S-ITS2-28S region and from 6.5 to 10.76% in the portion of the 28S gene. Blast searches (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) performed on these sequences demonstrated the highest matches with sequences of digenean trematodes of superfamily Echinostomatoidea. The new sequences were aligned with sequences of representative species of this superfamily. The aligned dataset of the 5.8S-ITS2-28S rDNA region included 35 sequences of the Echinostomatoidea and 408 sites after trimming the ends to match the shortest aligned sequences. This alignment without outgroups showed a high sequence divergence of ITS2 rDNA region and comprises 228 variable (56%) and 175 (43%) parsimony informative sites. The aligned dataset of the partial 28S gene included 33 sequences of the Echinostomatoidea and was comprised of 990 sites after trimming the ends to match the shortest aligned sequences. This alignment without outgroups comprises 341 variable (34.44%) and 250 (25.25%) parsimony informative sites.

Maximum likelihood, neighbor-joining and maximum parsimony analyses of these sequences, including representative species of superfamily Echinostomatoidea, produced identical topology of phylogenetic trees (Figs 2, 3). The *Echinochasmus* sp. Stanevičiūtė et al. 2008 clustered together with *S. pseudoechinata* in a 94–100% supported subclade in the ITS2 phylogenetic tree (Fig. 2) and a 100% supported clade in the 28S phylogenetic tree (Fig. 3). This subclade clustered together with other species from *Echinochasmus* genus and formed a well-supported monophyletic clade, clearly separated from clades containing other species of Echinostomatoidea families. *Echinoparyphium mordwilkoi* clustered in a 96–100% supported clade with *Echinoparyphium* spp. Species of these genera formed a 99–100% supported subclade without separate branch of *E. mordwilkoi* (Fig. 3).

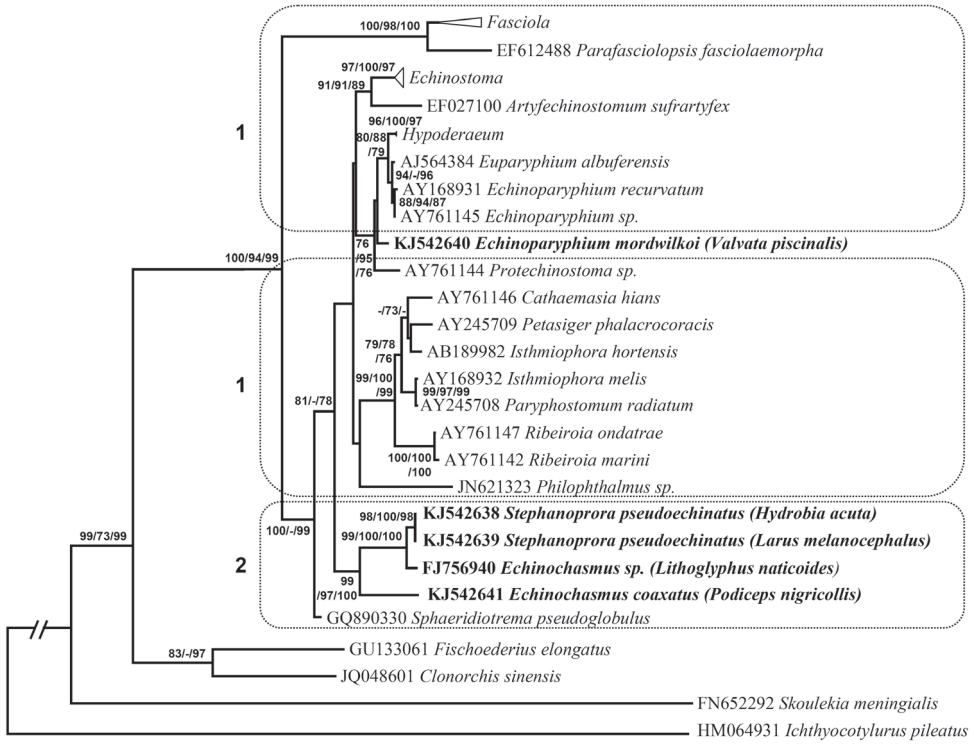

Figure 2. Phylogenetic ITS2 tree. Maximum likelihood phylogenetic tree based on analysis of ribosomal DNA sequences (5.8S-ITS2-28S). Bootstrap percentages refer to maximum likelihood / neighbor-joing / maximum parsimony analysis. Only bootstrap values above 70% are shown. GenBank accession numbers are indicated before species names. Names of the target species are in bold; their hosts are presented in parentheses. Compressed clades: *Fasciola* (comprised sequences under GenBank accession numbers AM900370, EF534995, EF612486, JF496715), *Echinostoma* (AF067850, AF067852, AJ564383, AY168930, EPU58100, ETU58097, ELU58099, GQ463131, GQ463132), *Hypoderaeum* (AJ564385, GQ463134). Dotted rectangles 1 indicate digeneans whose life cycles include Lymnaeoidea as first intermediate host; dotted rectangle 2 indicates digeneans whose life cycles include prosobranch snails as first intermediate hosts.

