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Chromosomal organization of repetitive DNAs in *Hordeum bogdanii* and *H. brevisubulatum* (Poaceae)

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Abstract

Molecular karyotypes of *H. bogdanii* Wilensky, 1918 (2n = 14), and *H. brevisubulatum* Link, 1844 ssp. *brevisubulatum* (2n = 28), were characterized by physical mapping of several repetitive sequences. A total of 18 repeats, including all possible di- or trinucleotide SSR (simple sequence repeat) motifs and satellite DNAs, such as pAs1, 5S rDNA, 45S rDNA, and pSc119.2, were used as probes for fluorescence in situ hybridization on root-tip metaphase chromosomes. Except for the SSR motifs AG, AT and GC, all the repeats we examined produced detectable hybridization signals on chromosomes of both species. A detailed molecular karyotype of the I genome of *H. bogdanii* is described for the first time, and each repetitive sequence is physically mapped. A high degree of chromosome variation, including aneuploidy and structural changes, was observed in *H. brevisubulatum*. Although the distribution of repeats in the chromosomes of *H. brevisubulatum* is different from that of *H. bogdanii*, similar patterns between the two species imply that the autopolyploid origin of *H. brevisubulatum* is from a *Hordeum* species with an I genome. A comparison of the I genome and the other *Hordeum* genomes, H, Xa and Xu, shows that colocalization of motifs AAC, ACT and CAT and colocalization of motifs AAG and AGG are characteristic of the I genome. In addition, we discuss the evolutionary significance of repeats in the genome during genome differentiation.

Keywords

Hordeum bogdanii, *Hordeum brevisubulatum*, autopolyploid, repetitive sequence, FISH

Introduction

Species in Triticeae have large genomes, 75% of which consists of repetitive sequences (Flavell et al. 1974, 1977). Many repetitive sequences, such as microsatellite and satellite DNA, can generate fluorescence in situ hybridization (FISH) patterns on individual chromosomes that are specific to a single species (Tsujimoto et al. 1997, Cuadrado et al. 2008). The FISH patterns generated by these repetitive probes always produce a stable and unique karyotype for a given species (Badaeva et al. 1996, 2002). The FISH patterns from a few repeats correspond to the heterochromatin regions in chromosomes (Cuadrado et al. 1995, Pedersen and Langridge 1997). Thus, the genomic organization of a given species can be characterized using FISH-based cytological analysis. Phylogenies can even be derived from repeat-based comparative FISH karyotyping (Cuadrado and Jouve 2002, Jiang and Gill 2006, Heslop-Harrison and Schwarzacher 2011).

The genus *Hordeum* Linnaeus, 1753 in the tribe Triticeae is divided into 32 species and is distributed in southern South America, South Africa, and the northern hemisphere (Bothmer et al. 1995). Cytotypes in this genus exist at three ploidy levels (diploid, tetraploid and hexaploid) with a basic chromosome number of $x = 7$ (Bothmer et al. 1995). Hybrid analysis and C-band analysis (Bothmer et al. 1986, 1995, Linde-Laursen et al. 1992) have revealed four genome groups in *Hordeum* that are designated as H (*Hordeum bulbosum* Linnaeus, 1756; *Hordeum vulgare* Linnaeus, 1753), Xa (*Hordeum marinum* Hudson, 1778), Xu (*Hordeum murinum* Linnaeus, 1753), and I (all the remaining species) (Blattner 2009). The H genome chromosomes of *H. vulgare* were first characterized using FISH probes of all possible classes of trinucleotide SSRs (simple sequence repeat). This provided detailed information on the sequence content of barley chromatin and saturated the physical map of all the barley chromosomes (Cuadrado and Jouve 2007a). Later, cytogenetic diversity between *H. vulgare* and *H. bulbosum*, was revealed using probes of SSRs, 5S rDNA, 45S rDNA, and 120-bp repeats from *Secale cereale* Linnaeus, 1753 (Carmona et al. 2013a). Karyotypes of the species of the *H. marinum* complex were determined using several tandem repeats. The results revealed the genome structure of different *H. marinum* taxa and demonstrated the allopolyploid origin of tetraploid forms of *H. gussoneanum* Parlatore, 1845 (Carmona et al. 2013b). In addition, the genomic constitution of *H. murinum* was characterized using multiple repetitive sequences. The results identified all the individual chromosomes within the *H. murinum* complex, elucidated its genomic structure and phylogeny, and explained the appearance of different cytotypes (Cuadrado et al. 2013). Thus far, detailed information on the chromosome organization of multiple repetitive sequences is available for the H, Xa, and Xu genomes. However, this information is still lacking for the I genome.

Hordeum bogdanii Wilensky, 1918, and *H. brevisubulatum* , should include the I genome based on the description of Blattner (2009). *H. bogdanii* is a rather common Asiatic species with a distribution that ranges from western Iran to eastern China and a diploid form of $2n = 2x = 14$ (Yang et al. 1987). Conversely, the *H. brevisubulatum* complex is distributed from Western Turkey to eastern China and consists of diploids,

tetraploids, and hexaploids (Bothmer 1979, Linde-Laursen et al. 1980). Polyploids of *H. brevisubulatum* are thought to be autoploid based on morphological (Bothmer 1979, Dewey 1979) and hybrid meiotic analysis (Landström et al. 1984).

In this paper, the molecular karyotype of the I genome is described in detail based on physical mapping of all possible dinucleotide and trinucleotide SSRs along with 5S rDNA, 45S rDNA, pSc119.2, and pAs1 repeats on mitotic chromosomes in *H. bogdanii* and *H. brevisubulatum*. The results provide more information on the genomic differentiation in *Hordeum* at the chromosomal level and will also help elucidate the functional and evolutionary implications of different repetitive sequences as genomes differentiate during speciation.

Material and methods

Plant material

Hordeum bogdanii was collected in Germu, Qinghai, China. *Hordeum brevisubulatum* ssp. *brevisubulatum* was collected in Tongde, Qinghai, China. More than 50 or more than 100 individuals of both species were collected in the field. Samples used for cytogenetic investigation were randomly selected from different individuals. Approximately 20 individuals were used for chromosome preparation. Only the investigated samples that displayed clear FISH patterns were present in this study.

Slide preparation

Seeds of *H. bogdanii* and *H. brevisubulatum* were germinated on moist filter paper in petri dishes at room temperature. Root tips with a length of approximately 1–2 cm were excised, pretreated in N_2O gas for 2 h as described by Kato (1999), and fixed in 3:1 (v/v) 100% ethanol:glacial acetic acid. Each root tip was squashed in a drop of 45% acetic acid.

DNA probes and labelling

Synthetic dinucleotide SSRs $(AT)_{15}$, $(AG)_{15}$, $(AC)_{15}$, and $(GC)_{15}$ and trinucleotide SSRs $(AAG)_{10}$, $(AAC)_{10}$, $(AAT)_{10}$, $(ACG)_{10}$, $(ACT)_{10}$, $(AGG)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$ and $(GGC)_{10}$ were end-labelled with fluorescein amidate (FAM, green, Sangon Biotech Co., Ltd., Shanghai, China). For the repetitive sequences pAs1 (Rayburn and Gill 1986), pSc119.2 (Bedbrook et al. 1980), and 45S rDNA, oligonucleotide probes described by Danilova et al. (2012) and Tang et al. (2014) were used. The oligonucleotides pSc119.2 and 45S rDNA were end-labelled with FAM (green), and pAs1 was end-labelled with TAMRA (red). The 5S rDNA was amplified by polymerase chain

reaction (PCR) using genomic DNA of *H. bogdanii* as described by Fukui et al. (1994) and was labelled with fluorescein-12-dUTP (green) using a random primer labelling method. Genomic DNA of *H. bogdanii* was labelled with tetramethy1-rhodamine-5-dUTP using the random primer method, as described by Dou et al. (2009).

FISH and microphotometry

FISH experiments were conducted using the method of Dou et al. (Dou et al. 2009) with minor modifications. Samples on prepared slides were denatured in 0.2 M NaOH in 70% ethanol at room temperature for 10 min, rinsed in 70% cold ethanol (stored at minus 20°C) for approximately 30 minutes, and air dried. The hybridization mixture, 10 µl per slide, was prepared by adding 50% de-ionized formamide, 50% dextran sulphate, 2 × SSC (0.3 M NaCl, 0.03 M Na3-citrate), 1 µg/µl denatured salmon sperm DNA and 10 ng of probe. The hybridization mixture with oligonucleotide probes was placed directly onto the denatured slide preparation. The hybridization mixture with the 5S rDNA probe was denatured in boiling water for 5 minutes. Hybridization was conducted overnight at 37°C in a moistened chamber. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured with a cooled CCD camera (Photometrics CoolSNAP) using a fluorescence microscope (Leica) and were processed with the Meta Imaging System (Universal Imaging Corporation). Finally, images were adjusted using Adobe Photoshop 6.0 for contrast and background optimization.

Results

Chromosomal organization of repeats in *H. bogdanii*

A stable chromosome number of 2n = 14 was detected in all tested samples of *H. bogdanii.* The repetitive sequence pAs1 produced multiple sites that were subtelomeric, intercalary, or pericentromeric on all chromosomes. The hybridization pattern of pAs1 was informative enough to distinguish each chromosome of *H. bogdanii.* Thus, chromosome localization of other repeats was conducted using pAs1 as a landmark (Fig. 1). The chromosomal distribution of each sequence was described in detail (Fig. 2).

The four possible dinucleotide SSR probes $(AG)_{15}$, $(AC)_{15}$, $(AT)_{15}$, and $(GC)_{15}$ were used to characterize the chromosomes of *H. bogdanii*. Only $(AC)_{15}$ produced detectable hybridization signals, which appeared in subtelomeric regions on all chromosomes and in pericentromeric regions on a few chromosomes. The signals were strongest in subtelomeric regions of chromosomes B, C and D (Fig. 2).

All 10 trinucleotide SSRs produced detectable hybridizations (Fig. 2). The probe $(AAC)_{10}$ revealed many intense and rich pericentromeric hybridization signals on all seven pairs of chromosomes, intercalary signals on chromosomes B and F, and subtelo-

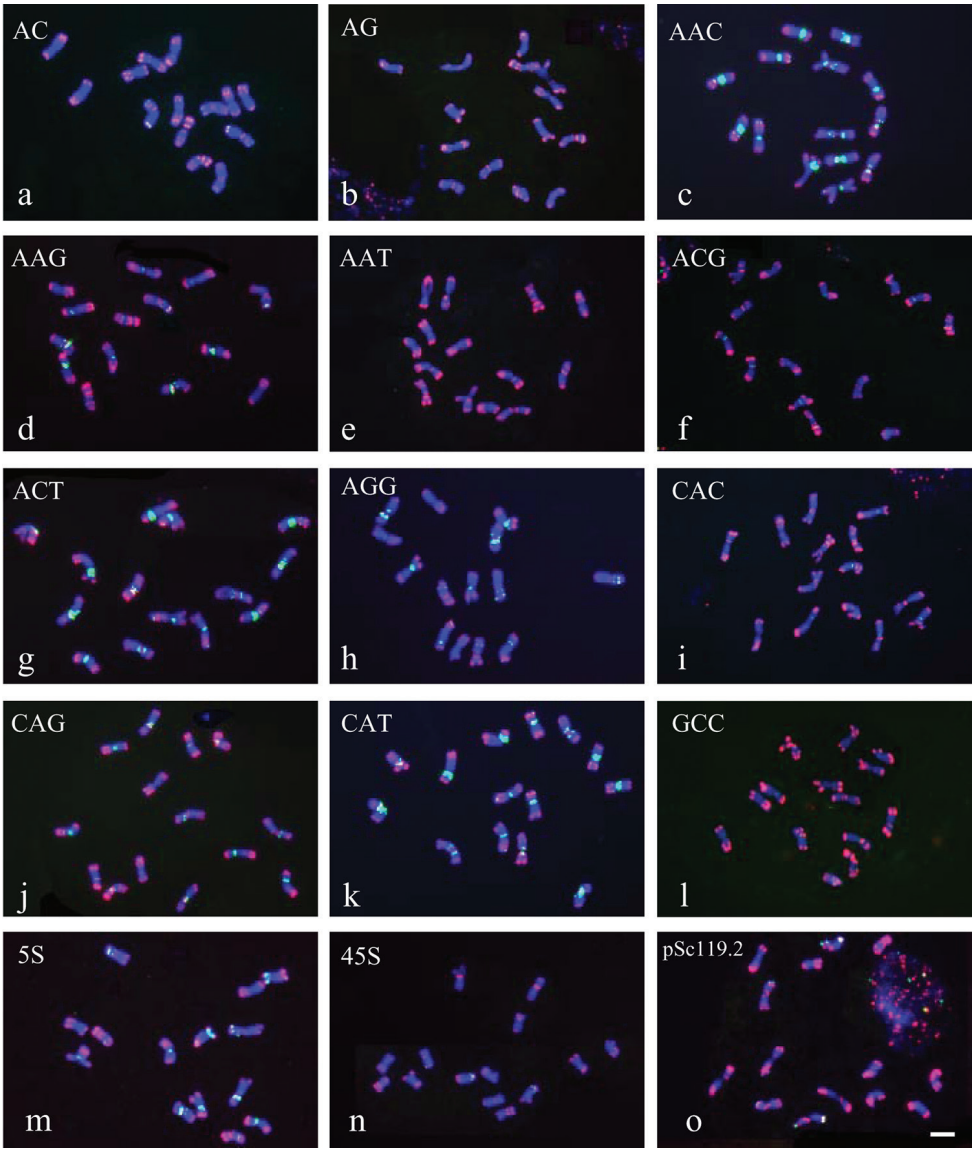

Figure 1. FISH patterns of mitotic metaphase chromosomes of *H. bogdanii* detected by pAs1 (red) combined with the several other repeats (green): **a** $(AC)_{15}$ **b** $(AG)_{15}$ **c** $(AAC)_{10}$ **d** $(AAG)_{10}$ **e** $(AAT)_{10}$ **f** $(ACG)_{10}$ **g** $(ACT)_{10}$ **h** $(AGG)_{10}$ **i** $(CAC)_{10}$ **j** $(CAG)_{10}$ **k** $CAT)_{10}$ **l** $(GCC)_{10}$ **m** 5S rDNA **n** 45SrDNA **o** pSc119.2. Bar =10 μm.

meric signals on chromosomes D and E. The FISH patterns of $(ACT)_{10}$ and $(CAT)_{10}$ appeared to be identical to those of $(AAC)_{10}$. An identical FISH pattern was shared by $(AAG)_{10}$ and $(AGG)_{10}$, which produced intense pericentromeric hybridizations, intercalary hybridizations, or both in six pairs of chromosomes. The $(CAC)_{10}$ probe produced faint signals in four chromosome pairs and strong signals in three pairs in peri-

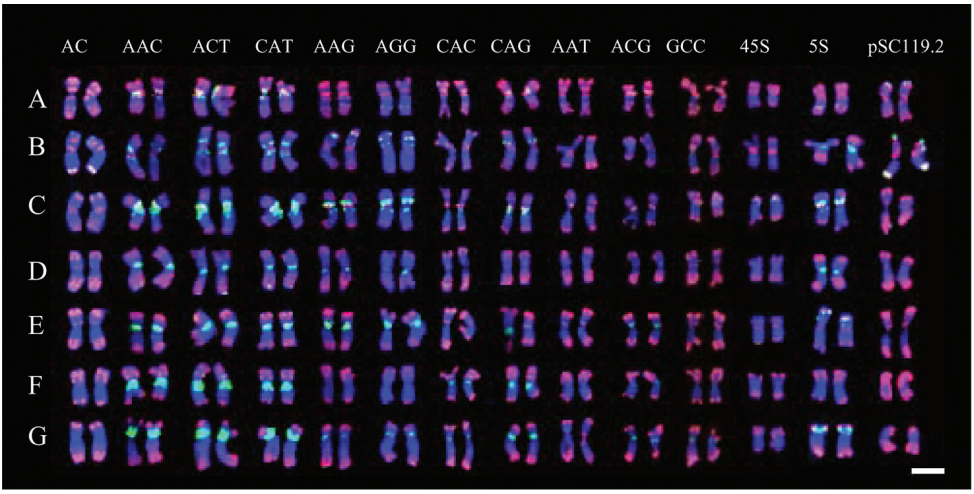

Figure 2. Molecular karyotypes of *H. bogdanii* probed by pAs1 (red) combined with the other several repeats (green). Seven pairs of chromosomes are designated from **A–G** for distinguishing them from the numerals of the 7 homologue groups used in barly. Bar =10 μm.

centromeric regions, intercalary regions, or both. The distribution of the $(CAG)_{10}$ signal overlapped with that of the $(CAC)_{10}$ signal, but the $(CAG)_{10}$ signal had a stronger hybridization intensity. $(AAT)_{10}$ produced faint signals in three pairs of chromosomes in pericentromeric, intercalary, and subtelomeric regions. Hybridization of $(ACG)_{10}$ was detected in six pairs of chromosomes and was localized primarily in centromeric regions. The distribution of $(GCC)_{10}$ was more dispersed; however, more intense hybridizations were still observed in centromeric regions on three pairs of chromosomes.

Three 45S rDNA sites were detected in two pairs of chromosomes. One carried a distinct site on the subtelomeric region of the short arm; the other harboured two faint hybridization sites in the ends of both arms. 5S rDNA was distributed in all chromosomes except for one. Nine distinct 5S rDNA sites were exclusively determined and were localized on centromeric, pericentromeric, intercalary, or subtelomeric regions. Three pairs of chromosomes carried only one 5S rDNA site, and another three harboured two 5S rDNA sites. Two 45S rDNA and three 5S rDNA sites were reported in an accession of *H. bogdanii* (Taketa et al. 2001). The fact that many more 45 S rDNA and 5S rDNA sites were revealed in this study suggests the presence of intra-specific polymorphisms. Four distinct hybridization sites were probed by pSc119.2, and these were localized on subtelomeric regions of three pairs of chromosomes.

Chromosomal organization of repeats in *H. brevisubulatum*

A chromosome number of 2n = 28 was detected in nearly all tested individuals of *H. brevisubulatum*. However, chromosome numbers of 2n = 26 and 2n = 27 (Fig. 3a, d)

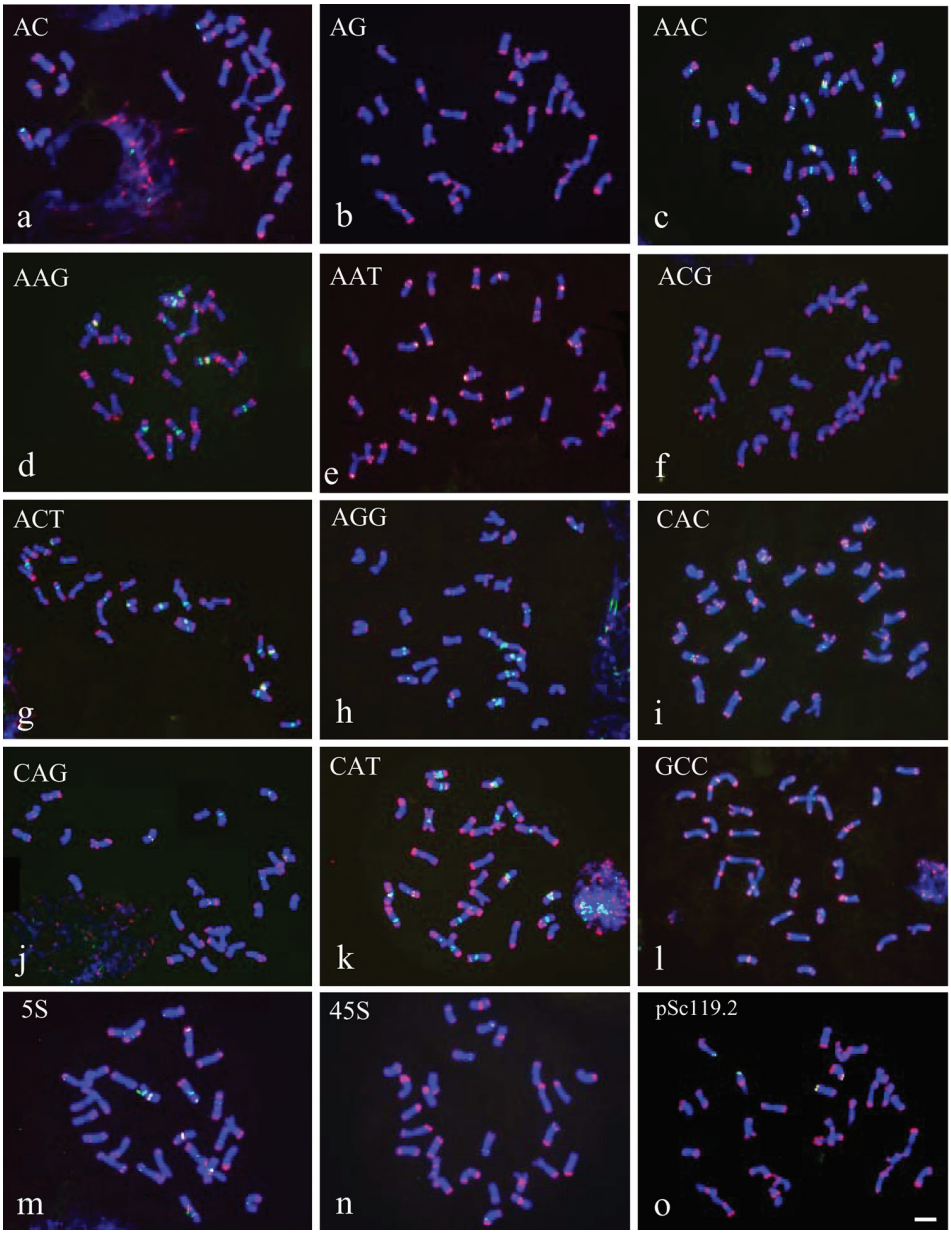

Figure 3. FISH patterns of mitotic metaphase chromosomes of *H. brevisubulatum* detected by pAs1 (red) combined with several other repeats (green): **a** $(AC)_{15}$ **b** $(AG)_{15}$ **c** $(AAC)_{10}$ **d** $(AAG)_{10}$ **e** $(AAT)_{10}$ **f** $(ACG)_{10}$ **g** $(ACT)_{10}$ **h** $(AGG)_{10}$ **i** $(CAC)_{10}$ **j** $(CAG)_{10}$ **k** $(CAT)_{10}$ **l** $(GCC)_{10}$ **m** 5S rDNA **n** 45SrDNA **o** pSc119.2. Bar =10 μm.

were also observed in a few cases. Moreover, a monotelosomic chromosome was clearly identified in one case (Fig. 3k). Karyotyping was tentatively conducted using pAs1 combined with other repeats. Highly variable karyotypes were revealed in *H. brevisubulatum*

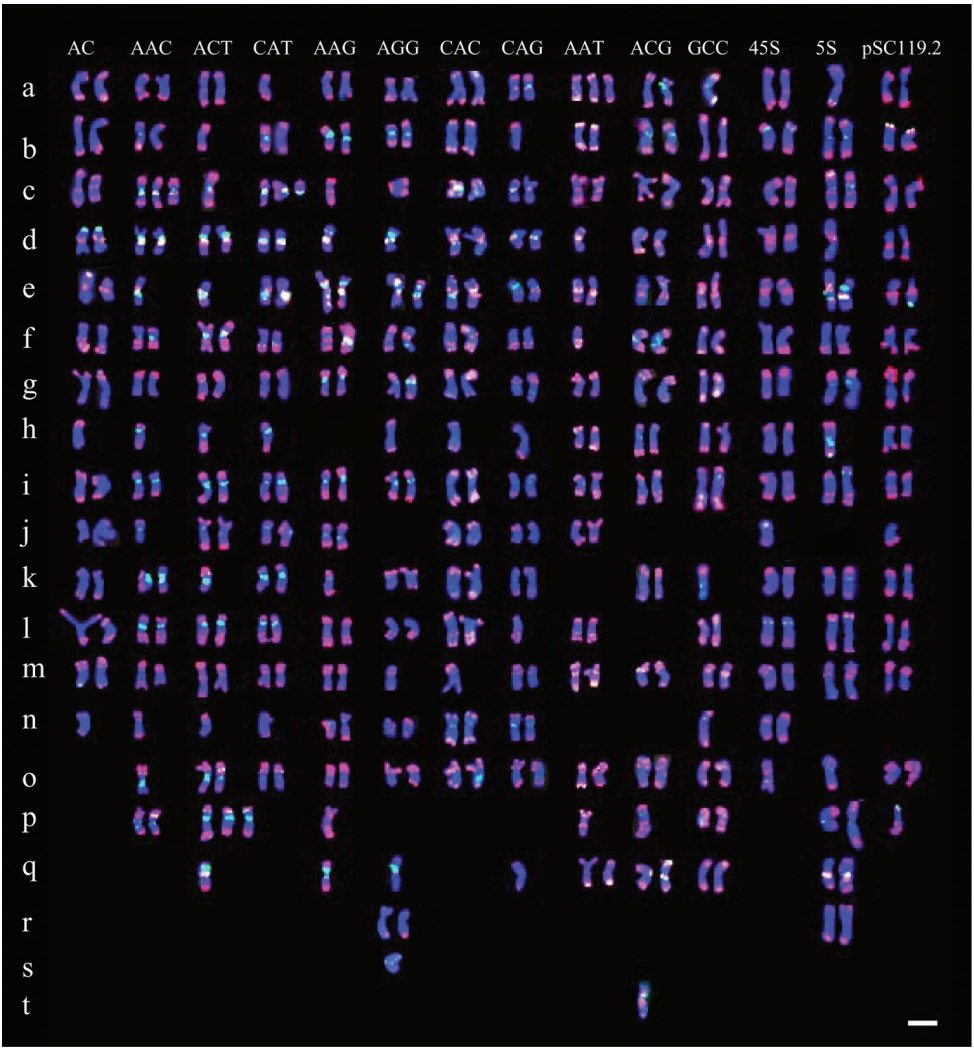

Figure 4. Molecular karyotypes of *H. brevisubulatum* probed by pAs1 (red) combined with several other repeats (green). Chromosome types are designated by lowercase roman letters from a to t to distinguish them from the symbols designating *H. bogdanii*. Bar =10 μm.