Discussion

Sequence divergence between *S. pseudoechinata* and *Echinochasmus* sp., 1.84% in the 5.8S-ITS2-28S rDNA region and 1.4% in the partial 28S gene, falls within the level of intragenus variability. Both taxa made up a strongly supported clade together with the type-species of the genus *Echinochasmus*, *E. coaxatus*. These results imply that macrocercous cercaria of *Echinochasmus* sp. may be attributed to the genus *Stephanoprora* Odhner, 1902. According to Kostadinova (2005a), data on the life histories of some Echinochasminae species (including, probably, *E. macrocaudatus* Ditrich, Scholz & VargasVazques, 1996) tend to support the affiliation of species to *Stephanoprora* rather

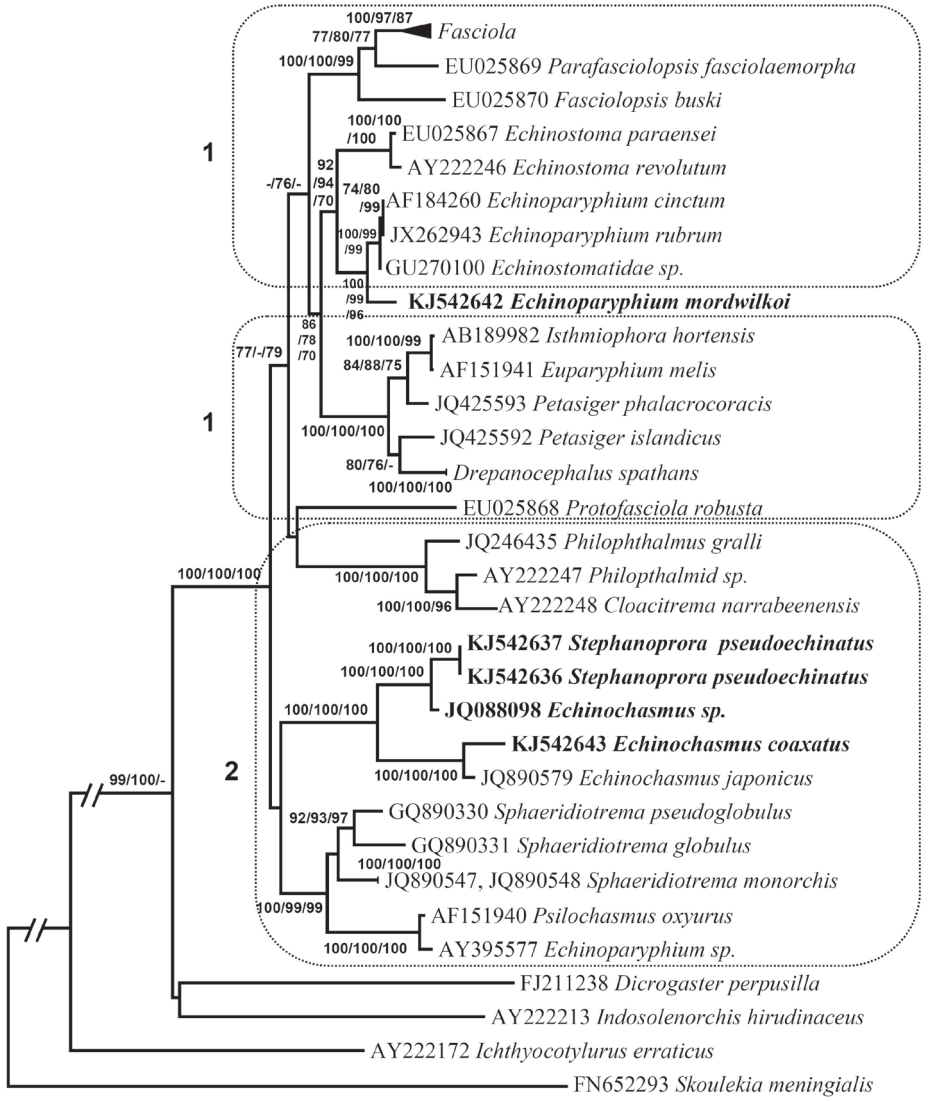

Figure 3. Phylogenetic 28S tree. Maximum likelihood phylogenetic tree based on analysis of ribosomal 28S gene DNA partial sequences. Bootstrap percentages refer to maximum likelihood / neighbor-joing / maximum parsimony analysis. Only bootstrap values above 70% are shown. GenBank accession numbers are indicated before species names. Names of the target species are in bold.Compressed clade *Fasciola* comprised sequences under GenBank accession numbers AY222244, EU025871, EU025872, HM004190). Dotted rectangles 1 indicate digeneans whose life cycles include Lymnaeoidea as first intermediate host; dotted rectangle 2 indicates digeneans whose life cycles include prosobranch snails as first intermediate hosts.