(Fig. 4). Variations in chromosome number, such as monosomy, nullisomy, and trisomy, were clearly shown to have occurred. Chromosome structural aberrations, such as deletions, translocations, or inversions, are likely to be present, although these aberrations were not evident from the FISH results alone. Thus, approximately 20 chromosomal variants designated from a to t were roughly identified in the individuals that were investigated (Fig. 4).

All possible di- and trinucleotide SSR probes except for $(AG)_{15}$, $(AT)_{15}$ and $(GC)_{15}$ produced hybridizations on chromosomes of *H. brevisubulatum*. The $(AC)_{15}$ probe

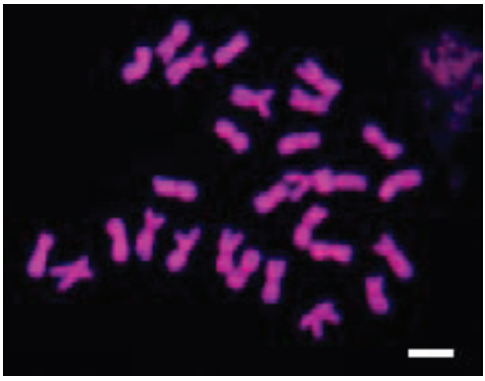

Figure 5. GISH patterns of mitotic metaphase chromosomes of *H. brevisubulatum* probed by the genomic DNA of *H. bogdanii*.

was primarily detected in subtelomeric regions. Nearly half of the total chromosomes carried detectable signals, and signals of seven or eight chromosomes appeared more distinct. The chromosomal distribution of $(AAC)_{10}$, $(ACT)_{10}$, and $(CAT)_{10}$ was similar to that in *H. bogdanii*. The $(AAC)_{10}$, $(ACT)_{10}$, and $(CAT)_{10}$ repeats were still colocalized and were detected on 22–26 chromosomes. $(AAG)_{10}$ and $(AGG)_{10}$ appeared to be colocalized and were primarily distributed in pericentromeric regions on 13–16 chromosomes. $(CAC)_{10}$ and $(CAG)_{10}$ had similar distributions to those observed in *H. bogdanii*. Signals of $(CAC)_{10}$ and $(CAG)_{10}$ were detectable in 19–22 chromosomes and were stronger on eight or nine chromosomes. The FISH pattern of $(AAT)_{10}$ in *H. brevisubulatum* was distinctly different from that in *H. bogdanii.* $(AAT)_{10}$ produced a stronger hybridization signal in *H. brevisubulatum* than in *H. bogdanii*, primarily in subtelomeric regions on nearly all chromosomes. Signals of $(ACG)_{10}$ were detectable in 13 chromosomes. As with $(AAT)_{10}$, stronger hybridization signals of $(GCC)_{10}$ were detected in *H. brevisubulatum* than in *H. bogdanii.*

45S rDNA produced hybridization signals in subtelomeric regions on seven or eight chromosomes. 5S rDNA was detected in centromeric, pericentromeric, intercalary, or subtelomeric regions on 24 chromosomes. The present karyotypes of 5S rDNA and 45S rDNA in *H. brevisubulatum* differed from those of *H. brevisubulatum* based on the accessions reported by Taketa et al. (1999). A heterogeneous composition of 5S and 45S rDNA was observed in a total of five 5S rDNA sites and 18 45S rDNA sites (Taketa et al. 1999). However, many more 5S rDNA sites than 45 S rDNA sites were detected in our study. This pattern suggests that intraspecific polymorphism may exist among different populations. Distinct pSc119.2 signals were detected in subtelomeric regions on four or five chromosomes.

Furthermore, the technique of genomic *in situ* hybridization (GISH) was used to determine the polyploidy origin of *H. brevisubulatum*. The result showed that the chromosomes of *H. brevisubulatum* were evenly painted by using labelled genomic DNA of *H. bogdanii* as the probe (Fig. 5).

Discussion

Chromosomal identification and genomic characterization of *H. bogdanii*

Fifteen of the 18 repetitive sequences produced detectable hybridization in mitotic metaphase chromosomes in *H. bogdanii*. A few repeats, including highly polymorphic sites, can be used to uniquely identify each chromosome. The satellite DNA pAs1 and 5S rDNA can be used to distinguish each chromosome in *H. bogdanii*. The SSRs AAC, ACT, CAT, AAG and AGG are also ideal markers for chromosome identification because of their abundance and their large number of polymorphic sites across individual chromosomes (Fig. 6). Thus, individual chromosomes of *H. bogdanii* can be easily identified using the above repeats. In this study, pAs1 repeats were used as a reference marker, and each repetitive sequence was accurately physically mapped.

Most of the repeats produced multiple hybridizations. However, high-intensity hybridizations were always observed on pericentromeric or subtelomeric regions. This implies that the *H. bogdanii* genome contains more repetitive sequences in subtelomeric and pericentromeric parts of the chromosome than in interstitial regions. The distributions of AAC, ACT, and CAT were revealed to be colocalized and were identical in intensity in this study, suggesting that AAC, ACT, and CAT may be evenly distributed in an intermingled way. The same was true for AAG and AGG. Although CAC and CAG were found to be colocalized, different hybridization intensities suggest that their distribution was close rather than intermingled.

Differences between the I and H genomes can be observed by comparing their distribution of their repetitive sequences. A distinct difference can be observed in a few trinucleotide SSR motifs. In *H. vulgare* (H genome), the AAC, AAG, and AGG motifs are colocalized around the centromere in all chromosomes; ACT produces multiple signals in six chromosome pairs; and CAT produces a strong signal and a weak signal in 4H and 5H, respectively (Cuadrado and Jouve 2007a) (Fig. 6). Another distinct difference is exhibited by the dinucleotide AG, which was intensely detected around the centromere in *H. vulgare* (Hagras et al. 2005, Carmona et al. 2013a) but was absent in *H. bogdanii.* Additional differences between *H. bogdanii* and *H. vulgare* can be seen in the distribution patterns of AAT, GCC, CAG, 45S rDNA, 5S rDNA and pSc119.2. Physical mapping of a few sequences in *H. marinum* (Xa genome) showed that AAC and AAG produced intense and rich patterns of multiple SSR signals that were particularly concentrated in the pericentromeric region and that ACT and CAT were weakly distributed (Carmona et al. 2013b). The pSc119.2 repeat produced subtelomeric signals on nearly all chromosomes in *H. marinum* (Taketa et al. 2000); however, fewer of these signals were present in *H. bogdanii*. Karyotype analysis of diploid *H. murinum* (Xu) showed that AG produced intense signals around the centromere in four chromosome pairs and that AAG produced more rich and intense signals than AAC, ACT, and CAT in pericentromeric regions (Cuadrado et al. 2013, Carmona et al. 2013b). In addition, no pSc119.2 hybridization signals were detected in diploid *H. murinum* (Taketa et al. 2000).

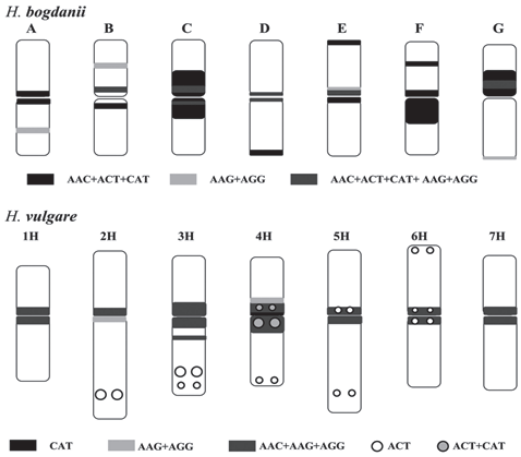

Figure 6. Idiogram of chromosomes of *H. bogdanii* (I genome) and *H. vulgare* (H genome) showing the distributions of the SSRs AAC, ACT, CAT, AAG and AGG. Chromosomal information of *H. vulgare* is taken from Cuadrado et al. (2007a)

Thus, the genomic composition of the I genome in *H. bogdanii* revealed by the distribution of several repeats was highly different from that of the H, Xa, and Xu genomes in *Hordeum*.

Autopolyploid origin of *H. brevisubulatum*

The *Hordeum brevisubulatum* species complex has a range of cytotypes and can occur in diploid, tetraploid, and hexaploid forms (Bothmer 1979, Dewey 1979, Linde-Laursen et al. 1980). Earlier studies have shown that polyploids in the *H. brevisubulatum* complex may be autoploids (Bothmer 1979, Dewey 1979). Data from meiotic pairing in the hybrids indicate that autoploidy characterizes the entire complex, with one "basic" genome (Landström et al. 1984). The karyotype of tetraploid *H. brevisubulatum* was not shown to be a strictly doubled karyotype from a diploid form. However, similar chromosomal distributions of many repetitive sequences between *H. bogdanii* and *H. brevisubulatum* were revealed in this study. Specifically, colocalized distributions of AAC, ACT, and CAT in pericentromeric regions in nearly all chromosomes and colocalized distributions of AAG and AGG in most chromosomes were observed in *H. brevisubulatum.* Nearly double the number of 45S rDNA and 5S rDNA sites in *H. bogdanii* were also detected in *H. brevisubulatum.* In addition, no genomic differentiation was detected in *H. brevisubulatum* by GISH with labelled genomic DNA of *H. bogdanii*. This pattern strongly suggests an autopolyploid origin of *H. brevisubulatum* from an I genome.

Several examples of structural rearrangements of chromosomes at the population level in the *H. brevisubulatum* complex have been reported (Landström et al. 1984). In

this study, high levels of chromosomal variation, including variation in chromosome number and chromosome structure, were observed in the population under investigation. This observation implies that there is a high degree of instability of the autoploid genome in *H. brevisubulatum.* Karyotype variations have recently been reported in resynthesized and naturally formed allopolyploid species and in natural hybrids produced by polyploid homoploid hybridization (Chester et al. 2012, Lipman et al. 2013). Aneuploidy, inter- and intragenomic rearrangements, and the loss of repeats were frequently detected in early generations. As a natural species, *H. brevisubulatum* should be the product of a long evolutionary history. *Hordeum brevisubulatum* has been considered to be self-incompatible (Bothmer 1979). Although autopolyploidy may result from genome doubling within a single individual, most natural autopolyploids likely formed via some degree of hybridization, involving, for example, individuals from genetically differentiated populations (Stebbins 1985, Soltis and Soltis 1989). Thus, more studies are needed to determine whether karyotype variation in *H. brevisubulatum* might be affected by hybridization between genetically differentiated biotypes.

Major genetic changes, including the loss of homologs and DNA sequences, have been documented in recently formed polyploid species (Soltis et al. 2004, Abbott and Lowe 2004, Matyášek et al. 2007). Compared with the composition of the I genome in *H. bogdanii*, the distributions of a few repetitive sequences in *H. brevisubulatum* might be strongly altered by increases or decreases in copy number. The trinucleotide SSR motifs AAC, ACT, and CAT are primarily located in pericentromeric regions in all the chromosomes in *H. bogdanii*, but they are absent in a few chromosomes in *H. brevisubulatum*, possibly because of decreases in copy number. The distributions of AAG and AGG are similar. In contrast, the higher abundance of ATT and GCC in *H. brevisulatum* indicates that there is an increase in copy number that accompanies polyploidization.

Evolutionary trends of repetitive DNA sequences during genome differentiation

Repetitive DNA sequences are the main components of heterochromatin and are subject to rapid change. Such changes in the distribution of repetitive DNA sequences are one of the driving forces of genome evolution and speciation. One proposed function of repetitive sequences may be related to higher-order molecular structure (Redi et al. 2001). However, the molecular mechanisms by which genomes change are unknown (Plohl et al. 2002).

The SSR motif AAC was shown to be distributed mainly in the pericentromeric regions of the I, H, Xa, and Xu genomes. In addition, the wide distribution of AAC has been detected in wheat and the genus *Secale* (Cuadrado et al. 2000 Cuadrado and Jouve 2002). This wide distribution suggests that AAC repeats have an ancient origin in Triticeae species. The distribution of AAC in the I genome was revealed to be evenly co-localized with ACT and CAT. However, the distributions of AAC, ACT, and CAT are largely differentiated in the H, Xa, and Xu genomes. Variation in the distribution of repetitive sequences may be related to the amplification and deletion of repeat copies.

AAC, ACT, and CAT motifs in the I genome may have been co-amplified or deleted as a single repetitive unit. However, AAC, ACT, and CAT in other genomes have evolved independently. AAG exhibits the most chromosomal variation both within and between taxa (Pedersen et al. 1996, Pedersen and Langridge 1997, Cuadrado et al. 2008, Carmona et al. 2013a). AAG has been suggested to be more predisposed to being amplified or deleted relative to other repetitive sequences as a consequence of independent events in different lineages (Carmona et al. 2013b). The fact that AAG is evenly colocalized with AGG in the I genome suggests that the same evolutionary mechanism also drives variation in the chromosomal distribution of AAC, ACT, and CAT.

AC repeat sites are remarkably similar and have been shown to exhibit uniformly dispersed hybridization along the euchromatic portion of metaphase chromosomes in humans and barley and in the metaphase and polytene chromosomes of *Drosophila melanogaster* (Meigen, 1830) (Cuadrado and Jouve 2007b). In this study, AC repeat sites had a dispersed distribution. High-intensity hybridization bands for AC repeats were observed in subtelomeric and pericentromeric regions on a few chromosomes in the I genome. This atypical distribution suggests that there is a more complicated organization or function of AC repeats in plant genomes.

The sequence pAs1 contains Afa family sequences. The Afa family sequence was abundant in the four basic genomes of the genus *Hordeum*, and the hybridization patterns differed among the diploid species (Taketa et al. 2000). The distribution of the Afa family is more dispersed than those of the other tandem repetitive sequences. This dispersed distribution implies that the Afa family may be more commonly distributed in gene rich regions. In our study, despite the autopolyploidy origin of *H. brevisubulatum* from an I genome species, the distribution of pAs1 sequences in *H. brevisubulatum* was highly variable relative to those in diploid *H. bogdanii*. The Afa family of sequences may play an important role in the differentiation of either genome or species in the genus *Hordeum*.

Variation in the abundance and distribution of repetitive sequences and the shared distribution of the same repeats between different genomes suggest that repetitive sequences play a key role in both the structure and function of the genomes of higher eukaryotes (Cuadrado et al. 2008). To date, most information on the distribution of repeats in *Hordeum* genomes comes from physical mapping analysis of mitotic metaphase chromosomes, which are highly packaged. Physical mapping of repetitive sequences on extended chromosomes, such as during the pachytene stage of meiosis, may provide more valuable information on the chromosomal distribution of repeats and may help elucidate the evolution and genomic function of repetitive sequences.

Conclusion

Fifteen repetitive sequences, including SSR motif AC and all possible trinucleotide motifs and satellite DNAs pAs1, 5S rDNA, 45s rDNA, and pSc119.2, were accurately physically mapped on individual chromosomes in the I genome in *H. bogdanii*. High

genome instability was revealed in tetraploid *H. brevisubulatum*. The similar distribution of the repeats in both species suggests an autopolyploid origin of *H. brevisubulatum* from an I genome species. Comparative cytogenetic analysis between the I genome and other genomes in *Hordeum* showed that the distribution of a few repeats differed. Colocalization of motifs AAC, ACT, and CAT and colocalization of motifs AAG and AGG is characteristic of the I genome.

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Comparative analysis of the circadian rhythm genes *period* and *timeless* in *Culex pipiens* Linnaeus, 1758 (Diptera, Culicidae)

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Abstract

Nucleotide sequences of the circadian rhythm genes, *period* and *timeless*, were studied for the first time in mosquitoes *Culex pipiens* Linnaeus, 1758. In this work we evaluated variations of the studied genome fragments for the two forms of *C. pipiens* (forma "pipiens" – mosquitoes common for aboveground habitats, forma "molestus" – underground mosquitoes). We compared *C. pipiens* from Russia with transatlantic *C. pipiens* and subtropical C*ulex quinquefasciatus* Say, 1823. Our results show that intraspecies variability is higher for the gene *period* than for the gene *timeless*. The revealed substitutions in nucleotide sequences and especially in amino acid sequences grouped the individuals of the two forms into distinct clusters with high significance. The detected fixed amino acid substitutions may appear essential for functioning of the circadian rhythm proteins in *C. pipiens*, and may be correlated with adaptations of the taxa within the group *C. pipiens*. Our results suggest that natural selection favors fixed mutations and the decrease in diversity of the genes *period* and *timeless* in mosquitoes of the *C. pipiens* f. "molestus" compared with the *C. pipiens* f. "pipiens", is probably correlated with adaptive features of *C. pipiens* f. "molestus". The studied genome regions may be considered as promising molecular-genetic markers for identification, population and phylogenetic analysis of similar species and forms of the *Culex pipiens* complex.

Keywords

Culex pipiens, circadian rhythm genes, *period*, *timeless*, natural selection

Introduction

The *Culex pipiens* Linnaeus, 1758 complex considered by some authors as a 'polytypic species' includes up to seven morphologically identical or very similar forms (Harbach et al. 1984, 1985, Vinogradova 2000). By the second half of the 20th century, the taxonomic status of these forms changed several times from species to subspecies and back. At present only two species, *Culex pipiens* Linnaeus, 1758, and *Culex quinquefasciatus* Say, 1823 have been left within the *Culex pipiens* complex based on morphological similarity (Harbach 2012). Both species are known as bridge-vectors of West Nile and Saint Louis encephalitis flaviviruses, the etiological agents of dangerous human diseases (Vinogradova 2000). The medical significance of the *Culex pipiens* complex generates much interest in its studies, including taxonomy.

Only one species of the *Culex pipiens* complex, *C. pipiens*, has been found in Russia. This species includes two forms, *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus", originally described as distinct species (Harbach et al. 1984, 1985). *C. pipiens* forms designation is provided in accordance with the rules of International Code of Zoological Nomenclature (http://www.iczn.org/iczn/index.jsp). The two forms are morphologically identical, but have notably distinct biological features. The mosquitoes *C. pipiens* f. "pipiens" are anautogenous (females require a blood meal to mature each egg raft), mate in swarms, oviposit in a wide variety of natural and manmade habitats, feed preferentially on avian hosts and enter diapause to overwinter (Vinogradova 2000). In contrast *C. pipiens* f. "molestus" are autogenous (females oviposit the first egg raft without bloodmeal), develop without winter diapause in urban flooded basements and tunnels, feed preferentially on mammal hosts and are able to mate in a confined space. The specific features of reproduction and development of the two forms has resulted in their spatial isolation in moderate climate areas, suggesting genetic isolation. This suggestion is confirmed by the isoenzyme analysis of autogenous and anautogenous populations of *C. pipiens* from England (Byrne and Nichols 1999), Russia (Lopatin 2000) and Germany (Weitzel et al. 2009) as well as by study of populations from Europe with CQ11 assay (Bahnck and Fonseca 2006). The results of these investigations showed that in these regions the forms are genetically distinct, with no or poor gene flow between populations of different forms. However, in the Mediterranean area, in N Africa and the Middle East, both autogenous and anautogenous specimens develop in the same pools. These populations display highly variable autogeny rates, from 10-90% in Egypt (Gad et al. 1995) to 4–55% in Israel (Nudelman et al. 1988), and both autogenous and anautogenous females were encountered in the progenies of autogenous or anautogenous female parents (Gad et al. 1995). Consequently, the question of divergence of the two forms in moderate climates remains still unclear.

Among the specific behavioral/physiological traits which remained up to now the important criteria for defining populations of *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus", differences in mating behavior are under the special interest. Mating ac-

tivity of *C. pipiens* f. "pipiens" is restricted within the crepuscular period when males aggregate in swarms where they copulate with virgin females attracted to a swarm (Ivanov 1984, Fyodorova and Serbenyuk 1999, Vinogradova 2000). In contrast, males of *C. pipiens* f. "molestus" never swarm and have irregular locomotor and mating activity (Shinkawa et al. 1994). Such temporal differences in mating activity may represent the temporal isolation between two forms.

In insects the rhythms of mating activity are controlled by endogenous circadian clocks, which are under genetic control (Konopka and Benzer 1971, Sakai and Ishida 2001, Tauber et al. 2003). The differences in the daily timing of mating activity are documented in many sympatric sibling insect species, e.g in tephritid fruit flies (An et al. 2002, 2004), in *Drosophila* Fallen, 1923 species (Sakai and Ishida 2001, Tauber et al. 2003), sand fly species (Rivas et al. 2008), in *Nasonia* Ashmead, 1904 wasps (Bertossa et al. 2013), in cricket species (Fergus and Shaw 2013). Intra-specific differences in the rhythms of mating activity were revealed also between populations or strains, e.g. in fly *Bactrocera cucurbitae* Coquilletl, 1849 (Fuchikawa et al. 2010) and mosquitoes of *Anopheles cruzii* Dyar and Knab, 1908 complex (Rona et al. 2010).

Clock genes, especially *period* and *timeless*, play an essential role in regulation of mating rhythms in insects. In *Drosophila*, null mutants of the clock gene *period* (*per*) lost the circadian rhythm in mating activity (Sakai and Ishida 2001). Similar effects have been described for gene *timeless* (*tim*) in *Drosophila* and for gene *per* in *Grillus bimaculatus* De Geer, 1773 (Sehgal et al. 1994, Moriyama et al. 2008). The analysis of mating activity in transformant lines carrying *per* transcription units derived from *Drosophila melanogaster* Meigen, 1930 or *Drosophila pseudoobscura* Frolova & Astaurov, 1929, showed that *per* controls species-specific mating rhythms, at least in flies (Tauber et al. 2003).

It may be suggested that differences in the rhythm of mating activity in two forms of *C. pipiens* resulted from the variations in circadian clocks genes. To test this hypothesis, we selected the genes *per* and *tim*. The aim of our work was to study variable nucleotide sequences in these genes, and to estimate the possible evolutionary significance of the detected variations.

Methods

The larvae of mosquitoes of both intraspecific forms were collected mostly in August 2006 in Volgograd City and nearby areas. The sampling sites, methods of larvae collection and rearing in lab, and methods of evaluating autogenity have been described earlier (Fedorova and Shaikevich 2013). The DNA of mosquitoes collected in the underground sampling sites in Nizhny Novgorod, Moscow and St Petersburg, as well as in aboveground sampling site Iksha, Moscow region, was used to analyze the diversity of the first exon of the gene *tim*. The methods of mosquito sampling at these sites have been described earlier (Vinogradova and Shaikevich 2007).