than to *Echinochasmus* on the presence of a long-tailed cercarial stage. On the other hand, *S. pseudoechinata* is a marine species, while *Echinochasmus* sp. Stanevičiūtė et al. 2008 is a parasite of freshwater organisms, a finding that shows a considerable ecological

plasticity in this group. Sudarikov and Karmanova (1977) stated that the ontogenetic character state of Echinochasminae species concerning the absence of well-developed collar with collar spines in the morphology of cercaria, indicates that echinochasmids is a more ancient group than other echinostomatids. The phylogenetic relationships estimated by ITS2 and 28S sequences partly support this hypothesis, because *Echinochasmus* sp. Stanevičiūtė et al. 2008 and *S. pseudoechinata* were clustered in one clade with *Sphaeridiotrema globulus* (Psilostomidae) in the 28S tree. Cribb et al. (2001) stated that from 144 known life cycles of Echinostomatidae species about two-thirds of the first intermediate hosts are lymnaeoid pulmonates but there are also significant numbers of species developing in prosobranchs. Ecological preferences of Echinostomatidae species suggest that there has been a strong co-evolution with the Lymnaeoidea and a less frequent association with a few prosobranch taxa. On the contrary, all 18 species of *Echinochasmus* with known life cycles are restricted to prosobranchs. *Echinoparyphium mordwilkoi*, that shows a separate position from *Echinochasmus* in the molecular analyses (Figs 2, 3), is restricted to the lower heterobranch *Valvata piscinalis* (Valvatoidea). Most of Psilostomidae species also admit for the first intermediate host a prosobranch snail (Grabda-Kazubska et al. 1991), except those ones belonging to the genus *Ribeiroia* Travastos, 1939, which position in this family is questionable (Wilson et al. 2005). The species of this genus originally have parasitized pulmonate snails. In the 28S phylogenetic tree, the clade uniting *Echinochasmus* spp. and *Stephanoprora* sp. clustered with Psilostomidae (*Psilochasmus oxyurus* (Creplin, 1825) and *S. globulus*), whose life cycles include prosobranch snails as first intermediate host. The isolate of redia gathered from the prosobranch snail *Gabbia vertiginosa* (Frauenfeld, 1862), despite being identified as *Echinoparyphium* sp. (unpublished data from Genbank), also clustered with *P. oxyurus* and *S. globulus*. Grabda-Kazubska et al. (1991) stated that the morphological data and chaetotaxy of *Echinochasmus* cercaria also show that this genus appears more closely related to the *Psilotrema* (Odhner, 1913) and *Sphaeridiotrema* (Odhner, 1913) than to *Echinostoma*. The Psilostomidae, apart from the absence of a circumoral head-collar armed with spines, closely resemble the Echinostomatidae in their general morphology (Kostadinova 2005b). Species of *Philophthalmus* Looss, 1899 (Echinostomatoidea: Philophthalmidae), whose life cycles include prosobranch snails as first intermediate hosts, formed a well-supported clade in the main clade uniting subfamilies of Echinostomatidae (Fig. 3).

The chromosome complement of *Echinochasmus* sp. with 2n=22 chromosomes gradually decreasing in size and with one-armed elements prevailing are characteristic for species of type-genus *Echinostoma* (Baršienė 1993; Mutafova 1994). The same chromosome morphology has been reported for species of the genus *Echinopharyphium*, *Neoacanthoparyphium*, *Moliniella*, *Hypoderaeum*, *Isthmiophora* (Echinostomatinae), but in these species the diploid chromosome number is lower, 2n = 20 (see Baršienė 1993 for review, Mutafova 1994). The chromosome number and morphology of *Echinochasmus* sp. resemble the karyotypic data of other representatives of Echinostomatinae (Baršienė 1993). Surprisingly, the other two known karyotypes of species of Echinochasminae are very different from that of *Echinochasmus* sp. Stanevičiūtė et al. 2008.