DNA isolation and analysis

The DNA was isolated using the kit DIAtom™ DNA Prep (Isogen Russia). Each of the amplification reactions used 0.1 μg of the total DNA. The polymerase chain reaction (PCR) was run on the thermocycler GeneAmpR PCR System 2700 (Applied Biosystems USA), with amplification Encyclo PCR kit (Evrogen Russia), following the manufacturer's instructions. For PCR and sequencing of amplification products, specific primers were constructed which were complementary to the conserved sequences of exons in the published sequences of the genes *period* and *timeless* from the total genome of a similar species *C. quinquefasciatus* (Vector Base Gene ID CPIJ007193 and CPIJ007082, respectively) (Arensburger et al. 2010). When the first sequences were obtained, the primers were constructed basing on DNA sequences of *C. pipiens*. The PCR conditions were adjusted using the program Oligo6 (http://www.oligo.net/): primary denaturing 95°C - 5 min; 35 cycles at 95°C - 30 s, Tm (for each primer pair) - 1 min, 72°C - 1,5 min; final synthesis at 72°C for 7 min. Primer sequences and annealing temperatures for the PCR are shown in Table 1. Higher temperature was used if two primers in the pair had different annealing temperatures. Negative control was run for all amplification reactions. The DNA of introns was analysed by direct sequencing of amplicons without cloning. Amplified fragments of the genes *per* and *tim* were purified from the gel using QIAquick Gel Extraction kit (Qiagen USA). The fragments were cloned using the kit pGEM-T Easy Vector Systems (Promega USA); the DNA of the three clones for each individual mosquito was sequenced using the equipment ABI PRISM 310 and the BigDye Termination kit (Applied Biosystems USA), according to the manufacturer's instructions and deposited to GenBank under accession numbers: KU133680-KU133745. The sequences of separate exons of each clone were combined into a single sequence. Nine combined sequences from individual *C. pipiens* f. "pipiens" and nine combined sequences from individual *C. pipiens* f. "molestus" were investigated for each of the two genes studied, *per* and *tim*. Extended study of the coding sequences of exon 1 of the gene *tim* in two forms of *C. pipiens* was performed using the DNA from the 26 individual mosquitoes *C. pipiens* f. "molestus" and 17 individual mosquitoes *C. pipiens* f. "pipiens". 21 new different haplotypes are submitted to GenBank (KU997646 - KU997666).

Data analysis

The DNA sequences were translated into amino acids sequences using ExPASy software (Swiss Institute of Bioinformatics), and compared with amino acids sequences of *C. quinquefasciatus* (Arensburger et al. 2010) and *C. pipiens* from the USA (Meuti et al. 2015) using programs MAFFT (http://mafft.cbrc.jp/alignment/server/) and MEGA6 (Tamura et al. 2013). Evolutionary analysis was run using MEGA6. Maximum Composite Likelihood model (Tamura et al. 2004) and Kimura 2-parameter model (Kimura 1980) were used to describe the nucleotide substitution pattern. Tables below show

Table 1. Primers constructed to study the genes *per* and *tim*.

primer	sequence	Tm (°C)	region
PerF2	5'-AGTTCCAAATCGCGCCACAG-3'	54	*per* exon 2
PerR2	5'-TTGGGTTTGCTCGCTTCGTTC-3'	54	*per* exon 2
PerF3	5'-ACAATGCATAGCCAACCGCAAG-3'	55	*per* exon 3
PerR3	5'-GTTCGTCCCTTGACCATGATC-3'	54	*per* exon 3
PerF4	5'-AACGGCTGTTATCTCGTACTG-3'	52	*per* exon 4
PerR4	5'-GCATCGCGTGGTACATCATCG-3'	56	*per* exon 4
TimF1	5'-AATGGTTGCTAGCGAATCCG-3'	52	*tim* exon1
TimR1	5'-AGTAGAGTTCTCGACACCCG-3'	54	*tim* exon1
TimF5	5'-GATTGGTCGGATTTGATTGAG-3'	50	*tim* exon5
TimR5	5'-GTATGTCATCAACCGCCTTG-3'	52	*tim* exon5
TimF5-1	5'-GGAAACCAGCAAAAGACTCG-3'	52	*tim* intron5-6, exon6, intron6-7
TimR7	5'-TACGAGAGCACGTTGAACTG-3'	52	*tim* intron5-6, exon6, intron6-7
TimF7	5'-ACATACTGTACAACATTGCCCTG-3'	53	*tim* intron7-8
TimR8	5'-TCAGGTCGAACTTGATGATG-3'	50	*tim* intron7-8
TimF9	5'-GCTGCGGCCGAAAGCGCCAG-3'	60	*tim* intron9-10
TimR10	5'-ATTTCCATCGCTCGTGTGCTG-3'	54	*tim* intron9-10

the data obtained using the Maximum Composite Likelihood model. The Kimura 2-parameter model produced somewhat higher estimates. The optimal model describing evolutionary patterns was found using the option 'Find best DNA/Protein substitution model' in MEGA6. For our data, the Jones-Taylor-Thornton (JTT) model (Jones et al. 1992) showed the lowest BIC (Bayesian Information Criterion) scores for amino acid sequences and was selected to describe the amino acids substitution pattern. For estimating polymorphism within each group and evolutionary divergence between each group the number of base substitutions per site from averaging over all sequence pairs was calculated, all positions containing gaps and missing data were eliminated. Codon positions included were 1st+2nd+3rd+Noncoding. For the estimation of Maximum Likelihood Estimate of Transition/Transversion Bias (R) substitution pattern and rates were estimated under the Kimura 2-parameter model.

Phylogeny analysis was run in MEGA6. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. All ambiguous positions were removed for each sequence pair.

Natural selection and the probability of rejecting the null hypothesis of strict-neutrality (dN = dS) was evaluated using MEGA6. For these purposes was used a codon-based Z-test (MEGA6). For a pair of sequences, this is done by first estimating the number of synonymous substitutions per synonymous site (dS) and the number of nonsynonymous substitutions per nonsynonymous site (dN), and their variances: Var(dS) and Var(dN), respectively. With this information, we tested the null hypothesis that there is no impact of selection (dN = dS) and the probability (P) of rejecting the null hypothesis of strict-neutrality. Also was tested an alternative hypothesis of pu-

rifying selection (*d*N < *d*S) and the probability of rejecting the null hypothesis of strict-neutrality in favor of the alternative hypothesis using a codon-based Z-test (MEGA6). Values of *P* determine statistical significance in a hypothesis test. A low *P* value suggests that sample provides enough evidence for the rejecting of the null hypothesis for the entire population. Values of *P* less than 0.05 are considered significant at the 5% level. The variance of the difference was computed using the analytical method (Kimura 1980). All ambiguous positions were removed for each sequence pair.

Results

The gene *period* (*per*) in two forms of *C. pipiens*

The structure of the gene *per* was studied in three individual *C. pipiens* f. "molestus" and in three individual *C. pipiens* f. "pipiens". Coding sequences of the three exons of the gene *per* were analysed: exon 2, 333 bp, exon 3, 738 bp, and exon 4, 1229 bp. In total, the 18 compared sequences spanned each 2300 bp (Suppl. material 1).

In the exon 2 of the gene *per* (333 bp) 11 variable sites were found; six of these substitutions resulted in amino acid substitutions in both forms of *C. pipiens* (Fig. 1). The exon 3 (738 bp) had 12 variable nucleotide sites, resulting in three amino acid substitutions (Fig. 1). The exon 4 (1229 bp) had 27 variable nucleotide sites, resulting in four amino acid substitutions (Fig. 1). In total, the nucleotide sequence of the three exons of the gene *per* for the both intraspecific forms had 50 (2.2%) variable nucleotide sites and 13 (1.7%) polymorphic amino acid sites, 48 nucleotide sites being parsimony-informative. The estimated Transition/Transversion bias (R) is 2.83. The DNA polymorphism of the gene *per* among individuals of the *C. pipiens* f. "pipiens" (0.003) and of the *C. pipiens* f. "molestus" (0.002) were both low, variability of the amino acid sequences also was low (Table 2). The genetic distances between two forms of *C. pipiens* from Volgograd were 0.010 based on nucleotide sequences and 0.011 based on amino acid sequences of the gene *per* (Table 2).

Comparison of the gene *per* for transatlantic *C. pipiens*

The obtained sequences of the gene *per* of *C. pipiens* from Volgograd and *C. pipiens* f. "pipiens" from the USA (GenBank acc. number KM355980) using BLAST software were compared. The identity of nucleotide sequences of *C. pipiens* f. "pipiens" mosquitoes from different continents is 98-99%; 4-16 amino acid substitutions were detected. Pairwise comparison showed that *C. pipiens* f. "pipiens" from the USA is slightly different from the Volgograd *C. pipiens* f. "pipiens" (0.008) and from *C. pipiens* f. "molestus" (0.013); these values are comparable with the differences between the studied *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus" (Table 2).

```
[                                   11  111111111223  5555556677777]

[                     2455566778999900  123345558581  1234793646666]

[                     5115658158012425  704922470845  6538609040124]

#molestus2            VMVSGAAMECASAGQN  MSKQVPVASSSK  DTSDGLNESTPTP

#molestus1            ................  ............  ..C.R..D.....

#molestus3            A...............  ........T...  .......D.....

#pipiens1             ....E....TTTT...  IN......T...  .........I...

#pipiens2             .........TTTT...  I...........  .........I...

#pipiens3             .........TTTT...  I...........  .........I...

#pipiensUSA_KM355980  .........TTTP..D  ............  E.......NI...

#quinq_CPIJ007193     .TAC.STLDT..TVP.  ..EAAMAP.TIR  .I.G.QHD..SI.
```

Figure 1. Variable amino acid sites of the gene *period* in *C. pipiens*. *C. pipiens* from the USA (KM355980) and *C. quinquefasciatus* (CPIJ007193) are taken for the comparison. Exons 2 (sites 1-111), 3 (113-358), and 4 (360-768) are separated with blank columns. Positions of the variable sites relative to combined sequences as presented in Suppl. material 1 shown on the top.

Table 2. Estimates of Evolutionary Divergence over *per* and *tim* sequence pairs between *Culex pipiens* complex members.

	AA\NA	gene *period*				gene *timeless*			
		1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	molestus		**0.010**	**0.010**	**0.027**		**0.012**	**0.025**	**0.028**
2	pipiens	0.011		**0.008**	**0.029**	0,008		**0.031**	**0.030**
3	pipiensUSA	0.013	0.008		**0.027**	0.018	0.018		**0.009**
4	quin	0.036	0.036	0.041		0.018	0.020	0.004	

In upper right section in bold: the number of nucleotide base substitutions (NA) per site from averaging over all sequence pairs between groups is shown. All results are based on the pairwise analysis of 20 sequences. There were a total of 2300 positions of *per* gene and 1560 positions of *tim* gene in the final dataset. In lower left section: the number of amino acid substitutions (AA) per site from averaging over all sequence pairs between groups are shown. The analysis involved 20 amino acid sequences. A total of 766 positions of the gene *per* and 520 positions of the gene *tim* were analysed as the final dataset.

Comparison of the gene *per* in *C. pipiens* and *C. quinquefasciatus*

The identity of the nucleotide sequences of the gene *per* for the two species was 97%. Comparison of the DNA from both forms of *C. pipiens* and *C. quinquefasciatus* (CPIJ007193) revealed 113-116 (4.9–5.5%) variable nucleotide sites (Suppl. material 1): 37 nucleotide substitutions were non-synonymous, resulting in amino acid substi-

tutions (Fig. 1). 64 nucleotide substitutions and 24 amino acid substitutions are specific for *C. quinquefasciatus,* with nine substitutions in each of the exons 2 and 3, and six in exon 4 (Fig. 1). The mean genetic divergence between *C. pipiens* and *C. quinquefasciatus* is 0.03 by both DNA and amino acid sequences. The difference between *C. pipiens* and *C. quinquefasciatus* is three times higher than the difference between the two forms of *C. pipiens* (Table 2).

Gene *timeless* (*tim*) in the two forms of *C. pipiens*

Using the DNA from the same three individual mosquitoes *C. pipiens* f. "molestus" and three individual mosquitoes *C. pipiens* f. "pipiens", the three longest coding sequences of the gene *tim* were studied: exon 1 (1037 bp), exon 5 (376-379 bp), and exon 6 (145 bp). In total, the 18 compared sequences each spanned 1557-1560 bp. (Suppl. material 2).

In exon 1 of the gene *tim* (1037 bp) 15 variable nucleotide sites and five variable amino acid sites were found, four of them showing variations only for the *C. pipiens* f. "pipiens" and were not found in *C. pipiens* f. "molestus" (Fig. 2). In exon 5 (379 bp) five DNA substitutions were found, and in exon 6 (145 bp) there were three variable nucleotide sites; all substitutions in the exons 5 and 6 were synonymous, resulting in similar amino acid sequences for *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus" (Suppl. material 2, Fig. 2). The estimated Transition/Transversion bias (R) is 2.79.

Comparing the nucleotide sequences of the gene *tim* for the specimens of *C. pipiens* f. "molestus" one variable DNA site was found, the detected nucleotide substitution does not result in amino acid substitution. The aligned DNA sequences of the *C. pipiens* f. "pipiens" had 11 variable sites, one mutation resulting in amino acid substitution (Fig. 2). The DNA polymorphism of the gene *tim* among specimens of the *C. pipiens* f. "pipiens" (0.0038) was higher than for *C. pipiens* f. "molestus" (0.0004), and variability of the amino acid sequences was 0.001 and 0.000, respectively. Comparing the total sequence of the three exons of the gene *tim,* between *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus" 23 (1.5%) variable nucleotide sites were found (all 23 sites were parsimony-informative) and six (0.4%) polymorphic amino acid sites. Genetic distance between the two forms was 0.012 for DNA sequences and 0.008 for amino acid sequences (Table 2).

Comparison of the gene *tim* for the transatlantic *C. pipiens*

The obtained sequences of the gene *tim* for *C. pipiens* from Volgograd and *C. pipiens* f. "pipiens" from the USA (KM355979) were compared using BLAST software. Identity of nucleotide sequences for mosquitoes of *C. pipiens* f. "pipiens" from different continents is 96-97%. We found 7-12 amino acid substitutions. Unexpectedly, we found a 60-bp deletion within the coding sequence of exon 1 in *C. pipiens* f. "pipiens" from the USA (KM355979), positions 263-282 in Fig. 2. No similar deletion was found in either of the studied *C. pipiens* forms from Volgograd and no similar deletions were

```
[                        112222222222222222222222233333   3]
[                       923246666666777777777888900223   7]
[                       196063456789012345678901240202 0   5]
#molestus2              YVNCDGDSLTLGWDHQCTQTTLLIPELTTHT   E
#molestus1              ...............................   .
#molestus3              ...............................   .
#pipiens1               ....E......................SQI   .
#pipiens2               ....E...............P.......SQI   .
#pipiens3               ....E...............P.......SQI   .
#pipiensUSA_KM355979    FIDF.--------------------DQSSQ.   .
#quinq_CPIJ007082       FI.F..................P..DQSSQ.   Q
```

Figure 2. Variations of amino acid sites in the gene *tim* from *C. pipiens*. *C.pipiens* from the USA (KM355979), and *C.quinquefasciatus* (CPIJ007082) are taken for the comparison. Dashes show deletion in exon 1 in *C. pipiens* from the USA (KM355979). Exon 1 (sites 1-345) and exon 5 (sites 347-472) are separated by blank columns. Positions of the variable sites in combined *tim* sequences shown on the top.

found in *C. quinquefasciatus* (CPIJ007082). The genetic distance between *C. pipiens* f. "pipiens" from Volgograd and *C. pipiens* f. "pipiens" from the USA (KM355979) is 0.025, two times higher than the distance between both forms of *C. pipiens* in Volgograd 0.012 (Table 2).

Comparison of the gene *tim* from *C. pipiens* and *C. quinquefasciatus*

Comparison of DNA sequences of exons 1, 5 and 6 of the gene *tim* between representatives of the two species, *C. pipiens* and *C. quinquefasciatus,* revealed 50 variable sites (see Suppl. material 2), which result in 8-14 amino acid substitutions, eight of which are found only in *C. quinquefasciatus* (Fig. 2). The genetic distance between the species is 0.029 in the DNA sequences and 0.019 in amino acid sequences (Table 2). A striking similarity should be noted for the gene *tim* from *C. quinquefasciatus* and *C. pipiens* f. "pipiens" from the USA (KM355979). Their amino acid sequences have only three variable sites: 136, 280, and 375 (Fig. 2), their DNA sequences differ in 13 single-nucleotide substitutions and one deletion. The genetic distance for the gene *tim* between *C. quinquefasciatus* and *C. pipiens* f. "pipiens" from the USA (KM355979) is 0.009 based on DNA sequences and 0.004 based on amino acid sequences, lower that the distance between the two forms of *C. pipiens* in Volgograd (Table 2).

Extended study of exon 1 of the gene *tim* in two forms of *C. pipiens*

Our results showed that exon 1 of the gene *tim* in *C. pipiens* f. "molestus" differ from that of in *C. pipiens* f. "pipiens" (Fig. 2). Contrary to the gene *per,* no shared polymorphisms were found in amino acid sequences of gene *tim* between two forms (Figs 1, 2). To confirm these findings we studied the structure of exon 1 (1037 bp) of the gene *tim* in 23 specimens of *C. pipiens* f. "molestus" and 14 specimens of *C. pipiens* f. "pipiens" in addition to 6 samples of gene *tim* described above. In total, 43 samples were examined.

The obtained nucleotide sequences showed overlapping peaks in one or more sites for 6 individuals. Four of them were identified as *C. pipiens* f. "molestus" and two as *C. pipiens* f. "pipiens". Exon 1 of the gene *tim* of these six samples was studied by cloning and the DNA of the five clones for each specimen was sequenced. In total 79 sequences were obtained for comparative analysis (Suppl. material 3). In five specimens one allele was identical to *C. pipiens* f. "pipiens" and other one was identical to *C. pipiens* f. "molestus", i. e. these mosquitoes represented hybrids. In one *C. pipiens* f. "molestus" (NN23) the alleles differed by two nucleotide substitutions in 3'end. All hybrids were collected in Volgograd, where both forms develop in the same pools in summer.

49 variable nucleotide sites and 23 distinct haplotypes were found in exon 1 of the gene *tim* (Fig. 3). *C. pipiens* f. "pipiens" showed 19 haplotypes. Four haplotypes were obtained in *C. pipiens* f. "molestus" (H1-H4). Haplotypes H1 and H2 detected in *C. pipiens* f. "molestus" from geographically remote locations (Volgograd, Nizhny Novgorod, Moscow and S.-Petersburg) differed by only one synonymous nucleotide substitution A-G at position 653 in Exon 1 of the gene *tim* (Fig. 3). H3 and H4 were detected only in two individuals: H3 combined with H1 (*C. pipiens* f. "molestus") in NN23 and H4 in combination with H11 (*C. pipiens* f. "pipiens") in V219 (Suppl. material 3). Amino acid sequences of *C. pipiens* f. "molestus" with H1 and H2 haplotypes differed from *C. pipiens* f. "pipiens" by two substitutions Serine (Ser)-Threonine (Thr) and *Glutamine* (Gln)-Histidine (His). Additional substitutions were detected in two specimens with H3 and H4 haplotypes namely the T968A and T968G substitutions in DNA sequences which resulted in Gln in amino acid sequence (Fig. 3, Suppl. material 3). In total, two variations of amino acid sequences were found in *C. pipiens* f. "molestus" and 8 in *C. pipiens* f. "pipiens" (Fig. 3).

The DNA polymorphism of the exon 1 of gene *tim* among specimens of the *C. pipiens* f. "pipiens" (0.007) was ten times higher than for the *C. pipiens* f. "molestus" (0.0006), and variability of the amino acid sequences was 0.0053 and 0.0001, respectively. Genetic distance between the two forms was 0.011 for DNA sequences and 0.009 for amino acid sequences. Genetic distance between *C. pipiens* of both forms and *C. quinquefasciatus* was 0.029 for DNA and 0.02 for amino acid sequences (Suppl. material 3). The DNA polymorphism, as well as genetic distances between the two forms in extended study of the exon 1 are very close to the results obtained for the three exons of the gene *tim* (see above) (Table 2).

```
[                                   1]
[         1112223 3445555667 77899990]  122333
[      5794460674 8671245572 47366690]  547223
[      3764940991 1178434315 00402814]  968020
#H1    CTATTCCCTT CGCTTTCAAG TAAAGTCT   PDTTHT
#H2    .......... .......G.. ........   ......
#H3    .......... .......... .....A.A   ....Q.
#H4    ..C...T... T..C.C.G.. .....G..   ....Q.
#H5    .A.CCTT..C .......G.. A..T.GT.   .E.SQI
#H6    .A.CCTTG.C .A.....G.. A.CTAGT.   .EPSQI
#H7    .A..C.TG.C .A.....G.. A.CTAGT.   .EPSQI
#H8    .A.C.TTG.C .A.....G.. A.CTAGT.   .EPSQI
#H9    .A.CCTTG.C .A.....G.. A.CTTGT.   .EPSQI
#H10   .A...TT... ..T.C..G.A .TCT.G..   S.PSQ.
#H11   .A.CCTT..C ..T...TG.A ...T.G..   S..SQ.
#H12   .A.CCTT... ..T.C..G.A .T.T.G..   S..SQ.
#H13   .A...TT.C. ..T.C..G.A .T.T.G..   S..SQ.
#H14   .A...TT..C ..T.C..G.A .T.TAGT.   S..SQI
#H15   ...CCTT..C .A.....G.. A.-T.GT.   .E-SQI
#H16   .....TT... ....C..G.. ...TTG..   ...SQ.
#H17   .A...TT.CC .......G.A ...T.G..   ...SQ.
#H18   .A...TT... ....C..GGA ...T.G..   ...SQ.
#H19   T..CCT...C .......G.A .T.TAG..   ...SQ.
#H20   .A...TT... .......G.. .T.T.G..   ...SQ.
#H21   .A.CCTT..C .......G.. ...T.GT.   ...SQI
#H22   .A.CCTT..C .A..C..G.. ..CT.G..   ..PSQ.
#H23   .....TT... .......GGA ..CT.G..   ..PSQ.
```

Figure 3. DNA haplotypes and variable amino acid positions in the exon 1 of the gene *tim* from *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus". Haplotypes numbers and variable nucleotide sites are shown on the left. Variable amino acid sites are shown on the right. Only variable haplotypes are shown, all 79 sequences are presented in Suppl. material 3. Positions of the variable sites shown on the top. Dash show deletion of 12 nucleotides (sites 831-842) in exon 1 in *C.pipiens* f. "pipiens" from Moscow region.

Variation in non-coding regions of the gene *tim*

The sequences of some non-coding regions were analysed, expecting to find differences not only in coding DNA structure but also in intron size between *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus". The primers were constructed for the conserved sites of the exons using the obtained sequences, and by homology with the gene *tim* from *C. quinquefasciatus* (CPIJ007082). The sequences of the introns 1-2 (7158 bp in length) and 10-11 (5189 bp), being too long for efficient PCR and sequencing and containing numerous repeats were not analysed. As for the other introns, sequencing of the PCR products showed no variability between two intraspecific forms in intron between exons 5 and 6 (59 bp). In intron 6-7 (61 bp) three variable sites and in intron 7-8 (160 bp) six variable sites were found. Studied introns showed no mutations common with either of the two *C. pipiens* forms. In the intron 9-10 (167 bp) seven variable sites were found, six of which differed between the two forms (Suppl. material 4). The length of all amplified intron sequences was identical for *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus".

Phylogenetic analysis

Phylogenetic dendrograms were constructed applying the Neighbor-Joining method to amino acid sequences of the three coding regions of genes *per* and *tim*, *C. quinquefasciatus* was used as an out-group. *C. quinquefasciatus* and *C. pipiens* form two well differentiated clusters. Basing on similarity of the gene *per* the individuals of the *C. pipiens* f. "pipiens" group together and form a joint cluster with a bootstrap coefficient of 97. The studied specimens of the *C. pipiens* f. "molestus" had more polymorphic amino acid sequences, but also are grouped into one cluster with bootstrap coefficient of 63 (Fig. 4). Based on the similarity of the gene *tim*, *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus" group into separate clusters with a bootstrap coefficient of 88 (Fig. 4B).

On the dendrogram for the exon 1 of the gene *tim*, constructed using the results of our extended study, most specimens of the *C. pipiens* f. "molestus" form separate clusters with a bootstrap coefficient of 96. A separate subcluster is formed by sequences of the hybrid V219 clones with haplotype H4. The studied specimens of the *C. pipiens* f. "pipiens" have polymorphic DNA sequences (Suppl. material 3). The dendrogram basing on amino acid sequences shows similar configuration.