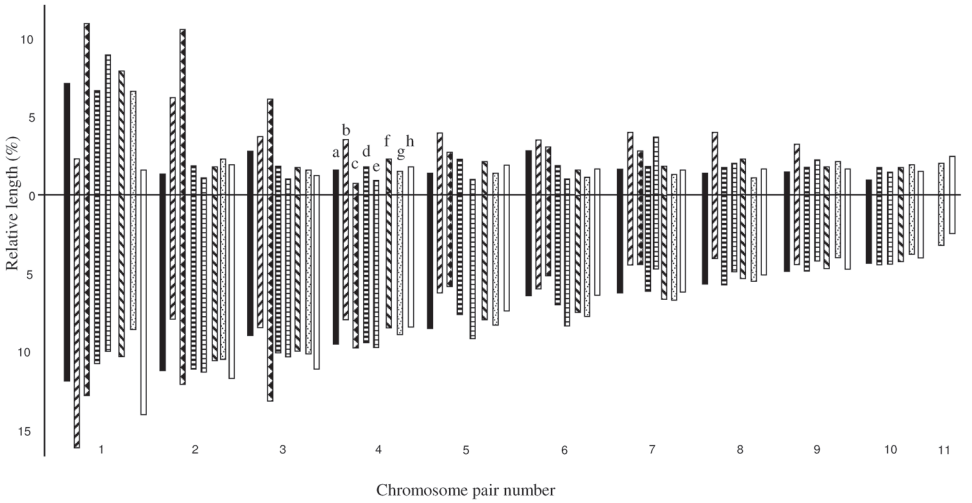

Figure 4. Idiograms representing the haploid chromosome sets. Idiogram representing the haploid sets of eight species: **a** *Echinochasmus* sp. **b** *Episthmium bursicola* **c** *Echinochasmus beleocephalus* **d** *Echinopharyphium aconiatum* **e** *Istmiophora melis* **f** *Hypoderaeum conoideum* **g** *Sphaeridiotrema globulus* **h** *Echinostoma revolutum* **b, c** - data of Baršienė and Kiselienė (1990) **d, e, f, h** data of Baršienė (1993) **g** data of Mutafova (2001).

The chromosome number of *E. beleocephalus* is 2n=14 and the karyotype consists of three pairs of large biarmed chromosomes and four pairs of smaller homologues. The chromosome set of *Episthmium bursicola* contains 2n=18 and is conspicuous by the presence of a large first pair of subtelocentric elements and the rest of biarmed chromosomes (Baršienė and Kiselienė 1990). The karyotype of Psilostomidae (Echinostomatoidea) – *Psilotrema* sp., *Psilotrema simillimum* (Mühling, 1898) (2n=16), *Psilotrema spiculigerum* (Mühling, 1898) (2n=24) and *Sphaeridiotrema globulus* (2n=14) also vary in their chromosome patterns (Baršienė 1993; Mutafova et al. 1998). Mutafova et al. (2001) studied *S. globulus* and found a quite different diploid karyotype (2n=22 instead of 2n=14), with similar characteristic to those found in species of the genus *Echinostoma* 2n=22 and chromosomes of similar relative length; likewise, the centromeric position also varied possibly due to pericentric inversions. A possibility of mistake in the identifications of some species was mentioned by Mutafova et al. (2001). The ideograms of karyotypes of *Echinochasmus* sp. and some discussed species were constructed (Fig. 4) based on the mean values presented in Table 1 and previously published data (Baršienė and Kiselienė 1990, Baršienė 1993, Mutafova et al. 2001). A notable variation in chromosome number and morphology suggest the occurrence of multiple chromosome changes: Robertsonian changes, translocations and pericentric inversions. Chromosome rearrangements in lineage of Echinostomatinae show a karyotypic trend towards reduction in chromosome number, but the main karyotypic changes occurring in a case of speciation in this lineage are multiple pericentric inversions and fit into category of karyotypic orthoselection according to White (1973).

Centric fusions could be a possible mechanism for changes in the chromosomal number in this family and in the other digenean groups (Grossman et al. 1981a,b, Baršienė 1993, Mutafova 1994). Pericentric inversions are also possibly involved in the karyotypic evolution of echinostomatids, since within the group of species with 2n=20 some of them have more biarmed chromosomes than others, while differences in relative length values are not so conspicuous. The notable differences found in the karyotypes of echinochasmine species show the need for further karyological analysis of this family.

The results of this study indicated that the phylogenetic branching of digeneans is related to the nature of their first intermediate host. Moreover, the mode of karyotype evolution correlates with the intermediate host: a remarkable karyotype variation was detected among species parasitizing prosobranch snails, whereas differences among karyotypes of the species parasitizing lymnaeoid pulmonates snails are not significant.

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RESEARCH ARTICLE

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First description of the karyotype and localization of major and minor ribosomal genes in *Rhoadsia altipinna* Fowler, 1911 (Characiformes, Characidae) from Ecuador

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Abstract

Karyotypic features of *Rhoadsia altipinna* Fowler, 1911 from Ecuador were investigated by examining metaphase chromosomes through Giemsa staining, C-banding, Ag-NOR, and two-color-fluorescence in situ hybridization (FISH) for mapping of 18S and 5S ribosomal genes. The species exhibit a karyotype with 2n = 50, composed of 10 metacentric, 26 submetacentric and 14 subtelocentric elements, with a fundamental number FN=86 and is characterized by the presence of a larger metacentric pair (number 1), which is about 2/3 longer than the average length of the rest of the metacentric series. Sex chromosomes were not observed. Heterochromatin is identifiable on 44 chromosomes, distributed in paracentromeric position near the centromere. The first metacentric pair presents two well-defined heterochromatic blocks in paracentromeric position, near the centromere. Impregnation with silver nitrate showed a single pair of Ag-positive NORs localized at terminal regions of the short arms of the subtelocentric chromosome pair number 12. FISH assay confirmed these localization of NORs and revealed that minor rDNA clusters occur interstitially on the larger metacentric pair number 1. Comparison of results here reported with those available on other Characidae permit to hypothesize that the presence of a very large metacentric pair might represent a unique and derived condition that characterize one of four major lineages molecularly identified in this family.

Keywords
18S and 5S ribosomal genes, C-bands, fishes, karyotype, NORs

Introduction

The study of fish chromosomes has become an active area of research in recent decades providing basic information on the number, size and morphology of chromosomes, nucleolus organizers regions (NORs), distribution of constitutive heterochromatin and other more specific markers, detected through the application of molecular techniques (Nirchio and Oliveira 2006a). These features has been of great importance in allowing the diagnose of species, identification of differentiate cryptic species and chromosomal races (Nirchio et al. 2003a, 2005, 2007), establishing the relationships between species within a genus or family (Nirchio et al. 2001, 2006b, 2008, Oliveira et al. 2003), clarifying the origin of natural hybrids (Nirchio et al. 2003b) and increasing the knowledge of evolutionary mechanisms and genetic question in fishes (Nirchio et al. 2014).

Characiformes are exclusively freshwater fishes distributed in America and Africa, with the greatest diversity in major Neotropical watersheds (Buckup 1998). Characiformes comprises 2,081 valid species grouped in 23 families: Characidae is the largest with 15 subfamilies and 1,086 valid species (Eschmeyer and Fong 2015). These fish have the larger geographic distribution within this order occupying almost all environments of freshwater, with distribution in the Americas, from southwestern United States to South of Argentina (Lucena 1993). In Ecuador, among the freshwater fishes, the Characiformes is the second largest order for number of species (345), after Siluriformes (365) (Barriga 2012) and although chromosome studies in the Neotropical area have been performed for 475 species of Characiformes (Oliveira et al. 2009) until now there is an absolute absence of data from Ecuador.

The Rhoadsiinae, belonging to Characidae, includes three nominal genera: *Rhoadsia* with two species (*R. altipinna*, *R. minor* Eigenmann & Henn, 1914), *Parastremma* with three species (*P. sadina* Eigenmann, 1912, *P. album* Dahl, 1960, *P. pulchrum* Dahl, 1960) and *Carlana* with only one species (*C. eigenmanni* (Meek, 1912)) (Cardoso 2003). In this work we present for the first time the cytogenetic description of *Rhoadsia altipinna* Fowler, 1911, which is characterized by a striking sexual dimorphism (Fig. 1). Species of *Rhoadsia* are distributed in Ecuador and Peru where they are relatively common and ecologically important. *R. altipinna* occurs at low altitudes in the southwest region from the South of the Guayas River to North of the Peru, while *R. minor* occurs at higher altitudes and in river systems in the Northwest of Ecuador (Barriga 2012). There are not cytogenetic data available for these species. The low diversity of species and peculiar geographical distribution of *Rhoadsia* species turn it in an interesting group from the evolutionary and conservation perspective, since in the western part of Ecuador, many areas within the range of the subfamily are under the condition of relatively serious threat (Loh et al. 2014).