Evolutionary analysis

One way to test whether natural selection is operating on a gene is to compare the relative abundance of synonymous and nonsynonymous substitutions within the gene sequences (Tamura et al. 2013). Analysing evolution of the nucleotide sequences, the

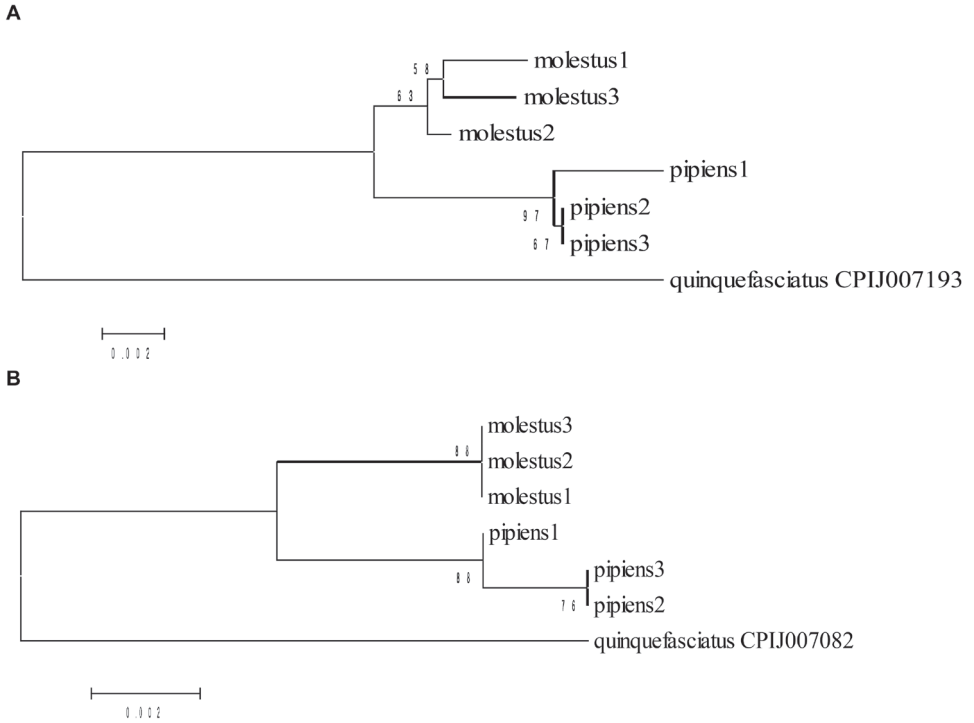

Figure 4. Evolutionary relationships of the studied taxa. Neighbor-joining trees of *C. pipiens* based on **A** *period* and **B** *timeless* inferred amino acid sequences with the *C. quinquefasciatus* (CPIJ007193) as the outgroup. Percent bootstrap support based on 1000 replicates. Seven amino acid sequences were analysed with a total of 766 positions of PERIOD (A) and 519 positions of TIMELESS (B) in the final datasets.

Codon-based Test of Neutrality rejected the null hypothesis of strict-neutrality with strong statistical support in both genes (Table 4). Though comparison of some haplotypes within the *C. pipiens* f. "pipiens" also shows deviation from neutrality, difference between the forms is considerably higher (Suppl. materials 5, 6). Analysis of *d*N-*d*S between the *per* nucleotide sequences of both intraspecific forms indicates that the probability of rejecting the null hypothesis of strict-neutrality ranges from 0 to 0.015 across the sequences with an overall average of 0.003. Between *tim* nucleotide sequences of the three exons of *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus", the probability of rejecting the null hypothesis of strict-neutrality ranges from 0.003 to 0.017 across the specimens with an overall average of 0.006. Table 4 shows average mean *d*N-*d*S and of the probability of rejecting the null hypothesis of strict-neutrality for each individual.

Analysis of *d*N-*d*S between the 79 nucleotide sequences of exon 1 of the gene *tim* indicates that the probability of rejecting the null hypothesis of strict-neutrality between intraspecific forms ranges from 0.002 to 0.15 across the sequences with an overall average of 0.05. The number of synonymous substitutions per site (*d*S) was higher that the number of non-synonymous substitutions per site (*d*N), indicating Purifying Selection. The probability of rejecting the null hypothesis of strict-neutrality (*d*N = *d*S)

Table 3. Comparison of exons and introns variability between *C. pipiens* f. "pipiens" and f. "molestus" from Russia.

Gene	Locus	size (bp)	Variable DNA sites	Differentiating DNA sites	Variable AA sites	Differentiating AA sites
Per	exon2	333	11	6	6	4
	exon3	738	12	2	3	1
	exon4	1229	27	9	4	1
Tim	exon1	1037	49	2	10	1
	exon5	376–379	5	1	0	0
	exon6	145	3	0	0	0
	intron5-6	59	0	0	-	-
	intron6-7	61	3	0	-	-
	intron7-8	160	6	0	-	-
	intron9-10	167	7	6	-	-

AA - amino acid

in favor of the alternative hypothesis of Purifying Selection ($dN < dS$) ranges for the *tim* nucleotide sequences of *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus" from 0.001 to 0.10 with an overall average of 0.025 (Suppl. material 7).

Discussion

For the first time the genetic structure of the circadian rhythm genes (*per* and *tim*) were analysed for mosquitoes *C. pipiens* f. "molestus". Our results have shown that DNA variation in individuals of *C. pipiens* f. "molestus" is smaller than in individuals of *C. pipiens* f. "pipiens". Extended study of exon 1 of the gene *tim* revealed 4 DNA haplotypes in *C. pipiens* f. "molestus" and 19 haplotypes in *C. pipiens* f. "pipiens". Decrease in DNA variability for the underground mosquitoes of *C. pipiens* f. "molestus" was also reported earlier in our study of mitochondrial DNA (Shaikevich and Zakharov 2010).

In coding sequences of both genes *per* and *tim*, variations between physiologically different forms of *C. pipiens* were found (Table 3). In the gene *per* we found nine polymorphisms shared between the two forms and four fixed differences between the two forms, taking into account *C. pipiens* f. "pipiens" from N America (Fig. 1). The gene *tim* had one shared amino acid polymorphisms and one fixed difference between the forms (Fig. 3). Higher variation of the gene *per* is also revealed by comparison of *C. pipiens* and *C. quinquefasciatus*: basing on the amino acid sequences, the genetic distances between the species are higher for the gene *per* (0.036) that for the gene of *tim* (0.02).

C. pipiens f. "pipiens" from N America clusters with *C. pipiens* f. "pipiens" from Volgograd basing on comparison of the gene *per* and with *C. quinquefasciatus* based on comparison of the gene *tim*. It remains unknown whether this is common for all American *C. pipiens* f. "pipiens", shown using microsatellite analysis to differ from the

Table 4. Codon-based Test of Neutrality between *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus".

specimen		gene *period*						gene *timeless*					
		1	2	3	4	5	6	1	2	3	4	5	6
1	pipiens1		-1.950	-2.829	-3.130	-3.682	-3.309		-2.307	-2.009	-2.830	-2.779	-2.679
2	pipiens2	0.108		-2.904	-3.326	-3.865	-3.349	0.034		-1.856	-2.906	-2.859	-2.766
3	pipiens3	0.010	0.008		-3.775	-4.284	-3.788	0.089	0.0663		-2.945	-2.899	-2.805
4	molestus1	0.001	0.003	0.000		-1.534	-0.586	0.0047	0.0023	0.005		-0.333	-0.998
5	molestus2	0.000	0.000	0.000	0.154		-1.427	0.0056	0.0027	0.0058	0.774		-0.665
6	molestus3	0.002	0.003	0.000	0.502	0.15		0.0073	0.0034	0.0075	0.320	0.547	

The test statistic (*d*N - *d*S) is shown above the diagonal. The probability of rejecting the null hypothesis of strict-neutrality (*d*N = *d*S) is shown. Values of *P* less than 0.01 are considered significant at the 1% level. There was a total of 766 positions of gene *per* and of 519 positions of gene *tim* in the final dataset. Evolutionary analyses were conducted in MEGA6.

European *C. pipiens* f. "pipiens" (Fonseca et al. 2004), or if it is a specific feature of the laboratory line, used to study the genes on circadian rhythm (Meuti et al. 2015).

Genetic structure of the studied genes is polymorphic. However, the revealed substitutions in nucleotide sequences and especially in protein sequences grouped the individuals of the two forms into distinct clusters with high significance, a longer genetic distance separating the cluster of *C. pipiens* from *C. quinquefasciatus*. Although the two studied genes differed in variability, the results of analysis of the gene *per*, as well as the gene *tim*, show that the difference between *C. pipiens* and *C. quinquefasciatus* are 2.5–3 times higher than the difference between the forms of *C. pipiens*. The genetic distances again confirm the order of evolutionary events in the *C. pipiens* complex: the divergence of the form *C. pipiens* f. "molestus" from *C. pipiens* occurred considerably later than the divergence of *C. pipiens* and *C. quinquefasciatus* (Barr 1967, Fonseca et al. 2004, Shaikevich and Zakharov 2014).

The non-coding genome sequences are considered to be highly variable. These sequences are often used to search for the markers to differentiate closely related organisms by size of the PCR products. For example, variation in spacers of the ribosomal genes cluster is a base for identification of some mosquito species of the genus *Anopheles* (Nicolescu et al. 2004, Gordeev et al. 2004). In sequences of three *tim* introns no significant difference was found between the forms. For *Aedes albopictus* Skuse, 1894, also no significant difference in the introns of the gene *tim* was reported (Summa et al. 2012).

The Test of Neutrality rejects the null hypothesis of strict-neutrality at $P < 5\%$ level and imply that both *per* and *tim* loci evolve under strong selective constraint during the divergence of intraspecific forms. Our results suggest that natural selection favored the fixed mutations and the decreased diversity of the genes *per* and *tim* in mosquitoes *C. pipiens* f. "molestus" compared with the *C. pipiens* f. "pipiens", probably preserving adaptive features of the form "molestus". Well-documented data have been reported showing that new native mutations sometimes are rapidly spreading in a population and that polymorphism in one locus may provide adaptive variations in behavioral and morphological phenotypes of the insects in nature (Tauber et al. 2007). The genes

involved in circadian rhythms are proved to coordinate seasonal responses, e.g. they initiate the reproductive diapause; malfunctioning of the genes *per* and *tim* was shown to interrupt diapausing of the *C. pipiens* females (Meuti et al. 2015). We can assume that mutations found in *per* and especially in *tim* genes are related with functioning of the circadian rhythm proteins and contributed to divergence of the forms of *C. pipiens.* The studied genes are promising candidates to evaluate the genetic basis of different behaviors of the two ecological forms within one subspecies. Further studies of the circadian rhythm genes in mosquitoes of the *Culex pipiens* complex would help to test this assumption.

Conclusions

Nucleotide sequences of the circadian rhythm genes were studied for the first time in mosquitoes *C. pipiens* f. "molestus" and compared with those for *C. pipiens* f. "pipiens" and *C. quinquefasciatus.* These results show that intraspecies variability is higher for the gene *per* than for the gene *tim.* Revealed substitutions in nucleotide sequences and especially in protein sequences grouped the individuals of the two ecological forms of *C. pipiens* into distinct clusters with high significance. The results suggest that natural selection favored the fixed mutations and the decreased diversity of the genes *per* and *tim* in mosquitoes of the *C. pipiens* f. "molestus" compared with the *C. pipiens* f. "pipiens". The detected fixed amino acid substitutions may appear essential for functioning of the circadian rhythm proteins in *C. pipiens*, and may be related with adaptations of the taxa within the group *C. pipiens*. Moreover, under natural selection mutations in the key genes of circadian pattern may provide some advantage to the underground *C. pipiens* f. "molestus". The studied genome regions may be considered as promising molecular-genetic markers for identification, population and phylogenetic analysis of similar species and forms of the *C. pipiens* complex.

Acknowledgements

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Supplementary material 1

Aligned nucleotide sequences of *per* gene.

Authors: Elena V. Shaikevich, Ludmila S. Karan, Marina V. Fyodorova

Data type: primary data

Explanation note: DNA sequences of three clones of each individual *C. pipiens* are presented and compared with sequences of *C. quinquefasciatus* (CPIJ007193) and *C. pipiens* from the USA (KM355980).

Supplementary material 2

Aligned nucleotide sequences of *tim* gene.

Authors: Elena V. Shaikevich, Ludmila S. Karan, Marina V. Fyodorova

Data type: primary data

Explanation note: DNA sequences of three clones of each individual *C. pipiens* are presented and compared with sequences of *C. quinquefasciatus* (CPIJ007193) and *C. pipiens* from the USA (KM355980).

Supplementary material 3

Analysis of the divergent between two forms of *C. pipiens* based on comparison of the exon 1 of the gene *tim* sequences.

Authors: Elena V. Shaikevich, Ludmila S. Karan, Marina V. Fyodorova

Data type: primary data

Explanation note: Nucleotide and amino acid sequences of the *tim* gene exon1 in compare with sequence of *C. quinquefasciatus*.

Supplementary material 4

Aligned *tim* nucleotide non-coding sequences.

Authors: Elena V. Shaikevich, Ludmila S. Karan, Marina V. Fyodorova

Data type: primary data

Explanation note: Intron's DNA sequences of individual *C. pipiens* f. "pipiens" and *C. pipiens* f. "molestus" are presented.

Supplementary material 5

Codon-based Test of Neutrality for analysis between *per* gene sequences of *C. pipiens* both forms.

Authors: Elena V. Shaikevich, Ludmila S. Karan, Marina V. Fyodorova

Data type: measurement of Z-test value

Explanation note: The test statistic (*d*N - *d*S) and the probability of rejecting the null hypothesis of strict-neutrality (*d*N = *d*S) are shown base on the differences between *per* gene sequences of *C. pipiens* both forms.

Supplementary material 6

Codon-based Test of Neutrality for analysis between *tim* gene sequences of *C. pipiens* both forms.

Authors: Elena V. Shaikevich, Ludmila S. Karan, Marina V. Fyodorova

Data type: measurement of Z-test value

Explanation note: The test statistic (*d*N - *d*S) and the probability of rejecting the null hypothesis of strict-neutrality (*d*N = *d*S) are shown base on the differences between *tim* gene sequences of *C. pipiens* both forms.

Supplementary material 7

Codon-based Test of of Purifying Selection for analysis between the exon1 of the gene *tim* sequences of *C. pipiens* both forms.

Authors: Elena V. Shaikevich, Ludmila S. Karan, Marina V. Fyodorova

Data type: measurement of Z-test value

Explanation note: The test statistic (*d*N - *d*S) and the probability of rejecting the null hypothesis of strict-neutrality (*d*N = *d*S) are shown base on the differences between the exon1 of the gene *tim* sequences of *C. pipiens* both forms.

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Karyological study of *Ololygon tripui* (Lourenço, Nascimento and Pires, 2009), (Anura, Hylidae) with comments on chromosomal traits among populations

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Abstract

To increase the number of cytogenetic characters used in *Ololygon tripui* systematics, we applied some cytogenetic techniques such as Giemsa, C- and NOR-banding, and fluorescence *in situ* hybridization (FISH) with 18S rDNA and repetitive microsatellite DNA probes to the study of four populations from Minas Gerais State (southeastern Brazil). All populations showed 2n = 24 and FN = 48, and chromosomal formula 8m + 10sm + 6st. Nucleolar organizing regions (NORs) were located on chromosome pair 6 in all populations, although in the Tripuí locality additional markings were observed on one homologue of chromosome pair 3. These patterns were partially congruent with results obtained using the 18S rDNA FISH probe. The microsatellites repetitive DNA $(GA)_{15}$ and $(CAT)_{10}$ probes accumulated predominantly in the terminal region of all chromosomes. Chromosome morphology and Ag-NOR were conserved among populations, a conserved pattern in *Ololygon* Fitzinger, 1843. Repetitive DNA FISH probes patterns were similar among populations, but they revealed species-specific differences when compared with other species of the genus *Ololygon*, suggesting that molecular cytogenetics are potentially more informative in karyologically conservative taxa.

Keywords
Cytotaxonomy, population cytogenetics, Ag-NOR, heterochromatic blocks, 18S rDNA, FISH, microsatellite DNA probes

Introduction

The genus *Ololygon* Fitzinger, 1843 belongs to Hylidae and currently includes 46 species (Duellman 2016, Frost 2016). In Hylidae, high level of karyotypical diversity has been reported in genera *Aplastodiscus* Lutz, 1950, *Hypsiboas* Wagler, 1830, and *Phyllomedusa* Wagler, 1830 (Carvalho et al. 2009, Ferro et al. 2012, Gruber et al. 2012, Gruber et al. 2013). On the other hand, karyotypes of *Ololygon* and *Scinax* Wagler, 1830 are highly conserved: all species show 2n = 24, and FN = 48, and morphological differences are restricted to slight variations between corresponding homeologous chromosomes (Nunes and Fagundes 2008, Cardozo et al. 2011, Nogueira et al. 2015, Peixoto et al. 2015). Within *Ololygon*, only 19 recognized species and four unnamed taxa have been karyologically studied. It's worth mentioning that the genus *Ololygon* was resurrected from the genus *Scinax* in a recent review made by Duellman et al. 2016. Before this, the species from the genus *Ololygon* were considered as *Scinax* (Frost 2016).

In the genus *Ololygon*, the NOR is observed on chromosome pair 6, with exception of *O. canastrensis* (Cardozo and Haddad, 1982), where NORs occur on chromosome pair 6 and 11 (Cardozo et al. 2011). C-banding pattern in this genus is predominantly centromeric, and some species have relatively large amount of heterochromatin (Cardozo et al. 2011, Peixoto et al. 2015). Until now, repetitive DNA probe patterns are known in only four species of this genus (reported as *Scinax* in Peixoto et al. 2015); all of them belong to the *Ololygon perpusilla* group (Peixoto 1987). In these species, repetitive DNA $(CA)_{15}$ probe accumulated in the terminal regions of most chromosomes and showed additional markings in the centromeric region of some chromosome pairs, while the $(CAT)_{10}$ repetitive DNA probe accumulated on the terminal or subtelomeric chromosomal regions (Peixoto et al. 2015).

Either as dispersed elements or repetitive elements organized in tandem, the repetitive DNA represents a large portion of the eukaryotic genome, and includes satellite DNA, microsatellites, minisatellites, telomeric sequences, multigene family, and transposable elements (Maxon et al. 1983, Charlesworth et al. 1994). Microsatellites sequences apparently evolve through slippage replication errors that remain unrepaired by the mechanism of DNA repair (Charlesworth et al. 1994, Jentzsch et al. 2013). Usually, these sequences accumulate close to regions characterized by low levels of recombination, such as heterochromatic regions found in terminal regions, centromeres and even in some sex chromosomes (Stephan and Walsh 2013, Cioffi et al. 2012).

Relatively a few systematic data are available for the taxonomically complex and diverse genus *Ololygon*. Thus, cytogenetic data are potentially informative for under-

standing the phylogenetic relationships of the species within the genus (Cardozo et al. 2011). The aim of this study was to characterize the karyotypes of four populations of *O. tripui* (Lourenço et al. 2009), to explore the information potential of standard cytogenetic techniques versus FISH using18S rDNA and repetitive DNA probes. Based on results obtained, evolutionary issues relevant for this species were discussed.

Material and methods

As a total, 32 specimens of *O. tripui* were collected from four populations from the state of Minas Gerais, Brazil: Estação Ecológica do Tripuí, Ouro Preto municipality (Tripuí – Type locality), Parque Estadual Serra do Brigadeiro, Ervália municipality (PESB), RPPN Mata do Sossego, Simonésia municipality (Sossego), and Usina da Fumaça, Muriaé municipality (Fumaça) (Table 1). Proceedings were carried out according to the Animal Welfare Commission of the Universidade Federal de Viçosa and the current Brazilian laws (CONCEA 1153/95). All vouchers were housed in the herpetological collection of the Museu de Zoologia João Moojen at the Universidade Federal de Viçosa (MZUFV), Viçosa municipality, in Minas Gerais State, Brazil (Table 1).

Mitotic chromosomes were obtained from gut epithelial cells according to Schmid (1978). Each specimen was injected intraperitoneally with 0.1% solution of colchicine (0.1 ml per 10 g of body weight) for 4 hours before euthanasia (carried out with 5% lidocaine). Best metaphases were photographed in digital Olympus BX53 light microscope with a DP73 Olympus camera. Chromosome pairing and measurements were performed using Image Pro Plus® (IPP Version 4.5) to determine the modal value (2n) and the FN for each population. Homologs were paired and grouped according to the centromere position, in decreasing size order. Approximately 20 metaphase spreads were analyzed per specimen, to determine the diploid chromosome number and karyotypic structure. The

Table 1. Sample sizes (N) per gender, sample locality and voucher identification of *Ololygon tripui* populations.

Population	Sample locality	N	Gender	Voucher (MZUFV)
PESB	Parque Estadual Serra do Brigadeiro, Ervália – MG (20°51'52"S, 042°31'17"W)	9	Male	9865, 9870, 9871, 9872, 10294, 10299-301, 11421
		3	Female	11511, 12103-104
Sossego	RPPN Mata do Sossego, Simonésia – MG (20°04'22"S, 042°04'12"W)	6	Male	11441-443, 12571, 12575-576
		2	Female	12573, 12577
Fumaça	Usina da Fumaça, Muriaé – MG (21°00'58"S, 042°26'36"W)	6	Male	11444-449
		3	Female	11438-440
Tripuí	Estação Ecológica do Tripuí, Ouro Preto – MG (20°23'22"S, 043°32'20"W)	3	Male	12447-449

chromosomes were classified according to their centromeric indices in metacentric (m), submetacentric (sm) and subtelocentric (st) following Green and Sessions (1991).

To identify heterochromatic regions, the C-banding technique followed Schmid et al. (1979). Active NORs in the preceding interphase were identified using silver nitrate precipitation (Ag-NOR) following Howell and Black (1980). The technique of FISH was performed according to Pinkel et al. (1986). The 18S rDNA probe was obtained from *O. tripui* via PCR and it was labeled by nick-translation with biotin-14-dATP. Signal detection and amplification were performed using isothiocyanate probe, fluorescein-conjugated avidin and anti-avidin-biotin. The $(GA)_{15}$ and $(CAT)_{10}$ repetitive DNA probes were thynilated with cy3 at the 5' position (Sigma-Aldrich). FISH images were captured in a BX53 Olympus microscope with a XM10 camera.

Results

All the individuals analyzed had a diploid complement of 24 chromosomes. All chromosomes were biarmed and their FN was 48 (Suppl. material 1 and Table 2). The chromosomal formulae of the *O. tripui* was 8m + 10sm + 6st, whereas chromosome pair 1, 2, 3, 5, and 8 were sm; 4, 6, and 7 were st, and 9–12 were m in all populations (Figs 1–4).

Heterochromatic blocks were detected in the centromeric and pericentromeric regions of chromosomes, only in the specimens from the Fumaça locality. Heterochromatic patterns were similar in both sexes (Fig. 1). The Ag-NOR region was pericentromeric and located on the short arm of chromosome pair 6. However, in the PESB specimens, the Ag-NOR was restricted to only one chromosome in females. In addition to marking the

Table 2. Comparative morphology and measurements of chromosome pairs in *Ololygon tripui* populations. m = metacentric, sm = submetacentric, st = subtelocentric, CR = centromeric ratio, CT = chromosome type.

Population	Gender		Chromosome pairs											
			1	2	3	4	5	6	7	8	9	10	11	12
PESB	Female	CR	2.41	2.53	2	3.38	2.46	3.25	4.55	2.4	1.25	1.11	1.23	1.19
		CT	sm	sm	sm	st	sm	st	st	sm	m	m	m	m
	Male	CR	2.11	2.32	2.3	3.57	1.99	3.4	3.19	2.31	1.1	1.1	1.1	1.17
		CT	sm	sm	sm	st	sm	st	st	sm	m	m	m	m
Sossego	Female	CR	2.38	2.28	2.41	3.91	2.04	3.96	3.63	2.17	1.49	1.35	1.15	1.26
		CT	sm	sm	sm	st	sm	st	st	sm	m	m	m	m
	Male	CR	2.17	2.18	2.09	3.49	2.1	3.45	3.53	2.05	1.23	1.32	1.19	1.14
		CT	sm	sm	sm	st	sm	st	st	sm	m	m	m	m
Fumaça	Female	CR	2.58	2.62	1.97	3.68	2.56	3.66	3.53	2.13	1.45	1.33	1.28	1.16
		CT	sm	sm	sm	st	sm	st	st	sm	m	m	m	m
	Male	CR	2.3	2.42	1.8	3.46	2.06	3.57	3.95	2.31	1.31	1.33	1.2	1.28
		CT	sm	sm	sm	st	sm	st	st	sm	m	m	m	m
Tripuí	Male	CR	2.39	2.44	2.51	3.76	2.31	3.67	3.67	1.81	1.35	1.19	1.15	1.14
		CT	sm	sm	sm	st	sm	st	st	sm	m	m	m	m

chromosome pair 6, males from the Tripuí population showed an additional Ag-NOR in the subtelomeric region in the long arm on one homolog of chromosome pair 3 (Fig. 4). The presence of a secondary constriction associated with Ag-NOR was also detected in the chromosome pair 6, and sometimes it was evident in only one of the homologs.