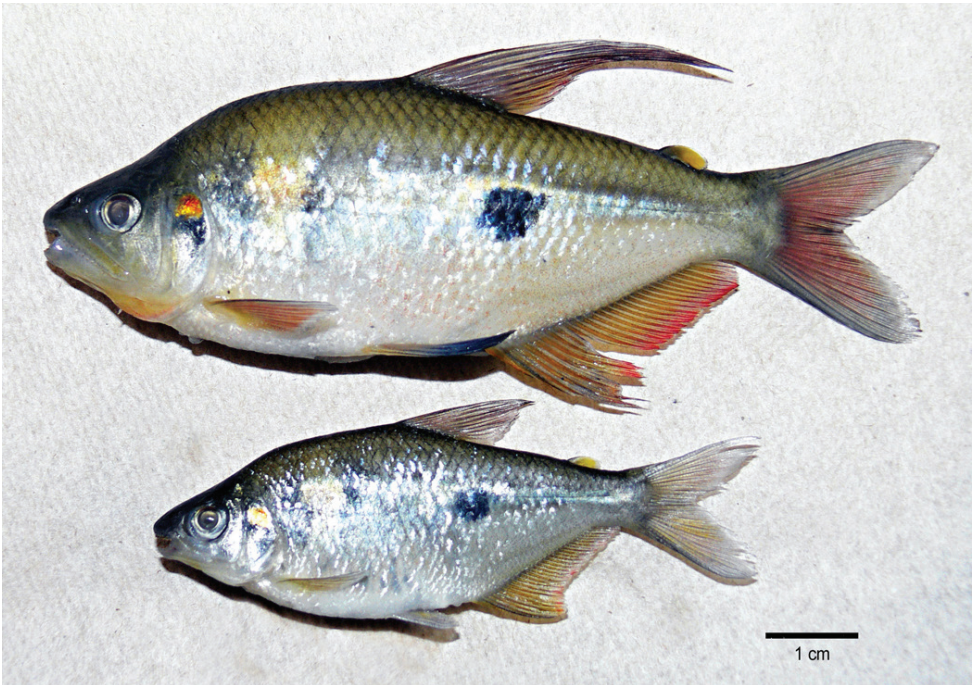

Figure 1. Male (**a**) and female (**b**) specimens of *R.altipinna*.

Methods

Twelve specimens of *R. altipinna* (6 males and 6 females) were collected at Dos Bocas (03°16'07.6"S 079°44'14.8"W) in the Province El Oro, Ecuador were analyzed. Kidney cells suspensions were obtained from fishes injected intramuscularly with yeast glucose solution for mitosis stimulation 24 hours before injecting colchicine (Lee and Elder 1980). Chromosome preparations were obtained injecting 0.0125% colchicine intraperitoneally (0.5 ml/100 g body weight) 50 min before sacrificing as described by Nirchio and Oliveira (2006a). Following the guidelines of the American Veterinary Medical Association for euthanasia of animals (AVMA 2013), fish were sacrificed by numbing them with an overdose of Benzocaine (250 mg/L) until the cessation of opercula movement. Kidney were removed, homogenized and hypotonised by KCl 0,075 M for 20 min at 37 °C. Suspensions were centrifuged at 1000 rpm for 10 min. Supernatant was removed and the cells were fixed by cold fresh Carnoy (3:1 methanol and glacial acetic acid). This process was repeated three times and the cold fresh Carnoy was replaced after each centrifugation. Slides were prepared by conventional air draying method and stained for 20 min with 10% Giemsa in phosphate buffer, pH 6.88. No less than 10 metaphases per sample were analyzed both in males and females using separately all investigated techniques. Silver-stained nucleolus organizer regions (Ag-NORs) were obtained according to Howell and Black (1980). C-bands were obtained following the method of Sumner (1972).

Vouchers specimens were fixed in 10% formalin and deposited in the fish collection of the Laboratório de Biologia e Genética de Peixes (LBP), UNESP, Botucatu (São Paulo State, Brazil) (collection numbers LBP 19362), and Universidad Técnica de Machala (UTMach-020, 021, 047-052).

Position of major and minor ribosomal genes onto the chromosomes was mapped by fluorescence *in situ* hybridization (FISH), following the method of Pinkel et al. (1986). Major (18S rDNA) and minor (5S rDNA) ribosomal probes were isolated from the genome of *Moenkhausia sanctaefilomenae* (Steindachner, 1907) by PCR. Probe for rDNA was obtained 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). This probe was labelled with Biotin-16-dUTP (Roche Applied Science) and hybridization signal detection of hybridization was performed using conjugated Avidin-Fluorescein (FITC). The 5S rDNA probe was obtained using the primer 5S F (5'TAC GCC CGA TCT CGT CCG ATC 3') and 5S R (5'CAG GCT GGT ATG GCC GTA ACG 3') (Pendás et al. 1994). This probe was labelled with Digoxigenin-11-dUTP (Roche Applied Science) and hybridization signal detection of hybridization was performed using Anti-Digoxigenin-Rhodamine (Roche Applied Science).

The mitotic figures were photographed using a Motic B410 microscope equipped with a Motic Moticam 5000C digital camera. Chromosomes were classified according to the arm ratio criteria (Levan et al. 1964). FISH metaphases were photographed with an Olympus BX61 photomicroscope equipped with a DP70 digital camera. Images were digitally processed with ADOBE PHOTOSHOP CS6 Extended.

Results

The analysis of 234 mitotic metaphase cells of *R. altipinna* revealed a diploid number of 2n=50 chromosomes. The karyotype consisted of 10 metacentric, 26 submetacentric and 14 subtelocentric elements, with a fundamental number FN=86 (Fig. 2a). The larger metacentric pair (number 1), is about 2/3 longer than the average length of the rest of the metacentric series. No differences between chromosome complements were found.

Heterochromatin is distributed in paracentromeric position near the centromere of 44 chromosomes (Fig. 2b). The first metacentric pair presents two well-defined heterochromatic blocks in paracentromeric position, near to the centromere. Impregnation with silver nitrate (Fig. 2c) showed a single pair of Ag-positive NORs located at terminal regions of the short arms of the subtelocentric chromosome pair number twelve.

Dual FISH with 18S and 5S rDNA probes (Fig. 3) confirmed the Ag-NOR sites and did not detect any further inactive major ribosomal clusters; in addition it showed that minor rDNA clusters occur interstitially on the larger metacentric pair number 1 and do not co-localize with the major rDNA clusters.

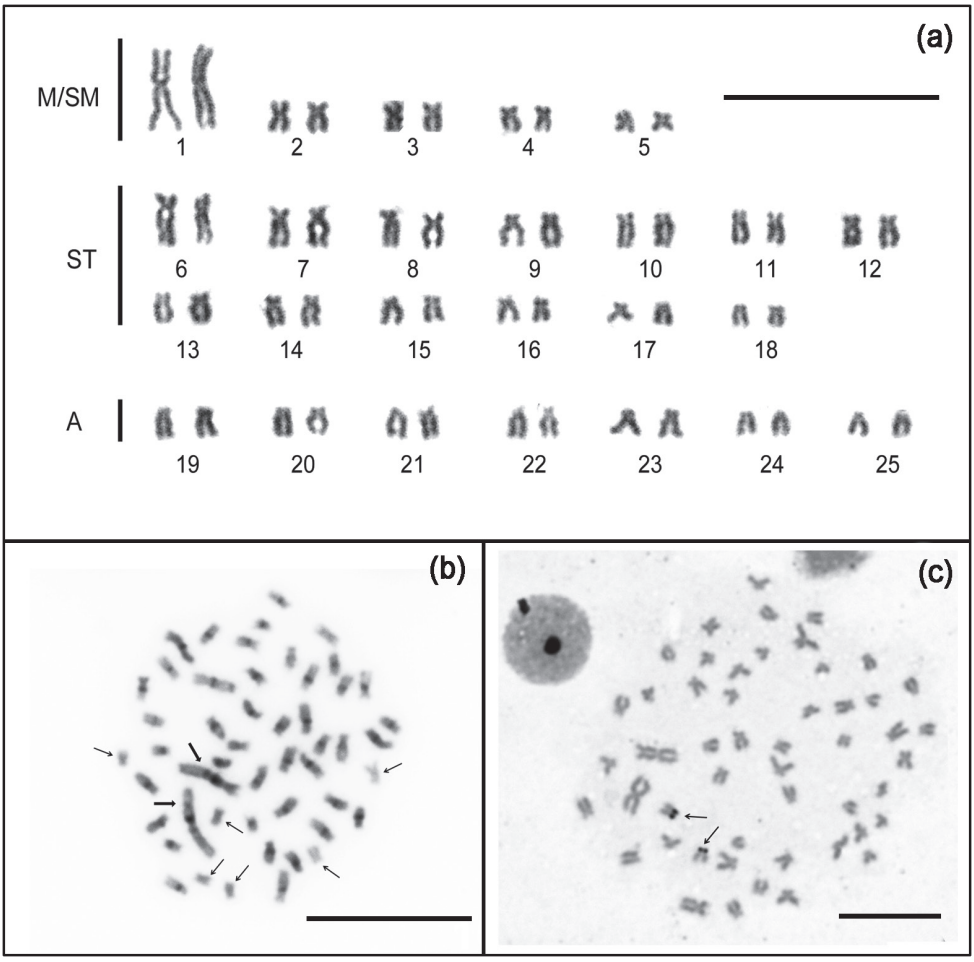

Figure 2. Chromosomes of *R. altipinna* (male). (**a**) Giemsa-stained karyotype, M/SM: Metacentric/Submetacentric; ST: Subtelocentric; A: Acrocentric; (**b**) C-band somatic metaphases - thin arrows indicate chromosomes without positive C-bands and thick arrows point to heterochromatin on the pair number 1; (**c**) Silver-stained metaphase. Arrows indicate Ag-NORs. Bar =10 μm.

Discussion

Cytogenetic studies in Characidae disclose great karyotype diversity related to the high variability of chromosome morphology among species and populations (Arai 2011), and the description of the karyotype of *R. altipinna* adds new data to this picture. Indeed within the family although modal diploid number is relatively constant (2n= 50–52), FN is scattered over a wide range: from 56 in *Aphyocharax dentatus* Eigenmann & Kennedy, 1903 (Souza et al. 1995) to 132 in *Astyanax scabripinnis* (Jenyns, 1842) (Fauaz 1994). According to Arefjev (1994), the high morphological variability of karyotypes with simultaneous relatively constant diploid chromosome numbers is due to