FISH showed 18S rDNA sites in chromosome pair 6 in males and females from the Fumaça, PESB, and Sossego populations. Multiple tags for 18S rDNA FISH were also observed in a male of the Tripuí population, with markings restricted to one homolog in chromosome pair 3 and 4, and on both homologs of chromosome pair 6. The microsatellites repetitive DNA probe $(GA)_{15}$ accumulated in the terminal region of all chromosomes (Fig. 5), whereas the $(CAT)_{10}$ probe showed conspicuous accumulation

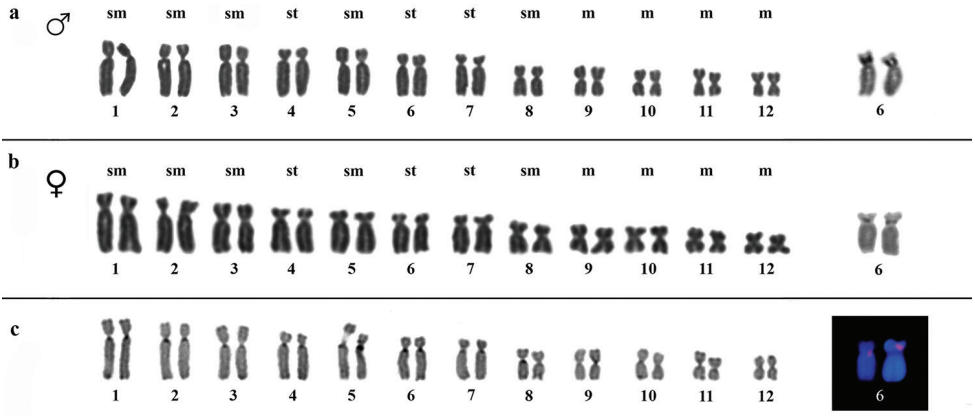

Figure 1. a–c. Karyotype of *Ololygon tripui* from the Fumaça locality. **a** Giemsa and Ag-NOR staining of male chromosomes **b** Giemsa and Ag-NOR staining of female chromosomes **c** C-banding and 18S rDNA markers on chromosome pair number 6. Bar = 10 μm.

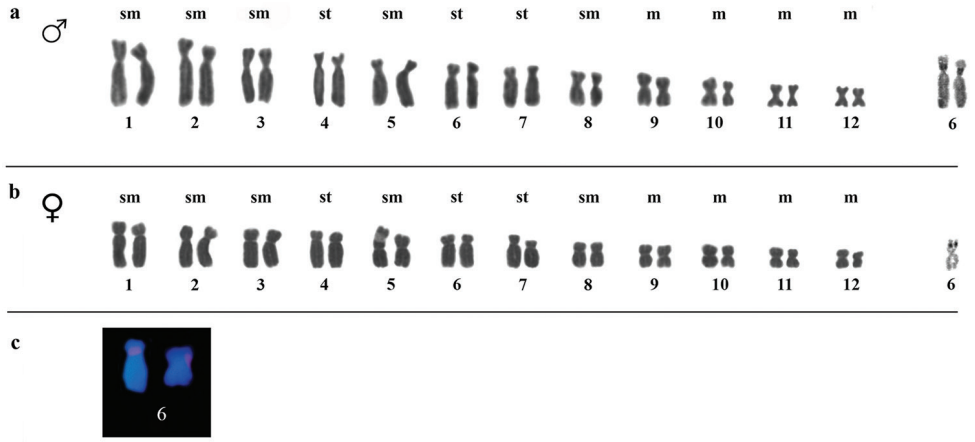

Figure 2. a–c. Karyotype of *Ololygon tripui* from PESB locality. **a** Giemsa and Ag-NOR staining of male chromosomes **b** Giemsa and Ag-NOR staining of female chromosomes **c** 18S rDNA sites on chromosome pair number 6. Bar = 10 μm.

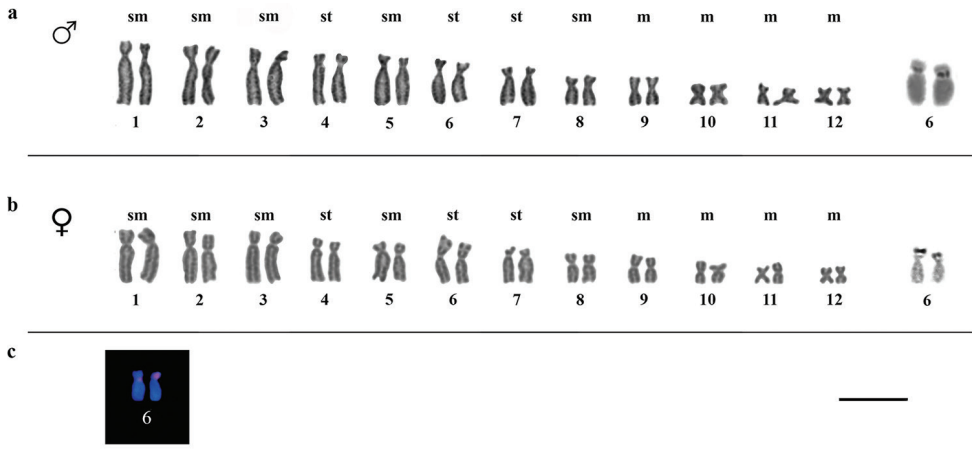

Figure 3. a–c. Karyotype of *Ololygon tripui* from the Sossego locality. **a** Giemsa and Ag-NOR staining of male chromosomes **b** Giemsa and Ag-NOR staining of female chromosomes **c** 18S rDNA markers on chromosome pair number 6. Bar = 10 µm.

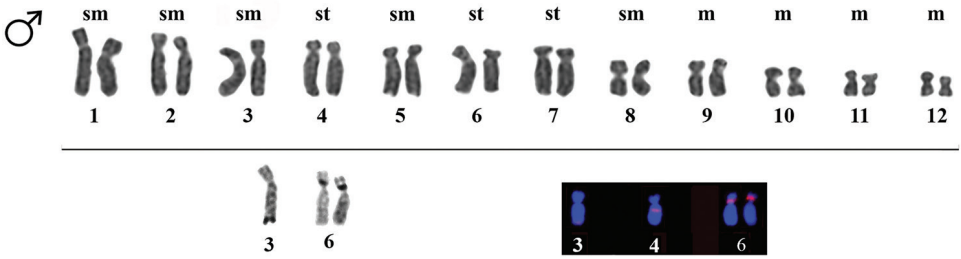

Figure 4. Karyotype of *Ololygon tripui* from the Tripuí locality. Giemsa, Ag-NOR staining, and 18S rDNA markers on multiple chromosomes of a male specimen. Bar = 10 µm.

in the terminal regions of all chromosomes, with additional markings in the centromeric regions of some chromosomes (Fig. 6).

Discussion

The diploid number of 2n = 24 and FN = 48 observed in *O. tripui* was similar to those reported for all studied species of the genus and considered as a highly conserved character, which is shared with other genera within Hylidae (i.e. *Scinax*, *Xenohyla* Izecksohn, 1998, and *Lysapsus* Cope, 1862) (Suárez et al. 2013). However, our results suggest that other characters associated with *Ololygon*, may represent putative synapomorphy of species within the genus *Ololygon*: i) *O. tripui* showed the chromosome pair 1 and 2 submetacentric-like and the chromosome pair 6 subtelocentric-like as well as all karyologically studied species of the genus. In *O. belloni* Faivovich, Gas-

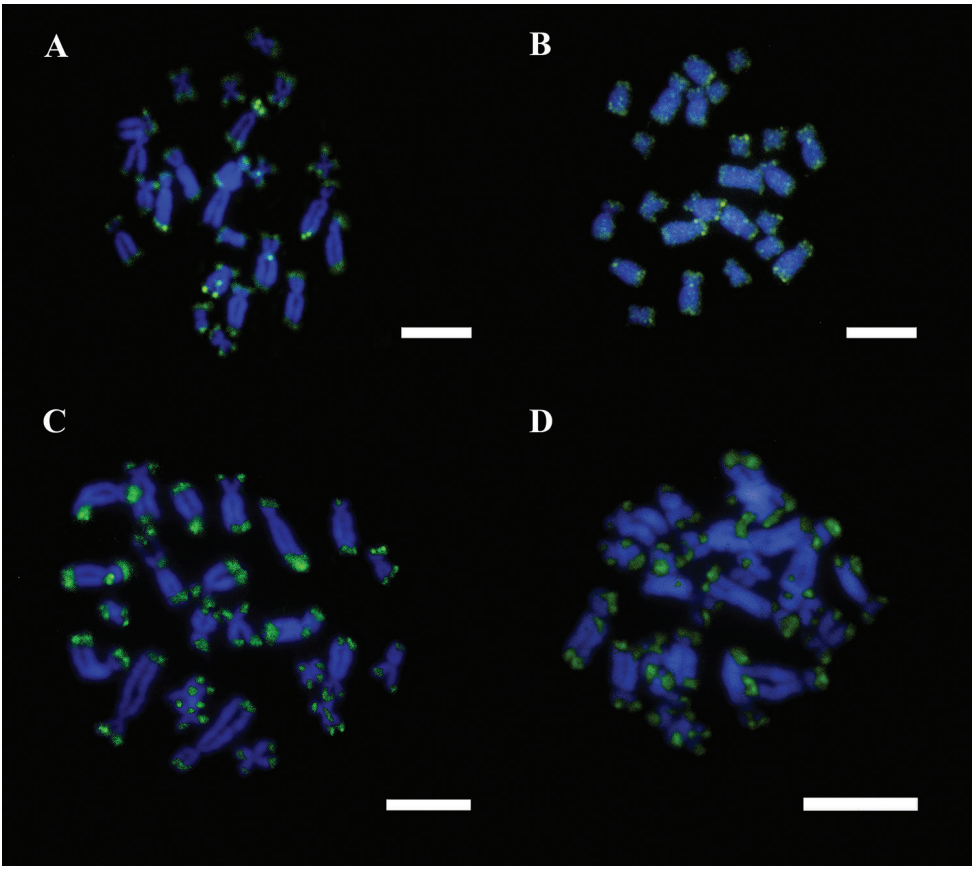

Figure 5. a–d. Male mitotic chromosomes of *Ololygon tripui* from different populations, labeled with the $(GA)_{10}$ repetitive DNA probe. **a** Fumaça **b** PESB **c** Sossego **d** Tripuí. Bar = 10 µm.

parini and Haddad, 2010, the chromosome pair 1 is metacentric, which is interpreted as a possible autapomorphy of this species (Peixoto et al. 2015); ii) Ag-NOR cistrons were identified on the chromosome pair 6 in all populations of *O. tripui*. These traits place the species of *Ololygon* as a chromosomally well-differentiated taxon from the species of *Scinax*. In the latter genus, the chromosome pair 1 and 2 are metacentric-like, the chromosome pair 6 is submetacentric-like; the Ag-NOR cistrons, as in most other hylids, usually occur on the chromosome pair 11. The only exception is *S. alter* (Lutz, 1973), which has terminal NORs on chromosome pair 3 (Nunes and Fagundes 2008, Cardozo et al. 2011, Peixoto et al. 2015). The genus *Ololygon* was recently resurrected from the genus *Scinax* (Duellman et al. 2016), based on molecular data. Our study demonstrates that chromosomal characters are also congruent with this taxonomical decision.

The polymorphic presence of more than one pair of Ag-NOR cistrons in the Tripuí population of *O. tripui* have also been reported in other species of this genus (Cardozo et al. 2011). For instance, in *O. rizibilis* (Bokermann, 1964) Ag-NORs are present on

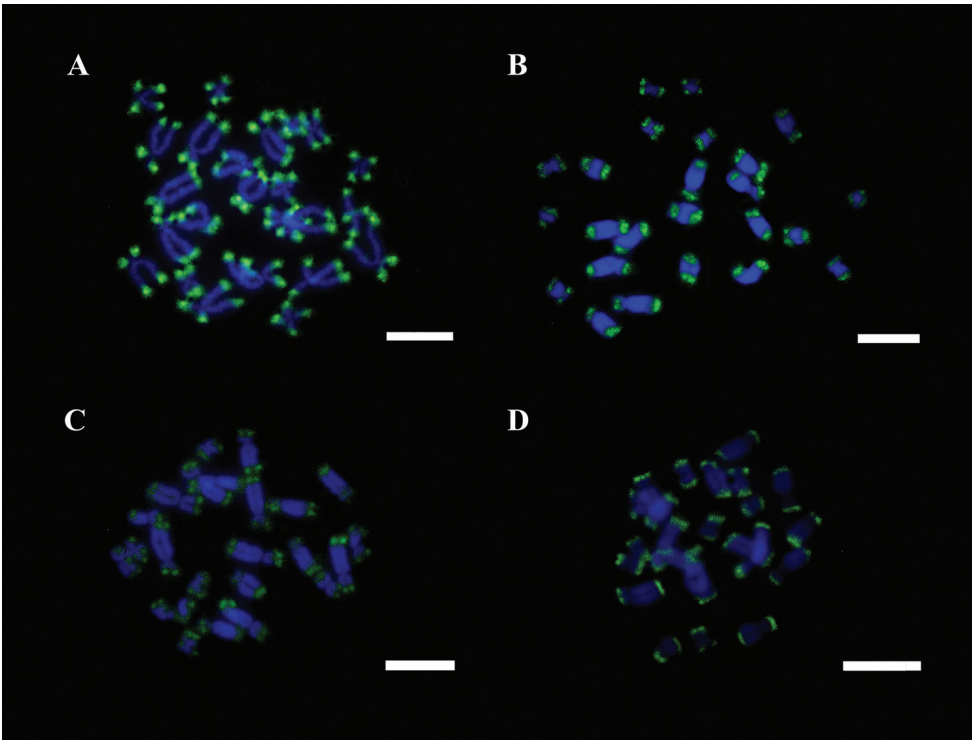

Figure 6. a–d. Male mitotic chromosome of *Ololygon tripui* from different populations, labeled with the $(CAT)_{15}$ repetitive DNA probe. **a** Fumaça **b** PESB **c** Sossego **d** Tripuí. Bar = 10 µm.

chromosome pair 5 and 6 from two different populations, whereas in *O. canastrensis*, Ag-NORs occurs on chromosome pair 6 and 11 (Cardozo et al. 2011). In *O. tripui*, the secondary constriction, associated with NORs, was found in the interstitial region close to the centromeric region of chromosome pair number 6, but sometimes it was restricted to one member of this chromosome pair. In anurans, the correlation of Ag-NORs with secondary constriction is commonly observed (Barth et al. 2009) and characterizes *Ololygon* (Cardozo et al. 2011).

Markings obtained with 18S rDNA FISH revealed some chromosomal aspects that were not evident using the Ag-NOR protocol. Although high correlation was observed in the Fumaça and Sossego populations between the FISH and NOR banding patterns, in the PESB population this correlation was partial: only one homolog showed Ag-NOR banding, whereas both homologs bore 18S rDNA sites. Notably, in the Tripuí population, 18S rDNA sites were detected in one homolog of chromosome pair 3 and 4 and in both homologs of chromosome pair 6; Ag-NOR-banding was evident on one homolog of chromosome pair 3, and on the chromosome pair 6. Unlike Ag-NORs, FISH exposure of rDNA regions occurs regardless of previous interphase activation of this region, allowing to detect actual levels of polymorphisms between

populations or species (Zurita et al. 1998). In fact, the results of the 18S rDNA FISH are more representative in PESB and Tripuí populations.

Partial correlation between results obtained by Ag-NOR and FISH techniques can be explained by the action of different mechanisms, such as: i) a limitation of the technique in detecting a rDNA sequences present in low copy number; ii) the mobility of rDNA sequences spread by transposable elements; iii) a real polymorphic condition; iv) the occurrence of chromosome rearrangements such as translocations; v) the physiological amplification of rDNA cistrons (Macgregor and Kezer 1973, Schubert and Wobus 1985, King et al. 1990).

Chromosome mapping using microsatellite sequences in hylids is scarce, however available data suggest that this kind of sequences accumulate in the vicinity of centromeres and telomeres (Peixoto et al. 2015). The accumulation of trinucleotide DNA probe $(CAT)_{10}$ in the terminal regions plus additional markings in the centromere region of some chromosomes of *O. tripui* populations, was similar to the pattern observed in bromeligenous species of *Ololygon* (*Ololygon* sp., *O. arduoa* Peixoto, 2002, *O. belloni*, and *O. cosenzai* Lacerda, Peixoto and Feio, 2012), where this probe are restricted to the terminal or subtelomeric regions of chromosomes (Peixoto et al. 2015). The dinucleotide $(GA)_{15}$ probe showed a conserved pattern in the populations of the *O. tripui* reported in this study. The $(CA)_{10}$ also showed accumulation in terminal regions of chromosomes, plus additional markings on centromeric regions of some chromosomes of the other *Ololygon* species (Peixoto et al. 2015).

Our results indicate that despite *O. tripui* populations were conservative in most of their cytogenetic characters such as diploid and fundamental number, Ag-NOR, repetitive DNA, and 18S rDNA patterns in relation to *Ololygon* sp., *O. arduoa*, *O. belloni*, and *O. cosenzai*, the former species differs in its chromosome morphology and in the repetitive DNA pattern, that probably occurs due to independent evolution of the species, thus corroborating its taxonomic status.

We concluded that although repetitive DNA patterns of variation are largely unknown in anurans, the cytogenetic mapping of different repetitive DNA sequences provided reliable chromosomal markers, revealing species-specific differences, when compared with other species of the genus. Our study showed initial insights on the use of repetitive DNA probes to discuss evolutionary issues.

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Supplementary material 1

Data on chromosomal and fundamental number, chromosomal formulae, C and NOR banding, 18S, and sample locality to all species from the genus *Ololygon* with some karyotypic study

Authors: Marco Antônio A. Peixoto, Marina P. C. Oliveira, Renato N. Feio, Jorge A. Dergam

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RESEARCH ARTICLE

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Further evidence for the variability of the 18S rDNA loci in the family Tingidae (Hemiptera, Heteroptera)

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Abstract

As of now, within the lace bug family Tingidae (Cimicomorpha), only 1.5% of the species described have been cytogenetically studied. In this paper, male karyotypes of *Stephanitis caucasica*, *S. pyri*, *Physatocheila confinis*, *Lasiacantha capucina*, *Dictyla rotundata* and *D. echii* were studied using FISH mapping with an 18S rDNA marker. The results show variability: the major rDNA sites are predominantly located on a pair of autosomes but occasionally on the X and Y chromosomes. All currently available data on the distribution of the major rDNA in the Tingidae karyotypes are summarized and shortly discussed. Our main concern is to clarify whether the chromosomal position of rDNA loci can contribute to resolving the phylogenetic relationships among the Tingidae taxa.

Keywords

Karyotype, FISH, major rDNA cluster, lace bugs, Cimicomorpha, Hemiptera

Introduction

The true bug family Tingidae is a relatively large and widespread group of phytophagous (sap-sucking) insects, some of which are important agricultural and forestry pests. The insects of this family are commonly known as the lace bugs due to a reticulation of the pronotum and fore wings. The family Tingidae is included in the true bug infraorder

Cimicomorpha (Hemiptera, Heteroptera) and considered as the closest relative to the family Miridae, lace bugs being either placed within the superfamily Miroidea (Drake and Davis 1960, Schuh and Štys 1991, Schuh et al. 2006, 2009, etc.), or taken as an the independent superfamily Tingoidea close to the Miroidea (Scudder 1959, Štys and Kerzhner 1975, Froeschner 1996, Golub and Popov 2016, etc.)

The relationships within the Tingidae are not entirely clear (Guilbert et al. 2014). The family currently comprises approximately 2200 species classified in 280 genera (Golub and Popov 2012, Golub et al. 2012). However, chromosome sets of only 31 species (1.5%) and 17 genera (6%) are known up to now (Grozeva and Nokkala 2001, Golub et al. 2015, for other references see Ueshima 1979).

Like other Heteroptera, lace bugs possess holokinetic chromosomes characterized by a non-localised centromere (Hughes-Schrader and Schrader 1961, Ueshima 1979). In spite of several studies, the karyological evolution of the family Tingidae remains poorly understood. The lace bugs' karyotypes seem to be highly conserved, with 12 autosomes reported for all so far studied species; the autosomes represent a series gradually decreasing in size. Most species have an XY type of sex determination while a few species have an X(0) system.

Until recently, only conventional chromosome staining techniques were used for the Tingidae. The first attempt to use a differential staining protocol was made by Grozeva and Nokkala (2001). They adapted C-banding to chromosomes of 13 species from 10 genera of lace bugs. This study revealed in karyotypes clear C-bands, which are useful for chromosome identification. Specifically, three species of the genus *Acalypta* Westwood, 1840, sharing the same karyotype of 2n = 12 + X(0), were demonstrated to differ in the number, size and location of C-heterochromatin blocks. These findings showed that C-heterochromatin distribution has had a role in the karyotype evolution of the family Tingidae.

A molecular hybridization technique such as fluorescence in situ hybridization (FISH) is a very useful method for studying molecular structure of chromosomes and differentiating separate chromosomes in different species. The chromosomal location of the rRNA genes is currently the most widely exploited marker in comparative cytogenetics of the Heteroptera (for a review see Grozeva et al. 2014). The nuclear genes coding for the ribosomal RNA are organized into the two distinct multigene families: the major rDNA repeats (genes for the 18S, 5.8S and 28S rRNAs) and the minor rDNA repeats (genes for the 5S rRNA). The major rDNA sites are often arranged in tandem arrays and undergo concerted evolution (the co-evolution of DNA sequences) being mapped to the same chromosomal region in the species karyotypes. Recently we (Golub et al. 2015) reported for the first FISH with an 18S rDNA probe in four lace bug species and discussed usefulness of the major rRNA gene cluster as a marker for revealing differences between species with similar karyotypes.

In the context of the above studies, we examined here the location of the 18S rDNA loci through FISH in six further species from the genera *Stephanitis* Stål, 1873, *Physatocheila* Fieber, 1861, *Dictyla* Stål, 1874 and *Lasiacantha* Stål, 1873. The standard karyotypes of four species, *Stephanitis caucasica*, *S. pyri*, *Physatocheila confinis* and *Dictyla rotundata* were studied for the first time.

Table 1. Material used for chromosome analysis.

Species	Number of males examined	Host plant, date and locality of collection
Dictyla echii (Schrank, 1782)	6	*Echium* sp., 22-26.07.2015, Teberda Nature Reserve, North Caucasus, Russia.
D. rotundata (Herrich-Schaeffer, 1835)	9	*Echium* sp., 27.07.2015, Teberda Nature Reserve, North Caucasus, Russia.
Lasiacantha capucina (Germar, 1837)	3	*Thymus* sp., 02.08.2015, Teberda Nature Reserve, North Caucasus, Russia.
Physatocheila confinis (Horváth, 1906)	3	*Crataegus* sp., 2.08.2015, Teberda Nature Reserve, North Caucasus, Russia.
Stephanitis caucasica Kiritshenko, 1939	12	*Rhododendron caucasicum* Pallas, 1786, 30.07.2015, Teberda Nature Reserve, North Caucasus, Russia.
S. pyri (Fabricius, 1775)	8	*Malus* sp., *Pyrus* sp., 15.08.2015, Voronezh Prov., Russia.

Materials and methods

The lace bug species used here were collected in 2015 in the Teberda Nature Reserve, North Caucasus and in Voronezh Province, Russia (Table 1). The species identification was made by V. Golub.

Only males were used in chromosome analysis. The specimens were fixed in the field in 3:1 Carnoy solution (96% ethanol: glacial acetic acid) and stored at 4°C. In the laboratory, testes were dissected out in a drop of 45% acetic acid and squashed on the slide. The cover slips were removed using dry ice. The preparations were stained using a Feulgen-Giemsa method by Grozeva and Nokkala (1996). To determine the number and chromosomal position of the major rDNA clusters, we carried out 18S rDNA FISH on meiotic chromosomes. In fluorescence *in situ* hybridization we followed Grozeva et al. (2014) protocol with some modifications described in Golub et al. (2015).

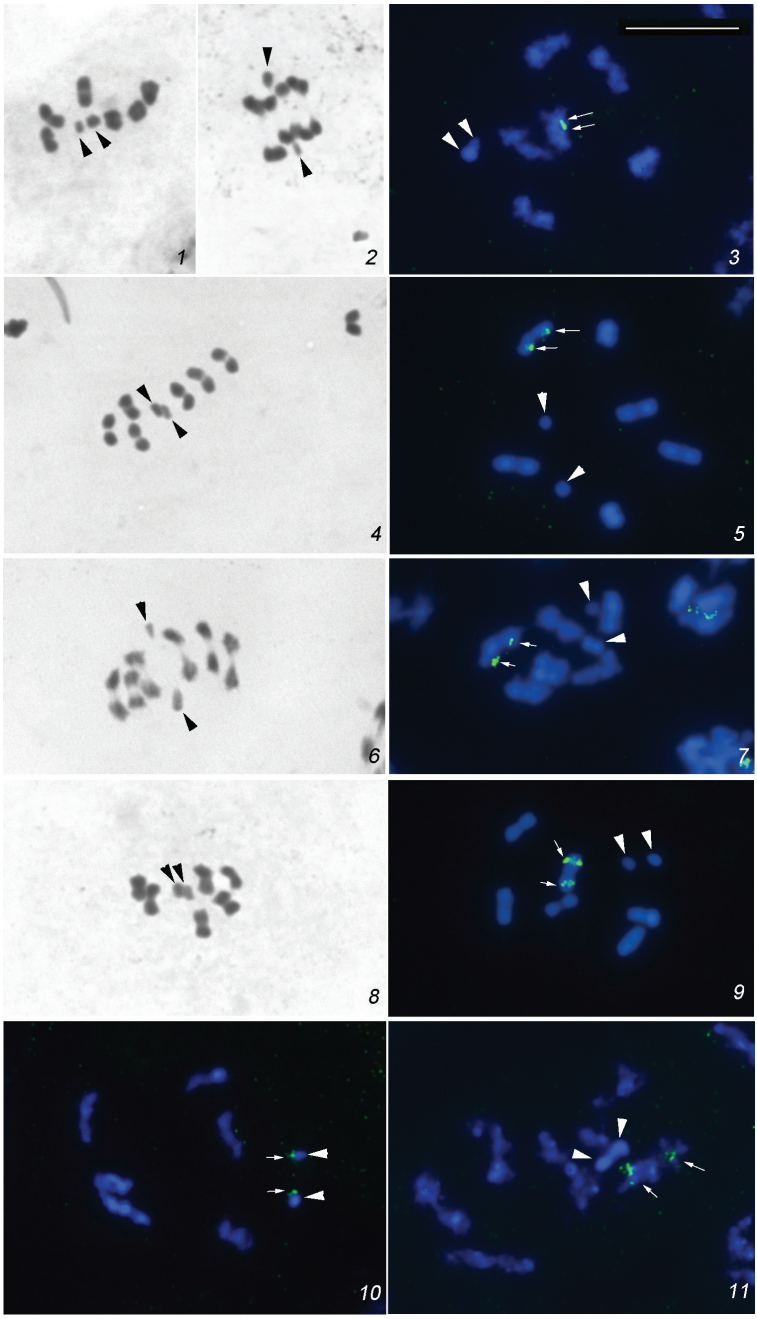
Chromosome slides were analyzed under a Leica DM 6000 B microscope. Images were taken with a Leica DFC 345 FX camera using Leica Application Suite 3.7 software with an Image Overlay module.

Results

Stephanitis caucasica, 2n = 14 (12 + XY)

Published data: absent

At spermatocyte metaphase I (MI), six bivalents of autosomes and X and Y univalent chromosomes are present suggesting diploid karyotype of 2n = 14 (12 + XY). All bivalents are of similar size. The sex chromosomes show different sizes, the larger being



Figures 1–11. Meiotic chromosomes of the Tingidae species with 2n = 12 + XY studied using conventional staining technique and 18S rDNA FISH. **1–3** *Stephanitis caucasica* **1, 2** conventional staining: MI (1), AI (2) **3** FISH: MI **4, 5** *Stephanitis pyri* **4** conventional staining: MI **5** FISH: MI **6, 7** *Physatocheila confinis* **6** conventional staining: MI/AI transition **7** FISH: early MI **8, 9** *Dictyla rotundata* **8** conventional staining: MI **9** FISH: MI **10** *Dictyla echii* FISH: early MI **11** *Lasiacantha capucina* FISH: prophase I. rDNA FISH signals are indicated by arrows. X and Y chromosomes are indicated by arrowheads. Bar = 10μm.

presumably the X, and are situated alongside each other (Fig. 1). During an anaphase I (AI) all chromosomes undergo segregation, with X and Y chromosomes segregating ahead of the autosomes (Fig. 2).

18S rDNA FISH resulted in bright signals on an autosomal bivalent at MI. The signals are most likely located subterminally on each homolog. Sex chromosomes are placed very close to each other (Fig. 3).

Stephanitis pyri, 2n = 14 (12 + XY)

Published data: absent

At spermatocyte MI, six bivalents of autosomes and X and Y univalent chromosomes are present suggesting diploid karyotype of 2n = 14 (12 + XY). All bivalents are of similar size. The sex chromosomes show slightly different sizes, the larger being presumably the X, and are situated alongside each other (Fig. 4).

18S rDNA FISH resulted in bright signals on an autosomal bivalent at MI. The signals are located interstitially on each homolog. The sex chromosomes are mutually co-orientated on the spindle (Fig. 5).

Phisatocheila confinis, 2n = 14 (12 + XY)

Published data: absent

At spermatocyte MI/AI transition, six bivalents of autosomes and X and Y univalent chromosomes are present suggesting diploid karyotype of 2n = 14 (12 + XY). All bivalents are of similar size. The sex chromosomes show distinctly different sizes, the larger being presumably the X. The sex chromosomes segregate ahead of the autosomes (Fig. 6).

18S rDNA FISH resulted in bright signals on an autosomal bivalent at MI. The signals are located interstitially on each homolog (Fig. 7).

Dictyla rotundata, 2n = 14 (12 + XY)

Published data: absent

At spermatocyte MI, six bivalents of autosomes and X and Y univalent chromosomes are present suggesting diploid karyotype of 2n = 14 (12 + XY). All bivalents are of similar size. The sex chromosomes show a similar size and are situated alongside each other (Fig. 8).

18S rDNA FISH resulted in bright signals on an autosomal bivalent at MI. The signals are located interstitially on each homolog (Fig. 9).

Dictyla echii, 2n = 14 (12 + XY)

Published data: 2n = 14 (12 + XY) in Grozeva and Nokkala (2001)

At early spermatocyte MI, there are six bivalents of autosomes and X and Y univalent chromosomes. All bivalents are of similar size. The sex chromosomes show a similar size and are placed not far from each other. Bright 18S rDNA FISH signals are located at one end of each sex chromosome (Fig. 10).

Lasiacantha capucina, 2n = 14 (12 + XY)

Published data: 2n = 14 (12 + XY) in Grozeva and Nokkala (2001)

At spermatocyte prophase I, there are six bivalents of autosomes which have diffuse structure at this stage. The X and Y chromosomes are positively heteropycnotic and placed very close to each other. Bright 18S rDNA FISH signals are located interstitially on each homolog of a bivalent (Fig. 11).

Discussion

Comparative karyotype analysis of six lace bug species was achieved using standard chromosome staining along with the 18S rDNA FISH marker. All species were found to have 2n = 14 (12 + XY). The karyotypes of *Stephanitis caucasica*, *S. pyri*, *Physatocheila confinis* and *Dictyla rotundata* were studied for the first time. The karyotypes of *Dictyla echii* and *Lasiacantha capucina* were previously studied by Grozeva and Nokkala (2001) who also reported 2n = 14 (12 + XY) for each of these species.

The results of this study confirmed the assumption of the high degree of karyotype conservation for the Tingidae (Ueshima 1979, Grozeva and Nokkala 2001, Golub et al. 2015). Including our new data, a total of 35 species from 17 genera were karyologically studied, but this represents less than 2% of known lace bug species of the world fauna. All studied species have the same number of autosomes, i.e., 12 in diploid karyotypes. The only exception might be *Acalypta parvula* (Fallén, 1807), for which different authors reported karyotypes of 2n = 12 + X(0) and 2n = 10 + XY discovered in populations from Finland and British Isles respectively (Southwood and Leston 1959, Grozeva and Nokkala 2001; for discussion, see Golub et al. 2015). Considering that Tingidae have holokinetic chromosomes, which are assumed to be susceptible to fission and fusion (Hughes-Schrader 1935), the conservation of the autosome number suggests that these rearrangements are not characteristic of lace bugs. This is supported also by the fact that in all tingid species the autosomes are of similar size, the pattern which can be considered as a ground plan feature of the family.

Despite the relative conservatism of the karyotype structure in general, some lace bug species clearly differ in size of sex chromosomes. For example, X and Y chromosomes appear noticeably heteromorphic in size in *Physatocheila confinis*, while they are evenly-sized in *Dictyla rotundata* and *D. echii* (Figs 6–10). Of particular interest, detectable size differences may provide an important criterion for identification of some closely related species. For example, *Stephanitis caucasica* possesses an enlarged X chromosome in comparison to the Y, whereas in *S. pyri* both sex chromosomes appear similar in size (Figs 1–5).

Some other true bug families also demonstrate interspecies difference in size of sex chromosomes (Bardella et al. 2014, Fairbairn et al. 2016). One of the important sources of chromosome size variability seems to be related to the constitutive heterochromatin variation (White 1973). A series of lace bug species studied by C-banding was shown to differ considerably in the C-heterochromatin content and its location. Most significant variation occurs in sex chromosomes, which appear variously heterochromatin-rich in different species (Grozeva and Nokkala 2001). Although no direct information is available, the X and Y chromosome variation might be a consequence of gain and loss of heterochromatic segments during the evolution of the sex chromosomes in the Tingidae.

In the Heteroptera, the major rRNA gene FISH has yielded a significant body of literature (Grozeva el al. 2011, Panzera et al. 2012, Pita et al. 2013, Bardella et al. 2013, Chirino et al. 2013, Grozeva et al. 2014). These studies have shown that the major rDNA cluster is localized variously in tested families (reviewed in Grozeva et al. 2014). However in the Tingidae, only 10 species have been analyzed to date (Golub et al. 2015, present paper). The mapping results are summarized in Table 2.

Despite the same chromosome number, the 18S rDNA clusters were found to vary in number (one or two in diploid karyotype) and location (sex chromosomes or autosomes) in lace bug species. The rDNA signals were observed either on the X chromosome as in *Agramma femorale*, or on both sex chromosomes as in *Tingis crispata* and *Dictyla echii*, or on a pair of autosomes as in the remaining species. The congeneric species can demonstrate both similarity and dissimilarity in the rDNA location pattern. For example, both studied *Stephanitis* species (*S. caucasica* and *S. pyri*) were found to have rDNA clusters on autosomes. A different situation arises with genera *Tingis* Fabricius, 1803 and *Dictyla*, where the congeneric species have rDNA either on autosomes or on sex chromosomes. Different mechanisms have been appointed to play a role in the rDNA evolutionary dynamics, particularly the transposition of the rRNA genes to new chromosome location in closely related species without changes in chromosome number (e.g., Granger et al. 2004, Cabrero and Camacho 2008, Nguyen et al. 2010, Panzera et al. 2012, Pita et al. 2013) and were mentioned in our previous publication (Golub et al. 2015).

Besides, the interspecific differences were found in the position of 18S rDNA clusters within chromosomes – subterminal or interstitial, and such differences are occurring likewise in congeneric species (Table 2). Specifically, subterminal clusters

Table 2. Distribution of the major rDNA loci in the Tingidae.

Species	Karyotype	18S rDNA- bearing chromosomes	The chromosomal location of 18S rDNA clusters	References
Agramma femorale Thomson, 1871	12 + XY	X	Subterminal	Golub et al. 2015
Dictyla echii (Schrank, 1782)	12 + XY	XY	Subterminal both on X and Y	Present paper
D. rotundata (Herrich-Schaeffer, 1835)	12 + XY	AA	Interstitial	Present paper
Elasmotropis testacea testacea (Herrich-Schaeffer, 1830)	12 + XY	AA	Subterminal	Golub et al. 2015
Lasiacantha capucina (Germar, 1837)	12 + XY	AA	Interstitial	Present paper
Physatocheila confinis (Horvath, 1906)	12 + XY	AA	Interstitial	Present paper
Stephanitis caucasica Kiritshenko, 1939	12 + XY	AA	Subterminal	Present paper
S. pyri (Fabricius, 1775)	12 + XY	AA	Interstitial	Present paper
Tingis crispata (Herrich-Schaeffer, 1838)	12 + XY	X,Y*	Interstitial on X, subterminal on Y	Golub et al. 2015
Tingis cardui (Linnaeus, 1758)	12 + XY	AA**	Interstitial	Golub et al. 2015

*X,Y – sex chromosomes; **AA – autosomal bivalent

appeared in autosomes of *Physatocheila confinis*, *Elasmotropis testacea testacea* and *S. caucasica*; in the X chromosome of *A. femorale*; in the Y chromosome of *T. crispate*; and in both sex chromosomes of *D. echii*. Furthermore, interstitial (intercalary) clusters appeared in the X chromosome of *T. crispata* but in autosomes of *T. cardui*, *D. rotundata* and *S. pyri* (Table 2). Differences observed within the genus *Stephanitis* indicate that in its evolution an inversion has occurred which changed the subterminal rDNA locus in *S. caucasica* to an interstitial position in *S. pyri* or *vice versa*.

The results presented here show that the major rDNA loci in the lace bug karyotypes may be considered as essential cytological markers to compare karyotypes of phylogenetically related species and to disclose chromosomal differentiation in species with similar karyotypes. This is likewise true for the species of the subfamily Triatominae (Reduviidae) which share the karyotype of 2n = 12 + XY and show extremely high dynamics of rDNA clusters, with the variation observed both between and within the species (Panzera et al. 2012, 2014, Bardella et al. 2013, Pita et al. 2013). Because of this, the chromosomal position of rDNA loci might be a useful marker for identifying recently diverged species or populations (Pita et al. 2013).

Based on the currently available data, the autosomal major rRNA gene location appears prevalent in the Tingidae being found in 6 genera out of the 7 genera tested. The occurrence of major rDNA sites in autosomes of the Tingidae is similar to the pattern that is most frequent in the order Heteroptera (e.g., Panzera et al. 2012, Pita

et al. 2013, Bardella et al. 2014, Grozeva et al. 2014). Because lace bugs have holokinetic chromosomes (without morphological markers such as centromeres), rather small chromosome size and similar karyotype structure (with all the autosomes being of similar size, so that in conventionally stained preparations the bivalents cannot be recognized on the basis of their size), it is uncertain whether an rDNA-bearing pair of autosomes is the same (homeologous) in different species. The resolution of this important issue will have to await further study based on new approaches and new discriminatory chromosomal landmarks.

In summary, the interspecific similarities and differences in the distribution of the major rDNA clusters make them promising markers for the further study of chromosome evolution in lace bugs. However, because of insufficient taxon sampling, the currently available data are inadequate to clarify the phylogenetic relationships within the Tingidae.

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RESEARCH ARTICLE

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Physical mapping of immune-related genes in Yesso scallop (*Patinopecten yessoensis*) using fluorescent *in situ* hybridization

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Abstract

The innate immune system plays a pivotal role in defending invasion of microorganisms for scallops. Previous studies on immune-related genes in the Yesso scallop, *Patinopecten yessoensis* (Jay, 1857) have mainly focused on characterization and expression pattern in response to bacterial challenge, no research has been carried out on the cytogenetic level yet. In the present study, eight fosmid clones containing the sequences of key immune-related genes (*Py*NFkB, *Py*TRAF2, *Py*TRAF4, *Py*TRAF7, *Py*Myd88-1, *Py*Myd88-3, *Py*MKK-7 and *Py*TNFR) were isolated and seven of them were successfully mapped on chromosomes of *P. yessoensis* utilizing fluorescence *in situ* hybridization. Wherein, *Py*Myd88-1, *Py*Myd88-3 and *Py*MKK-7 located on the same chromosome pair with adjacent positions and the other genes were mapped on four non-homologous chromosome pairs, showing a similar distribution to another five model species. The isolation and mapping of such genes of the Yesso scallop will lay a foundation for studies such as assignment of interested genes to chromosomes, construction cytogenetic maps and so on.

Keywords

Cytogenetic map, immune-related genes, FISH, fosmid

* These authors contributed equally to this work

Introduction

Studying chromosomal distribution pattern of important functional genes at the cytogenetic level, which can be useful for discussion of gene evolution, chromosome rearrangement and so on, is a crucial part of genome research in any species. For example, by mapping Antennapedia (ANTP) class homeobox genes to the chromosome in a lophotrochozoan protostome, Hui et al. (2012) elucidated an aspect of the genome organization of the protostome–deuterostome ancestor and revealed high levels of macrosynteny between *Platynereis dumerilii* (Audouin & Milne-Edwards, 1834) and chordates. In scallops, through comparing chromosome location of histone H3 genes in four species, it was suggested that gene duplication/diminution as well as chromosome rearrangements by inversion and translocation may have played important roles in the genomic evolution of Pectinidae (Zhang et al. 2007).

However, limited researches on chromosomal localization of functional genes have been conducted in mollusk species as yet. This is mostly due to the short probe length and the technical limitations, genes and sequences with low copy numbers tend to be more difficult to obtain positive signals after fluorescence *in situ* hybridization (FISH) (Wang et al. 2005, Zhao et al. 2015). To tackle this obstacle, large insert clones were introduced to FISH mapping and used as the probes. Bacterial artificial chromosome (BAC), bacteriophage P1 and fosmid have been already successfully applied in bivalve to achieve the goal of chromosome mapping (Wang et al. 2005, Zhang et al. 2008, Feng et al. 2014). What is more, in the Zhikong scallop, *Chlamys farreri* (Jones & Preston, 1904), Toll-Like receptor signaling pathway genes were unambiguously mapped to the chromosomes utilizing gene-containing BAC clones (Zhao et al. 2015). Such studies brought a novel method to investigating chromosomal distribution pattern of functional genes in mollusk.

The Yesso scallop, *Patinopecten yessoensis* (Jay, 1857), is a cold water bivalve and is naturally distributed along the coastline of northern Japan, the Far East of Russia and the northern Korean Peninsula (Shumway 1991). It is a species of great economic importance in China and Japan. Over the last decade, the population of the Yesso scallop is decreased sharply due to various infections. Like other scallop species, *P. yessoensis* lacks adaptive immunity and has evolved a series of sophisticated strategies to recognize and eliminate various invaders (Song et al. 2015). Several studies about immune-related genes in the Yesso scallop were published during 2015 to 2016 (Li et al. 2015, Ning et al. 2015, Wang et al. 2015, Zhang et al. 2015, Zou et al. 2015, Xing et al. 2016). These researches identified and characterized of *Py*MyD88, *Py*NFkB, *Py*TRAF, *Py*MKK, *Py*Tollip and *Py*TNFR genes in *P. yessoensis* and their distinct expression pattern in response to bacterial challenge. There is no doubt these results provided novel insight into immune-related genes of the Yesso scallop and the specific role and response of certain pathways in host immune responses against different bacterial pathogens. Yet, no early study focused on discussing this type of genes on cytogenetic level and mapping them to the chromosomes.

Cytogenetic studies of the Yesso scallop showed it possessed 38 chromosomes with a karyotype formula of 3m+5sm+8st+3t (Huang et al. 2007b). Yet, most of these studies were mainly focusing on the multi-copy genes or sequences such as rDNA sequences and histones genes (Huang et al. 2007b, Zhang et al. 2007). Recently, our study of mapping tandem repeats using fosmid clones demonstrated such probes provide a high successful rate for FISH (Li et al. 2016). Hence, with genome information of immune-related genes, it is an approachable way to locate genes of *P. yessoensis* using the fosmid clones as probes.

In present study, to investigate the chromosome distribution of immune-related genes of the Yesso scallop, we selected 8 gene-containing fosmid clones as probes for FISH. As a result, we successfully mapped 7 immune-related genes to the chromosomes of the Yesso scallop. Fosmid clones were proved to have high efficiency in chromosome mapping of single or low copy genes in the Yesso scallop. Our results provided the first physical mapping of immune-related genes in *P. yessoensis*, aiding to understanding chromosomal assignment and evolution of these genes.

Methods

Chromosome preparation

Trochophore larvae of *P. yessoensis* was treated for 2h at room temperature with 0.02% colchicine in sea water, the larvae were exposed to potassium chloride (KCl) solution (0.075M) for 30 min. Then it were fixed three times (15 min each) in fresh Carnoy's fixative (ethanol:glacial acetic acid = 3:1 v/v). Chromosome spreads were obtained by dissociating fixed larvae in 50% acetic acid and dropping the cellular suspension onto slides heated to 56°C.

Selection of fosmid clones and probe labeling

A fosmid library including 122, 880 clones of *P. yessoensis* has been constructed in our lab recently. The restriction fragments of two-dimensionally pooled fosmid clones were sequenced and generated sequences by a physical mapping technology based on next-generation sequencing, whole genome profiling (WGP) (Oeveren et al., 2011). The strategy for pooling clones was that clones in eight 384-microwell plates were mixed for a super pool. And two kinds of type IIB restriction endonucleases named BsaXI and FspEI were used to generate digested tags. These sequence tags are assigned to individual fosmid clones with a decoding rate over 88%. Sequences of 19 immune-related genes were identified by our lab (Li et al. 2015, Ning et al. 2015, Wang et al. 2015, Zou et al. 2015, Xing et al. 2016). These sequences were then cross checked with those sequence tags in order to locate genes to mono-clone.

Plasmid DNA from gene-containing fosmid clones, with an average insert size of 30-45 kb, was extracted by standard laboratory method (Sambrook and Russell David 1989) and labeled with digoxingenin-11-dUTP or biotin-16-dUTP using Dig- or Biotin-Nick Translation Mix (Roche) following the manufacturer's instruction. Labeling reaction mix with a total volume of 20μl contained 1μg plasmid DNA and 4μl nick-translation mix and was incubate under 15°C for 90 min. After adding EDTA (Ethylenediaminetetraacetic acid) to a final concentration of 25mM and heating to 65°C for 10 min, aliquots of 2μl reaction mix were sampled and run on 1% agarose gels to monitor fragment size. The length of the probes should be between 100 bp and 600 bp. Labeled probes were purified by SanPrep PCR products purify kit (Sangon Biotech) and then resolved at a concentration of 5-10 ng/μl in a hybridization solution of 2×SSC (sodium chloride/sodium citrate), 50% deinoized formamide and 10% dextran sulphate. 18S-28S rDNA probe were obtained by PCR amplification and labeled with biotin-16-dUTP based on previous study (Huang et al. 2007b).

FISH and Co-hybridization

FISH experiments were performed following methods previously published (Huang et al. 2007b). Chromosomes were denatured in a mixture containing with 70% formamide and 2×SSC at 76°C for 2 min 30 sec, dehydrated with a series of precool ethanol (70%, 90%, 100%; 5 min each) and air-dried. The hybridization mix was denatured at 90°C for 5 min and cooled rapidly. After incubating with the hybridization mix for 16h at 37°C in a moist chamber, slides were washed once in 50% formamide and 2×SSC for 5 min, three times in 2×SSC at 37°C (5 min each). Signal detection was performed using anti-digoxigenin-rhodamine (Roche) and fluorescein avidin DOS (Vector). Slides were counterstained with DAPI (4', 6-diamidino-2-phenylindole) in antifade solution (Vector). Microscopic analysis and capture of chromosome images were carried out using a Leica DM4000B microscope equipped with an epifluorescence system and the appropriate filter sets for fluorescein, rhodamine and DAPI as well as CCD camera. The signals were collected and processed with FISH software (Leica CW4000 CytoFISH Version Y 1.3.1). Karyotype analysis was carried out according to criteria defined by Levan et al. (1964).

Co-hybridization was conducted to investigate the relative chromosomal positions between each two fosmid clones. The protocol follows the same procedure of regular hybridization. And the hybridization mix with a total volume of 30μl contained 5-10ng/μl of each probe, 50% formamide, 10% dextran sulphate and 2×SSC.

Sequencing and sequence analysis

The partial sequences of genes which were successfully located by FISH were amplified and sequenced to make sure those are gene-containing fosmid clones. Primers

were published previously (Li et al. 2015, Ning et al. 2015, Wang et al. 2015, Zhao et al. 2015, Xing et al. 2016). Because the total sequence length of *Py*TRAF4 gene are over 40k, the plasmid of fosmid clone did not cover the entire *Py*TRAF4 sequence so that early published primers of this gene were not applicable for present study. Thus, primers of *Py*TRAF4 were redesigned using Primer5 software (Lalitha 2000). The amplification mixture contained 50ng genomic DNA, 0.2μM of each primers, 0.2μM of dNTP, 2mM $MgSO_4$ and 0.5U Platinum Taq DNA Polymerase. Cycling conditions were as follow: 2 min at 94°C (denaturation); 30 cycles of 15s at 94°C, 30s at 60°C, and 1min/kb at 68°C for extending. Detailed information about the primers can be found in Table 2. The products were purified with SanPrep PCR products purify kit (Sangon Biotech) for double end-DNA sequencing by ABI3730. Sequences were subjected to sequence similarity searches using BLASTN.