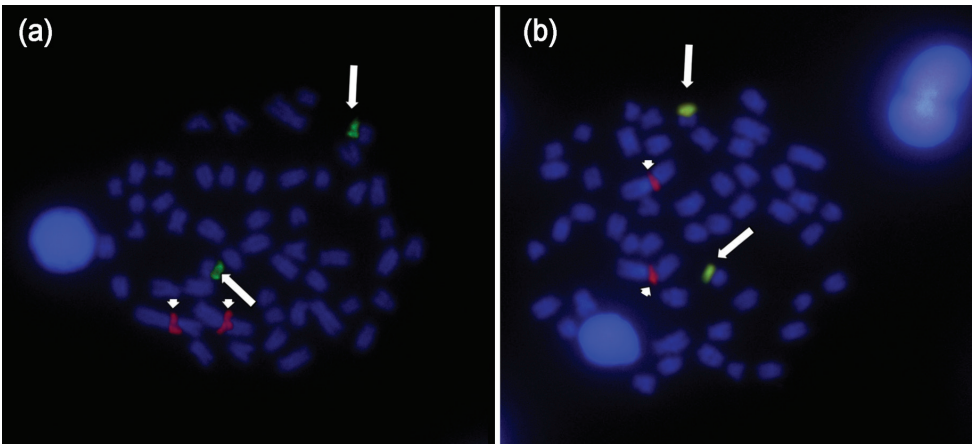

Figure 3. Dual Fluorescence *in situ* hybridization of 18S and 5S rDNA in male (**a**) and female (**b**) of *R. altipinna*. Arrows point to hybridization signal of 18S rDNA, arrowheads indicate hybridization signal of 5S rDNA. Chromosomes are counterstained with DAPI.

the occurrence of numerous chromosome inversions during the karyotype evolution in the group. A study performed from 1,135 living species contained in 12 families of the order Characiformes (Pazza and Kavalco 2010) revealed that Characidae are characterized by the highest rate of chromosomal changes.

Since this work reports the first description of the chromosome complement for *R. altipinna* and karyotype description for its sister species, *R. minor*, is not available yet, it is not possible to make more in-depth comparisons. Within the subfamily Rhoadsiinae, the karyotype of *Nematobrycon palmeri* Eigenmann, 1911 was published by Arefjev (1990) and, although the chromosomes are very condensed in his paper, their gross morphology is very similar to the observed here in *R. altipinna*.

Dual FISH with 18S and 5S rDNA probes showed that in *R. altipinna* minor ribosomal clusters occur interstitially on the larger metacentric pair number 1 and do not co-localize with the major rDNA clusters that are found in terminal position in an acrocentric pair. The presence of a single major rDNA cluster is the most common feature observed in fishes (Martins and Galetti 2001, Arai 2011). Although multiple 5S rDNA sites have been observed in a few species, such as *A. scabripinnis* (Ferro et al. 2001) and *Hoplerythrinus unitaeniatus* (Spix & Agassiz, 1829) (Diniz and Bertollo 2003) the occurrence of single minor rDNA cluster close to centromeres is the most common feature in fish chromosomes (Martins and Galetti 2001, Mariguela et al. 2011) and it has been suggested that this position would be optimal for its organization in fish, since it has been recorder in most species of several orders (Martins and Wasko 2004).

In the more recent and comprehensive study on the phylogeny of the order Characiformes Oliveira et al. (2011) identified four major lineages in Characidae: (1) a clade composed by the single genus *Spintherobolus* Eigenmann, 1911 (without available cytogenetic information); (2) a clade named A, corresponding to Stevardiinae; (3) a clade

named B composed by the subfamilies Tetragonopterinae, Characinae, Cheirodontinae, Aphyocharacinae and some small genera; (4) a clade named Clade C that includes also the subfamilies Rhoadsiinae, Stethaprioninae and many genera. Cytogenetic information is not available for *Spintherobolus* and in species of Clade A (Guimarães et al. 1995, Krinski et al. 2008, Pazian et al. 2012, Piscor et al. 2013) and Clade B (Martins-Santos and Tavares 1986, Souza et al. 1995, Alberdi and Fenocchio 1997, Mariguela et al. 2011) karyotypes do not show the big metacentric pair observed in *R. altipinna*. On the contrary, all the Characidae species belonging to Clade C are characterized by the presence of the first large metacentric chromosome pair as shown by many reports on *Astyanax* Baird & Girard, 1854 (Carvalho et al. 2002), *Oligosarcus* Günther, 1864 (Shuhei et al. 2007), *Hollandichthys* Eigenmann, 1910 (Carvalho et al. 2002), *Hemigrammus* Gill, 1858 (Arefjev 1990), *Moenkhausia* Eigenmann, 1903 (Foresti et al. 1989), *Hyphessobrycon* Durbin, 1908 (Arefjev 1990, Carvalho et al. 2002, Mendes et al. 2011), among others. Thus the large metacentric chromosome pair seems to represent a unique and derived character of Clade C, which could reinforce its monophyly.

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