Results

FISH mapping

In total 19 genes were used for fosmid clones searching. Eight of them were managed to find matched fosmid clones (Table 1). Positive and stable FISH signals were observed in at least 25 analyzed metaphases for each probe. After FISH, 7 of the 8 fosmid clones were successfully mapped to chromosomes of the Yesso scallop (Table 1). Wherein, Clone PF126M18 was mapped to a single metacentric chromosome pair 1 on interstitial positions. (Fig. 1a). Clone PF123H24 was mapped to a single submetacentric chromosome pair 8 on interstitial positions (Fig. 1b). Interestingly, both clone PF109F4 and PF106B20 showed its loci on 3 chromosomes pairs and both have two pairs of loci located on the telomeric position of subtelocentric chromosome pair 11, 13 (Fig. 1c, d). However, the third pair of loci of PF109F4 were found on the telocentric chromosome pair 18, that of PF106B20 were proven to be located on the subtelocentric chromosome pair 14. Clone PF118H7, PF123J11 and PF120E14 were respectively located on a single subtelocentric chromosome pair (Fig. 1e, f, g).

In previous study, clusters of 18S-28S rDNA were localized on 2 different pairs of subtelocentric chromosomes of the Yesso scallop (Huang et al. 2007a). We found some of the mapping results of clone PF106B20 and PF109F4 were highly similar with that of the 18S-28S rDNA, therefore, these two fosmid clones were separately co-hybridized with 18S-28S rDNA. As in Fig. 2a, b, the positions of signals of rDNA obviously matched two pairs of signals of PF106B20 or PF109F4 and the color of red blended with green emerging a yellow look. This suggested that the sequences of clone PF106B20 or PF109F4 were partially similar with 18S-28S rDNA sequences and the extra signals represented the chromosome loci of gene *Py*Myd88-3 and *Py*NFkB.

In order to find out the chromosome position relations of the genes which were successfully mapped, karyotype analysis of FISH results were carried out firstly (Fig. 1). The results showed clones PF126M18, PF123H24 and PF109F4 were located on a

Table 1. Information of genes and fosmid clones and FISH mapping results.

Clone name	Gene name	Chromosome type*	Location of signals
PF118H7	*Py*MKK7	st	On telomeric region of 14q
PF123J11	PyMyd88-1	st	On middle region of 14q
PF106B20	PyMyd88-3	st	On telomeric region of 11p, 13p and 14q
PF123H24	PyTRAF7	sm	On centromeric region of 8q
PF109F4	PyNFkB	t	On telomeric region of 11p and 13p, centromeric region of 18q
PF120E14	*Py*TRAF4	st	On telomeric region of 12q
PF126M18	PyTNFR	m	On middle region of 1q
PF118E11	*Py*TRAF2	N/A	No positive signals were found

*m: metacentric, sm: submetacentric, st: subtelocentric, t: telocentric

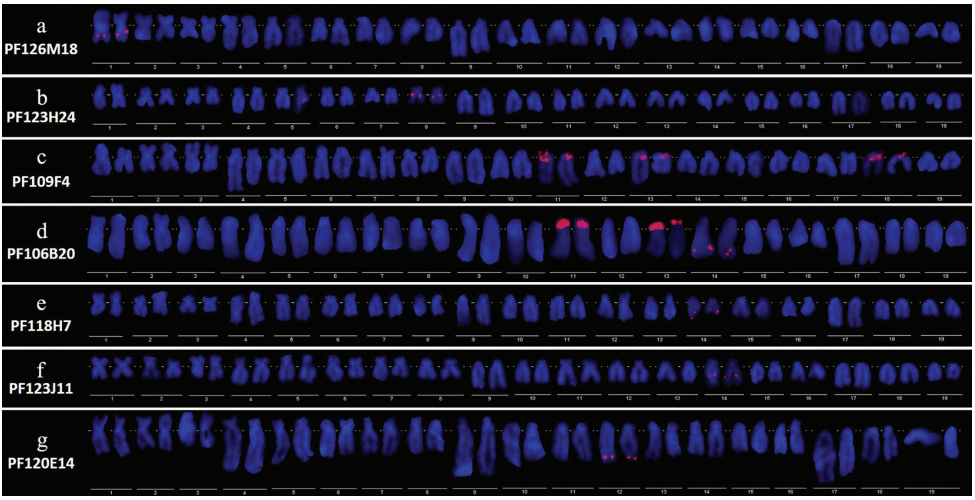

Figure 1. Karyotype analysis results of mapped gene-containing fosmid clones: PF126M18 (**A**); PF123H24 (**B**); PF109F4 (**G**); PF106B20 (**D**); PF118H7 (**E**); PF123J11 (**F**); PF120E14 (**G**). Chromosome numbering is based on chromosome type and relative length. Wherein, chromosome pair 1, 2, 3 represent the metacentric (m) chromosomes. Chromosome pair 4, 5, 6, 7, 8 represent the submetacentric (sm) chromosomes. Chromosome pair 9, 10, 11, 12, 13, 14, 15, 16 represent the subtelocentric (st) chromosomes. Chromosome pair 17, 18, 19 represent the telocentric (t) chromosomes.

pair of metacentric chromosomes, a pair of submetacentric chromosomes and a pair of telocentric chromosomes respectively (Fig. 1a, b, c). Thus gene *Py*TNFR, *Py*TRAF7 and *Py*NFkB must not cluster with other mapped genes according to the significant morphological differences of those chromosomes.

For those fosmid clones whose position relationship couldn't justified by karyotype, co-hybridization was conducted (Fig. 2). As it can be seen in Fig. 2c, d, clone PF106B20, PF123J11, PF118H7 were mapped to the long arms of the same pair of subtelocentric chromosomes which indicated *Py*Myd88-3, *Py*TRAF7, *Py*MKK7 be-

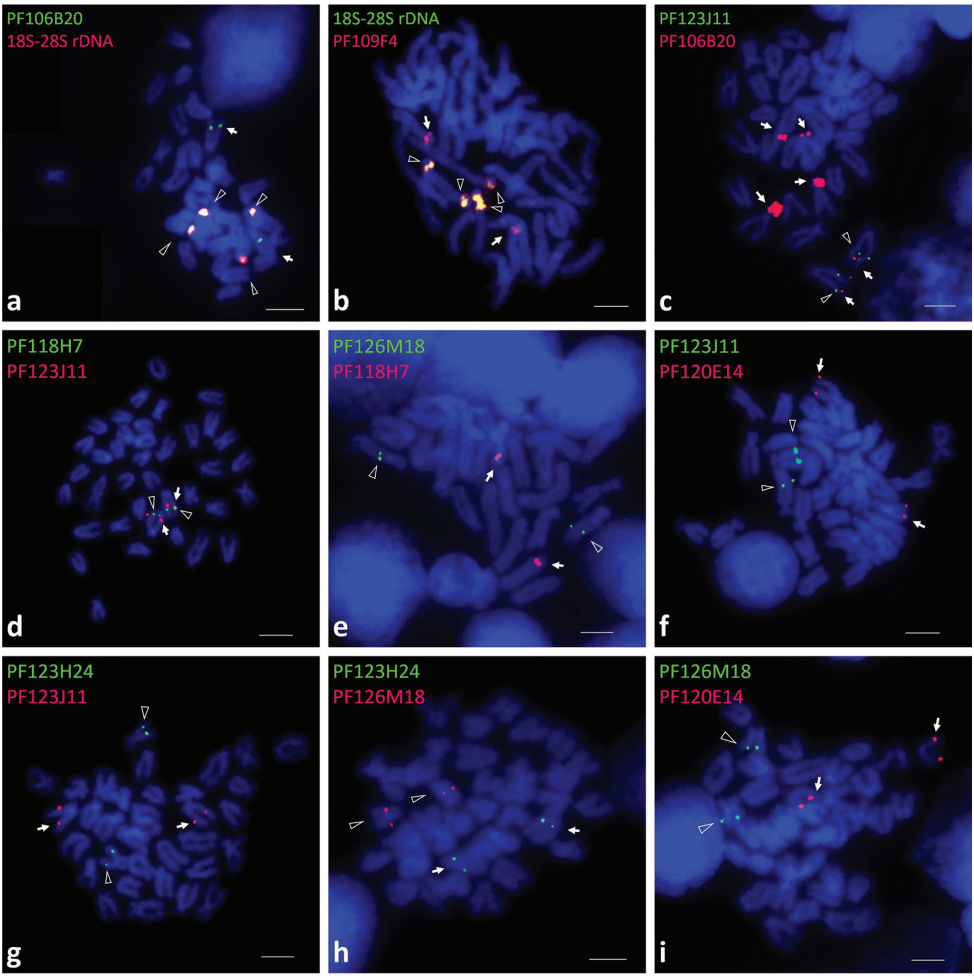

Figure 2. Co-hybridization results of fosmid clones with positive signals on mitotic metaphase chromosomes of *P. yessoensis* (A-I): Mapping of clone PF106B20 & 18S-28S rDNA (**A**), clone PF109F4 &18S-28S rDNA (**B**), clones PF123J11 & PF106B20 (**C**), clones PF118H7 & PF123J11 (**D**), clones PF126M18 & PF118H7 (**E**), clones PF123J11 & PF120E14 (**F**), clones PF123H24 & PF123J11 (**G**), clones PF123H24 & PF126M18 (H), clones PF126M18 & PF120E14 (I). The arrows and the open triangles indicate positive signals of different probes. Bars=10 μm.

longed to the same chromosome pair. Moreover, the co-hybridization results showed the positive signals of clone PF126M18 (*Py*TNFR), PF120E14 (*Py*TRAF4) and PF123H24 (*Py*TRAF7) all distributed in the different chromosome pair with clone PF123J11 or PF118H7 (Fig. 2e, f, g). Clone PF126M18 was also separately proven to be located in different chromosome pair with clone PF123H24 and PF120E14 (Fig. 2h, i). As a result, besides of clone PF106B20, PF123J11, PF118H7, no other clones were found to be mapped to the same chromosomes.

Table 2. Primers used for PCR and BNALSN results of the mapped clones.

Gene name (Clone name)	Primer	Primer sequence(5'-3')	Identities
*Py*Myd88-1 (PF123J11)	F- PF123J11 R- PF123J11	TTGCACATGCTCTGTCGCC GGACGCAGTTCGCTTTTGAT	679/684 (99%)
PyMyd88-3 (PF106B20)	F- PF106B20 R- PF106B20	GAGTGTCGAGTGCGACTTCATG CGCCTTCAGTAGACGTTTCCAG	944/946 (99%)
*Py*TRAF7 (PF123H24)	F- PF123H24 R- PF123H24	CCAGATTGTCACGCTGAAAGG CCAGATTGTCACGCTGAAAGG	76/77 (99%)
*Py*MKK7 (PF118H7)	F- PF118H7 R- PF118H7	TCAAAGGCTAAGACGAGGAGTGC CAACCAATGTGATGCCCAGG	773/779 (99%)
PyNFkB (PF109F4)	F- PF109F4 R- PF109F4	TGCCCGTGTTGTGGTAACCTTGG CGTGAGAGAGTTTTGTCCGCCCTT	90/92 (98%)
PyTNFR (PF126M18)	F- PF126M18 R- PF126M18	AACAACCTACCTGAAACGGAACA CGGTTAGGATTTGGACAAGGAC	54/56 (96%)
PyTRAF4 (PF120E14)	F- PF120E14 R- PF120E14	GGACTTATCGCTGTCAACC AAATGTGGCACCTTACTCG	187/189 (99%)

Sequencing results

The partial sequences of genes from the 7 mapped fosmid clones were amplified and products were successfully sequenced. A BLASTN analysis of the 7 sequences against the *P. yessoensis* genome sequencing data showed significant sequence matches as we expected and confirmed the existence of the genes in the fosmid clones (Table 2).

Particularly, because of the similarity between mapping results of clone PF106B20 and 18S-28S rDNA, PF109F4 and 18S-28S rDNA, plasmids of these two clones were used as template for amplification of 18S-28S rDNA. The obtained PCR products were successfully sequenced and the sequences were identical to the 18S-28S rDNA sequence of the Yesso scallop.

Discussion

The innate immune system plays a vital role in scallop species defending against various invaders because of lacking of adaptive immunity (Song et al. 2015). Focusing on studying such subject would not only provide us insights of molecular mechanism of the strategies that scallop species take for immune defense, but also play a guiding role in scallop farming industry. In 2015, a series of studies which genome-widely identified and characterized immune-related genes in the Yesso scallop were published (Li et al. 2015, Ning et al. 2015, Wang et al. 2015, Zhang et al. 2015, Zou et al. 2015). There is no doubt these studies provide insights into the versatile roles and responses of genes like TRAF genes, Myd88 genes, MKK genes and so on in the innate immune system against Gram-negative bacterial pathogens in bivalves. In addition, by conducting phylogenetic analyses, such studies also investigated evolutionary relationships of immune-related genes of *P. yessoensis*. Compared with genes from other invertebrate

and vertebrate species, it was shown that most of immune-related genes of the Yesso scallop like *Py*Tollip, *Py*NFkB, *Py*MKKs, *Py*TRAFs and so on were found to have relatively conserved structural features. Indubitably, the phylogenetic tree provided valuable information such as orthologous and paralogous relations among genes. At the same time, investigation of chromosomal distribution pattern of genes is another approach to gain a better understanding of gene evolutionary issue.

In present study, *Py*NFkB, *Py*TRAF4, *Py*TRAF7, *Py*Myd88-1, *Py*Myd88-3, *Py*MKK-7 and *Py*TNFR genes were successfully mapped to chromosomes of the Yesso scallop. After co-hybridization, *Py*MKK-7, *Py*Myd88-1 and *Py*Myd88-3 were found to be located on the same chromosome pair with very adjacent positions. Previous studies have shown that genes with similar expression patterns tend to cluster more frequently than those with different expression patterns (Chen et al. 2010, Liu and Han 2009). And looking back to the relative expression level of *Py*MKK-7, *Py*Myd88-1 and *Py*Myd88-3 genes after Gram-negative (*V. anguillarum*) bacterial challenge, it was obvious that the expression levels of all these three genes went through the pattern of first dropping and then increasing (Ning et al. 2015, Zou et al. 2015). Combining the adjacent chromosomal location and expression patterns of these three genes, we proved the hypothesis that the genes clustered on short distance along chromosomal may have similar expression patterns.

The genome-wide identification of five *Py*MyD88 duplication genes in the Yesso scallop showed that all five genes were located on the same linkage group. In present study, fosmid clones of two *Py*MyD88 genes (*Py*Myd88-1 and *Py*Myd88-3) were pinpointed and were successfully mapped to the same chromosome pair of the Yesso scallop. What is more, *Py*MKK7 gene was found to locate on the adjacent chromosomal position with that of *Py*MyD88 genes. It was thought that the higher number of *Py*MyD88 duplications in the Yesso scallop may be relevant to their specific and cooperative functions in the corresponding innate immune system and in *Py*MyD88-dependent and independent pathways against bacterial infection (Ning et al. 2015). With the present outcome, it seems *Py*MKK7 gene may also have cooperative function with *Py*MyD88 genes in the Yesso scallop.

According to genome sequencing data (BioProject number PRJNA259405), *Py*MKK-7, *Py*Myd88-1 and *Py*Myd88-3 genes were contained in three different scaffold, which were scaffold7441 (*Py*Myd88-1), scaffold5789 (*Py*Myd88-3), scaffold11045 (*Py*MKK-7) respectively. Based on the established SNP linkage map (unpublished), scaffold7441 and scaffold5789 belonged to the linkage group 1. However, which linkage group was scaffold11045 distributed to was not clear. Through co-hybridization, the present study successfully mapped *Py*MKK-7 and *Py*Myd88-1 genes into the same chromosome pair and anchored scaffold11045 to the linkage group 1 just like scaffold7441 and scaffold5789 which helped genome assembly of the *P. yessoensis*.

Apart from the three genes mapped in the same chromosome pair mentioned above, no other genes were found to have the similar pattern in the present study. In the Zhikong scallop, five key TLR signaling pathway genes (*Cf*TLR, *Cf*Myd88, *Cf*TRAF6, *Cf*NFkB and *Cf*IkB) were mapped in five non-homologous chromosome pairs which was similar with the FISH results of *Py*TRAF7, *Py*NFkB, *Py*TRAF4 and *Py*TNFR

Table 3. Chromosomal localization of immune-related genes in five model organisms.

Organism	NFkB	Myd88	MKK	TNFR	TRAF4	TRAF7
Homo sapiens	NFkB2(4791*) Chr. 10	MyD88 (4615*) Chr. 3	MKK7(5609*) Chr. 19	TNFRSF18(8784*) Chr. 1	TRAF4(9618*) Chr. 17	TRAF7(84231*) Chr. 16
Drosophila melanogaster	Dorsal(35047*) Chr. 2L	MyD88(35956*) Chr. 2R	MKK7(32256*) Chr. X	TNFR(32849*) Chr. X	TRAF4(33638 *) Chr. 2L	N/A
Danio rerio	NFkB3(425099*) Chr. 7	MyD88(403145*) Chr. 24	MKK7(560913*) Chr. 1	N/A	TRAF4b(404035 *) Chr. 21	TRAF7(563746*) Chr. 3
Mus musculus	NFkB3(5970*) Chr. 11	MyD88(17874*) Chr. 9	MKK7(26400*) Chr. 8	TNFRSF1B(21938*) Chr. 4	TRAF4(22032 *) Chr.11	TRAF7(224619*) Chr. 17
Gallus gallus	NFkB2(386574*) Chr. 6	MyD88(420420*) Chr. 2	N/A	TNFRSF1B(395083*) Chr. 21	TRAF4(417577*) Chr. 19	TRAF7(416555*) Chr. 14

* Gene ID in NCBI GENE database; Chr.: Chromosome.

(Zhao et al. 2015). What's more, for comparison purpose, we obtained the distribution information of same immune-related genes in five model species (Table 3) through the NCBI database (NCBI Map Viewer, http://www.ncbi.nlm.nih.gov/mapview/). In four species, all the known NFkB, Myd88, MKK, TNFR and TRAF genes locate on the non-homologous chromosome pairs. Only in *Drosophila melanogaster* Meigen, 1830, there are genes locating on same chromosome pairs, such as gene Dorsal and TRAF4 are on the chromosome 2L and gene MKK7 and TNFR are on the chromosome X. Thus, as it established, the candidate immune genes NFkB, Myd88, MKK, TNFR and TRAF are distantly linked in the not only scallops but also other species. This suggested such phenomenon might be universal among organisms.

Moreover, fosmid clone PF109F4 and PF106B20 were mapped to three different chromosome pairs separately. The FISH mapping results of the two clones both showed two pair positive signals located on two subtelocentric chromosome pairs which were quite alike with the FISH mapping image of the 18S-28S rDNA. With PCR as well as sequencing results, it was proved that these two clones did contain partial 18S-28S rDNA sequence. The average insert fragment of fosmid clones applied within this ranged from 30kb to 45kb. As a result, beside covered the target sequences that needed for FISH mapping, it inevitably included other sequences from the Yesso scallop genome. Early study showed 18S-28S rDNA sequence was highly repetitive and also had quite high hybridization efficiency during FISH. As a result, possessing partial homologous sequences of 18S-28S rDNA was believed to be the reason of appearance of multiple positive signals using clone PF109F4 and PF106B20 as probes. The co-hybridization result of PF109F4 and 18S-28S rDNA, PF106B20 and 18S-28S rDNA also proved these phenomena.

FISH is a powerful tool for chromosomal localization of DNA sequences and have already been used for cytogenetic study in scallop species. Comprehensively speaking, published study were mostly focusing on the multi-copy sequencing mapping such as histone H3 gene , ribosomal genes and satellite DNA (Leitão and Chaves 2008, Huang et al. 2006, 2007b, Zhang et al. 2007, Feng et al. 2014). In Zhikong scallop, five Toll-Like receptor signaling pathway genes were mapped into five non-homologous chromosomes and hereby, it was considered that the co-expression of TLR signaling pathway genes in the *C. farreri* may not act in a distance-dependent way (Zhao et al. 2015).

The present study revealed chromosomal distribution pattern of seven immune-related genes and enriched the number of chromosome markers in the Yesso scallop. These results will lay a foundation for the upcoming genome and cytogenetic research in *P. yessoensis*.

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RESEARCH ARTICLE

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Towards a FISH-based karyotype of *Rosa* L. (Rosaceae)

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Abstract

The genus *Rosa* Linnaeus, 1753 has important economic value in ornamental sector and many breeding activities are going on supported by molecular studies. However, the cytogenetic studies of rose species are scarce and mainly focused on chromosome counting and chromosome morphology-based karyotyping. Due to the small size of the chromosomes and a high frequency of polyploidy in the genus, karyotyping is very challenging for rose species and requires FISH-based cytogenetic markers to be applied. Therefore, in this work the aim is to establish a FISH-based karyotype for *Rosa wichurana* (Crépin, 1888), a rose species with several benefits for advanced molecular cytogenetic studies of genus *Rosa* (Kirov et al. 2015a). It is shown that FISH signals from 5S, 45S and an *Arabidopsis*-type telomeric repeat are distributed on five (1, 2, 4, 5 and 7) of seven chromosome pairs. In addition, it is demonstrated that the interstitial telomeric repeat sequences (ITR) are located in the centromeric regions of four chromosome pairs. Using low hybridization stringency for ITR visualization, we showed that the number of ITR signals increases four times (1–4 signals). This study is the first to propose a FISH-based *R. wichurana* karyotype for the reliable identification of chromosomes. The possible origin of *R. wichurana* ITR loci is discussed.

Keywords

Cytogenetic markers, fluorescence in situ hybridization, interstitial telomeric repeat (ITR), 5S rDNA, 45S rDNA, *Rosa wichurana*

Introduction

Rosa Linnaeus, 1753 is an economically important ornamental genus belonging to the Rosaceae. Of the approximately 200 described *Rosa* species (Wissemann and Ritz 2005), only 8 to 15 species contributed to the original germplasm of the modern rose cultivars. *Rosa* is one of the most widely cultivated ornamental plants worldwide, but few basic molecular cytogenetic studies in *Rosa* have been performed, including chromosome counts and karyotyping (Wylie 1954, Price et al. 1981, Liu and Li 1985, Subramanian 1987, Ma et al. 1997, Fernandez-Romero et al. 2001, Akasaka et al. 2002, 2003, Jian et al. 2013a, 2013b). Performing molecular cytogenetics in roses is a big challenge due to their very small genome size (the diploid genome size is 0.83 to 1.30 pg/2C, Roberts et al. 2009), small chromosomes (Kirov et al. 2014a), low mitotic index in roots and shoots, and weak root development (Ma et al. 1996). Moreover, most wild roses are polyploids (Vamosi and Dickinson 2006), ranging from diploid (2n = 2x = 14) to decaploid (2n = 10x = 70) (Roberts et al. 2009, Jian et al. 2010).

Rosa wichurana (Crépin, 1888) is a valuable model species for molecular cytogenetic studies in *Rosa* genus (Kirov et al. 2015b). It is a diploid species (2n = 2x = 14) with suitable apical and root meristems that can be used for chromosome preparations. *Rosa wichurana* is involved in the origin of modern rose cultivars and is one of the parental species used for the construction of several rose genetic maps (Crespel et al. 2002, Dugo et al. 2005, Shupert et al. 2007, Spiller et al. 2011, Moghaddam et al. 2012). To increase the efficiency of FISH experiments, we recently developed the "SteamDrop" protocol for the preparation of high quality chromosome slides (Kirov et al. 2014b). Using this "SteamDrop" protocol and Tyramide-FISH it was possible to physically map several single-copy genes on the mitotic and meiotic chromosomes of *R. wichurana* (Kirov et al. 2014a, Kirov et al. 2015a) and to anchor three linkage groups of the genetic map (Moghaddam et al. 2012) to three *R. wichurana* chromosomes.

Because the chromosomes are difficult to distinguish, further progress in cytogenetic mapping depends on the development of cytogenetic markers useful for chromosome identification. The conservative tandemly organized repetitive sequences 5S and 45S rRNA genes are valuable sources of cytogenetic markers, and have been used for chromosome identification in many plant species including *Rosa* species (Ma et al. 1997, Fernandez-Romero et al. 2001, Akasaka et al. 2002, 2003, Lim et al. 2005, Jian et al. 2012, Kirov et al. 2014a). Other conservative repeats, such as the *Arabidopsis*-type telomeric repeat (Fuchs et al. 1995, He et al. 2013) might be used for chromosome identification. Typically, telomeric repeats (TRs) occupy the end (telomere) of the chromosomes (Fuchs et al. 1995). However, the location of TRs on plant chromosomes is not restricted to the telomere ends and telomere-like sequences have been found in centromeric, subtelomeric and interstitial regions in several genera (Fuchs et al. 1995, Uchida et al. 2002, Tek and Jiang 2004, Mlinarec et al. 2009, Mandakova et al. 2010, Gong et al. 2012, He et al. 2013, Sousa et al. 2014). The unique position of these interstitial telomeric repeats (ITRs) on some chromosomes and their high copy number make them valuable cytogenetic markers. The position of ITR on chromosomes can also reflect ancient chromosomal rearrangement as telomeric sequences and

their remnants are involved in chromosomal rearrangements via illegitimate recombination between centromeric/telomeric repeats (Murat et al. 2010) and can be associated with fragile sites of chromosomes (Grabowska-Joachimiak et al. 2015). In addition, the chromosomal location of ITR can be used to detect descending dysploidy (Sousa and Renner 2015).

Development of an effective cytogenetic marker system is an important step in answering many biological questions (Jiang and Gill 2006). FISH-based markers have shown their effectiveness and ease-to-use. The modern methods of probe labeling and the application of directly labeled oligonucleotides make FISH-based chromosome identification a robust and fast procedure (Kato et al. 2004, Fu et al. 2015, Tang et al. 2014, Cuadrado et al. 2009). Up-to-date FISH based karyotyping was established for many plant species including wheat, maize, rice, soybean, common bean and others (Cheng et al. 2001, Kato et al. 2004, Findley et al. 2010, Iwata-Otsubo et al. 2015). Cytogenetic markers are widely used to trace individual chromosomes in hybrids accelerating transferring of desirable traits from wild relatives (Szinay et al. 2010). FISH-based karyotyping is used to shed light on speciation and allopolyploid formation (Badaeva et al. 2016). And a relatively new application came with the development of a FISH-based chromosome sorting procedure, allowing individual chromosome identification, sorting and further sequencing (Giorgi et al. 2013). These and other applications clearly demonstrate the importance of having a system of cytogenetic markers enabling chromosome identification.

This study aims to explore the opportunities of ITRs, 5S and 45S rDNA as cytogenetic markers allowing to distinguish individual chromosomes of *Rosa*. FISH with 5S rDNA, 45S rDNA and the *Arabidopsis*-type telomeric repeat was performed. These FISH results were combined with chromosome morphology measurements (Kirov et al. 2014a), in order to identify all seven mitotic chromosomes of *R. wichurana*. In addition, we also attempted to identify pachytene bivalents by FISH using the 45S rDNA and *Arabidopsis*-type telomeric repeat probes.

Materials and methods

Plant material

Rosa wichurana plants were grown in the field. For chromosome slide preparations, cuttings were made. Rooted cuttings were transferred to terracotta stone pots and grown in the greenhouse (moderate climatic conditions, East Flanders, Belgium). To prepare mitotic chromosome slides, young meristems were harvested. For meiotic (pachytene) chromosome slides, flowers buds with a hypanthium size of 3 mm were harvested.

Probe labeling

Plasmids containing 5S rRNA genes of rye (pSCT7, Lawrence and Appels 1986) and 45S rRNA genes of wheat (pTA71, Gerlach and Bedbrook 1979) were labeled by Digoxigenin-

and Biotin- Nick Translation Mix (Roche, Germany), respectively, according to the manufacturer's protocol. The *Arabidopsis*-type telomere repeat $(CCCTAAA)_3$, labeled by TAMRA at the 5' end (Syntol, Russia) was used.

Chromosome preparation and fluorescence *in situ* hybridisation

Pachytene and mitotic chromosomes were prepared according to the "SteamDrop" protocol (Kirov et al. 2014b).

For FISH we used the protocol described in Heslop-Harrison et al. (1991) with some modifications. Briefly, slides were incubated overnight at 37°C. Chromosomes were pretreated with 4% paraformaldehyde in 2xSSC (pH 8.3–8.5) for 6 min and dehydrated in ethanol (70%, 90% and 100%). Hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 2xSSC, 0.25% sodium dodecyl sulphate, 2.00 ±1.00 ng/µl probe DNA. The mixture was denatured at 75°C for 5 min, placed on ice for 5 min and 60 µl was applied on each slide. Slides were denaturated at 75°C for 5 min and incubated in a humid chamber for 15–16 hours at 37°C (the common hybridization condition) or at 23–25°C (the low stringency hybridization condition). For stringency washing 0.1xSSC solution was used at 48°C (2 times 7 minutes). Biotin and digoxigenin labeled probes were detected by Streptavidin-Cy3 (Sigma-Aldrich, USA), diluted 1:200 in TNB buffer, and anti-digoxigenin-FITC (Roche, Germany), diluted 1:200 in TNB buffer, respectively.

For sequential FISH experiments, the slides were washed in the series of ethanol (70%, 90% and 100%) after the first round of FISH and then the above-mentioned FISH procedure was applied.

Microscopy and image analysis

Images were acquired using a Zeiss AxioImager M2 fluorescence microscope (400× and 1000× magnification) equipped with an AxioCam MRm camera and Zen software (Zeiss, Belgium). Final image adjustments were performed using Photoshop (Adobe Inc., USA). Measurements of chromosome lengths and karyotyping was done in MicroMeasure version 3.2 (Reeves and Tear 2000) for at least 10 well-spread metaphases.

Results

FISH using Arabidopsis-type telomere repeat, 5S rDNA and 45S rDNA allows unambiguous identification of 3 *Rosa wichurana* mitotic chromosomes

FISH using the common hybridisation temperature of 37°C with 45S rDNA revealed a signal on chromosome 7, while the *Arabidopsis* type telomere-based probe hybridized on chromosome 5 (Fig. 1A).

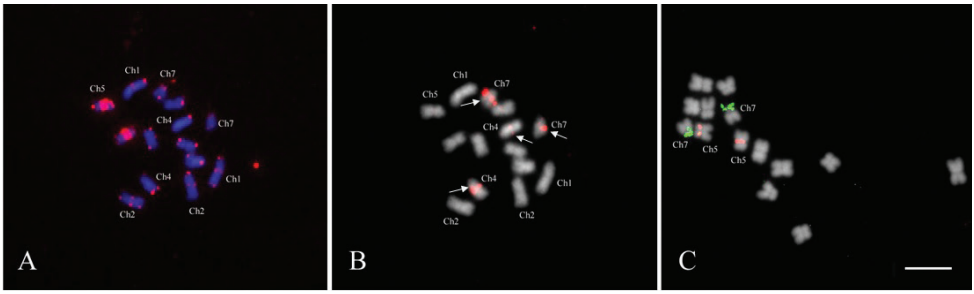

Figure 1. FISH on the chromosomes of *R. wichurana*. **A** FISH with *Arabidopsis*-type telomere probe (red) and 45S (green) under hybridization at 37°C **B** FISH with *Arabidopsis*-type telomere probe under the low hybridization stringency condition (23-25°C). Arrows indicate the major ITRs on chromosome 5 and arrowheads show the ITRs which are visible under the low hybridization stringency condition **C** The same metaphase as in 1B rehybridized with 5S rDNA under the common hybridization stringency (37°C). Arrows indicate the 5S rDNA signals. Sacale bar: 5 μm.

To further evaluate the value of the telomeric repeat (TR) as a cytogenetic marker, FISH was carried out at room temperature (the low hybridization temperature). We observed the *Arabidopsis*-type TR signals on all chromosome ends (Fig. 1B). Besides the telomeric signals, a bright fluorescent signal in the centromeric region on chromosome 5 and weak signals in the centromeric region on three other chromosomes 1, 2 and 7 were observed. Remarkably, the weak centromeric signals on chromosomes 1, 2 and 7 were not observed when performing a hybridization at 37°C (Fig. 1A). No ITRs were present on chromosomes 3, 4 and 6. FISH with 5S rDNA using the common hybridization temperature of 37°C showed fluorescent signals on the long arm of chromosomes 4 and 7 (Fig. 1C) but the signal frequency across the metaphases was low (20–40%).

Sequential FISH at the low hybridization temperature with the *Arabidopsis*-type telomere-based probe and 5S rDNA showed co-localization of these signals on chromosome 7. We also performed double-color FISH with the *Arabidopsis*-type telomere repeat-based probe and the 45S rDNA probe under the low temperature of hybridization (Fig. 2) which confirmed the identification of four (1, 2, 5 and 7) out of seven chromosomes.

A summary of the karyotypic features and distribution of FISH probes is given in Fig. 3. Taken together, three chromosomes (4, 5 and 7) of *R. wichurana* could be unambiguoulsy identified by 5S rDNA, 45S rDNA and the *Arabidopsis*-type TR using common FISH hybridisation conditions (Fig. 3).

All the other chromosomes can only be distinguished at this time based on their morphological parameters. Differentiation between chromosome 1 and 2 is possible by their centromeric indices which are 46.00 ±1.2% and 40.30 ±1.3%, respectively (Kirov et al. 2014a) and by the presence of an ITR when using FISH at low temperature hybridization conditions. Chromosomes 3 and 6 have centromeric indices on the level of 44.3 ±1.0% and 41.8 ±1.1%, respectively (Kirov et al. 2014a). However, these chromosomes still remain very difficult to distinguish from each other.

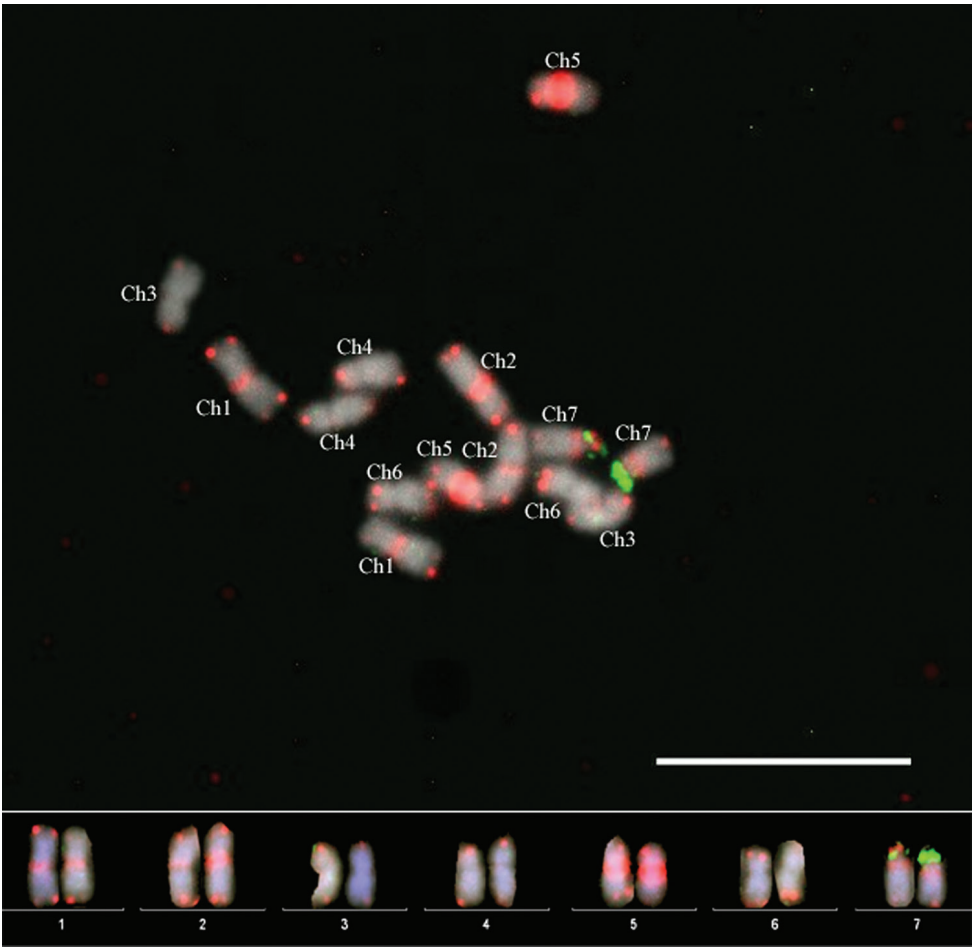

Figure 2. Double-color FISH under the low hybridization conditions using the *Arabidopsis*-type telomere repeat-based (red) and 45S rDNA (green) probes to *R. wichurana* mitotic chromosomes. Scales bar: 10 μm.

ITRs are located on the centromere of chromosome 5

FISH experiments with 5S rDNA, 45S rDNA, and the *Arabidopsis*-type TR on rose pachytene chromosomes provide a much higher resolution of the mapped sequences. 5S rDNA-FISH on pachytene chromosomes did not reveal any reliable signals, while FISH with the 45S rDNA probe resulted in a clear signal at the subtelomeric region of the NOR-bearing chromosome (Fig. 4). FISH with the *Arabidopsis*-type TR probe resulted in signals on all ends of pachytene chromosomes and one bright signal on the centromeric region of chromosome 5 (Fig. 4). Since centromeres of rose pachytene bivalents are clearly visible after DAPI staining as being the weakest part of the chromosomes (Kirov et al. 2015a), comparison between the DAPI stained chromosomes (Fig. 4B') and the ITR signal positions (Fig. 4A') revealed that the ITRs are located exactly on the centromere of chromosome 5.

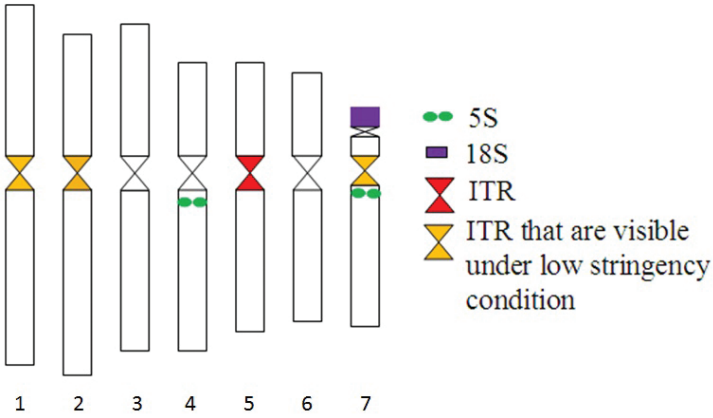

Figure 3. Distribution of the repetitive sequences on the mitotic *R. wichurana* chromosomes. [1] – ITR1: signals that are visible under hybridization at 37°C as well as at low temperature (23–25°C). [2] – ITR2: signals that are visible only under hybridization at low temperature (23–25°C).

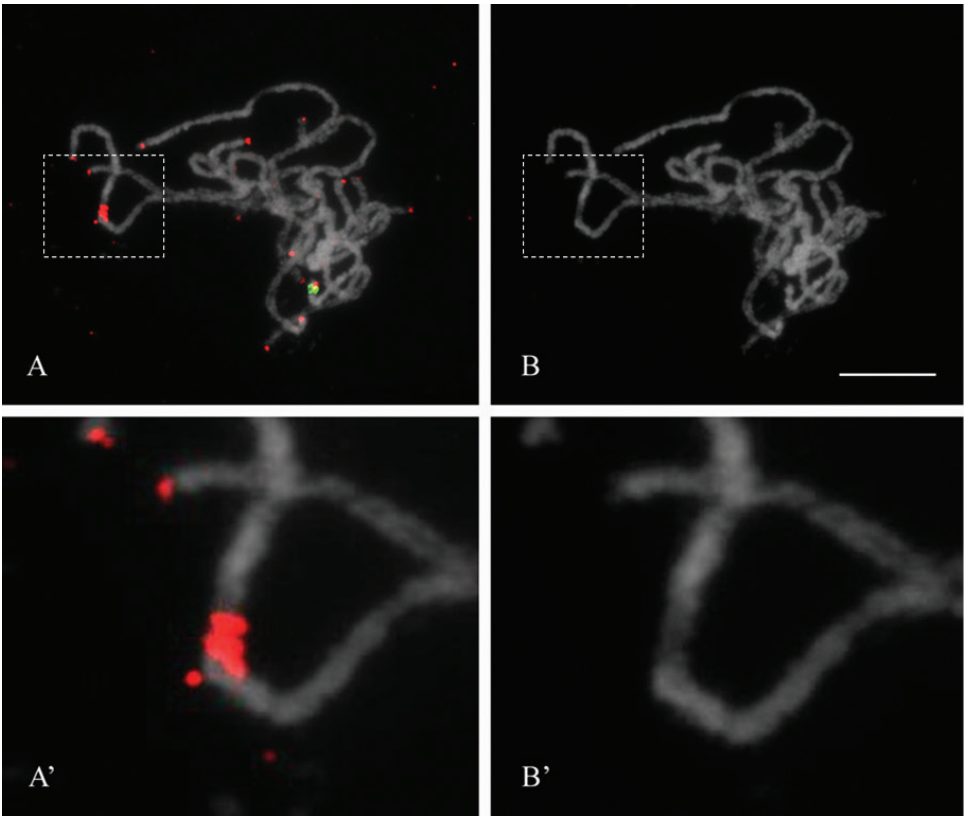

Figure 4. High resolution physical mapping of ITR on *R. wichurana* pachytene chromosomes. FISH with the *Arabidopsis*-type telomere repeat probe (red) and 45S (green). Merged (**A**) and the DAPI gray scale (**B**) pictures are shown. FISH was performed under the low hybridization stringency condition. Dotted lines show the regions that were digitally enlarged (A' and B'). Scales bar: 5 µm.

Discussion

Rosa mitotic and meiotic chromosomes are difficult to distinguish by common karyotype analysis (Kirov et al. 2014, Kirov et al. 2015a). The development of cytogenetic markers is necessary for individual chromosome identification and further cytogenetic studies in *Rosa*. In our study, we positively evaluated the use of the conservative tandem repeats, *Arabidopsis*-type telomere, 45S and 5S probes, as FISH-based cytogenetic chromosome markers for *R. wichurana*. However, the 5S rDNA probe cannot be considered as a good cytogenetic marker for *R. wichurana* chromosomes due to the low reliability of the FISH-signals. Application of FISH with the 5S rDNA probe to chromosome slides prepared by an alternative method (spread protocol of Pijnacker and Ferwerda (1984)) and using FAM labeled 5S oligos or a *R. wichurana* 5S clone as probes, did not improve FISH results (data not shown). Thus the reason for weak 5S rDNA FISH signals on *R. wichurana* chromosomes remains unclear. FISH with the *Arabidopsis*-type TR under low hybridization conditions (hybridization at 23-25°C instead of 37°C) provided us an additional tool for identification of *Rosa* chromosomes.

In this study, FISH with the 45S rDNA and the *Arabidopsis*-type telomere probe, reliably identified 2 (chromosome 5 and 7) of the 7 pachytene bivalents of *R. wichurana*. These markers will accelerate the ongoing physical mapping of pachytene chromosomes of *R. wichurana* as their identification by morphological parameters or specific heterochromatin patterns is impossible (Kirov et al. 2015a).

ITRs can be used to trace ancient chromosomes rearrangements such as chromosome fusions, Robertsonian translocations and duplications resulting in dysploidy (Mandakova et al. 2010, Sousa et al. 2014). However, *Rosa* species have a basic chromosome number n = 7, suggesting that no descending dysploidy, which usually results in basic chromosome number changes, has occurred. Therefore, it seems unlikely that the observed ITRs are the indications of such chromosome fusions or translocations. ITRs might also be the traces of intrachromosomal rearragements implicating telomeres (e.g., inversions and duplications) (Murat et al. 2010). In our study, the *Arabidopsis* telomere-like motif was found in centromeric repeats of *Rosa wichurana*, as is also observed in several other genera (Tek and Jiang 2004, He et al. 2013, Emadzade et al. 2014). The FISH signal from ITRs on chromosome 5 is significantly stronger than those observed in the telomeres of *R. wichurana* chromosomes. Thus, we hypothesize that the occurrence of ITRs in the centromeric regions of *R. wichurana* chromosomes is the result of insertion of *Arabidopsis* telomere-like sequence into centromeric sequence followed by massive amplification of centromeric tandem repeat(s) containing an *Arabidopsis* telomere-like motif. To check this hypothesis identification of centromeric repeats of *R. wichurana* should be done (Tek and Jiang 2004). The events leading to insertion of ITR sequences into centromere are unknown.

Interestingly, FISH under the low hybridization temperature – and thus low stringency – revealed more chromosomes possessing the telomeric repeat compared to FISH performed under the common hybridization temperature. This result suggest that these chromosomes (1, 2 and 7) may contain truncated or diverged telomere motifs. As a consequence for our experiments, the telomeric probe may be much more informative as cytogenetic marker when hybridized at a lower temperature than at 37°C (Fuchs et al.

1995, Tek and Jiang 2004, Sousa et al. 2014, Sousa and Renner 2015). However, the application of ITR markers under the low-hybridization stringency and simultaneous mapping of other probes (e.g. genes) can be challenging as non-specific hybridization signals may occur due to low stringency. In this case sequential FISH can be applied.

High-resolution FISH on pachytene chromosomes with the telomere probe resulted in a signal in the centromere of chromosome 5, indicating that the telomere-like motifs may be the components of the *R. wichurana* functional centromere as it has been shown for potato (Tek and Jiang 2004).

This is the first report describing valuable cytogenetic markers for four mitotic chromosomes and two pachytene bivalents of *R. wichurana*. Moreover, by combining our FISH results with the chromosome morphology measurements (Kirov et al. 2014a), all 7 mitotic chromosomes of *R. wichurana* could be identified. Because *R. wichurana* has many advantages as a model species for cytogenetic studies of the *Rosa* genus, the development of a complete set of cytogenetic markers should facilitate the physical mapping of its genome. Designing new DNA probes based on NGS data covering all chromosomes of *R. wichurana* is a scope for our future research. These markers will be indispensable for high-resolution physical mapping experiments (Kirov et al. 2015a) that are currently ongoing for this species.

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RESEARCH ARTICLE

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Co-located 18S/5S rDNA arrays: an ancient and unusual chromosomal trait in Julidini species (Labridae, Perciformes)

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Abstract

Wrasses (Labridae) are extremely diversified marine fishes, whose species exhibit complex interactions with the reef environment. They are widely distributed in the Indian, Pacific and Atlantic oceans. Their species have displayed a number of karyotypic divergent processes, including chromosomal regions with complex structural organization. Current cytogenetic information for this family is phylogenetically and geographically limited and mainly based on conventional cytogenetic techniques. Here, the distribution patterns of heterochromatin, GC-specific chromosome regions and Ag-NORs, and the organization of 18S and 5S rDNA sites of the Atlantic species *Thalassoma noronhanum* (Boulenger, 1890), *Halichoeres poeyi* (Steindachner, 1867), *Halichoeres radiatus* (Linnaeus, 1758), *Halichoeres brasiliensis* (Bloch, 1791) and *Halichoeres penrosei* Starks, 1913, belonging to the tribe Julidini were analyzed. All the species exhibited 2n=48 chromosomes with variation in the number of chromosome arms among genera. *T. noronhanum* has 2m+46a, while species of the genus *Halichoeres* Rüppell, 1835 share karyotypes with 48 acrocentric chromosomes. The *Halichoeres* species exhibit differences in the heterochromatin distribution patterns and in the number and distribution of 18S and 5S rDNA sites. The occurrence of 18S/5S rDNA syntenic arrangements in all the species indicates a functionally stable and adaptive genomic organization. The phylogenetic sharing of this rDNA organization highlights a marked and unusual chromosomal singularity inside the family Labridae.

Keywords
Chromosome evolution, *Halichoeres*, rDNA, syntenic genes, wrasses

Introduction

Wrasses (Labridae) are one of the most abundant and ecologically diversified fish groups in tropical reefs (Choat and Bellwood 1998). Their biodiversity is highlighted by nine tribes with 82 genera and 530 species (Westneat and Afaro 2005, Eschmeyer and Fong 2016), which exhibit extensive biological interactions in tropical reefs and temperate regions around the world (Choat and Bellwood 1998, Wainwright et al. 2004, Nelson 2006).

Cytogenetic analyses in Labridae have revealed particular trends in the karyotypic evolution of their clades (Sena and Molina 2007). In fact, pericentric inversions stand out as the major chromosomal rearrangements in the evolution of the tribes Hypsigenyini, Scarini and Julidini (Sena and Molina 2007, Molina et al. 2012). In turn, in the Novaculini, Cheilini, Pseudocheilini and Labrini tribes, both pericentric inversions and chromosome fusions have contributed for their karyotypic diversification (Ueno and Takai 2000).

In general, Labridae clades can be differentiated into four karyotypic patterns. The first one is characterized by conserved karyotypes, with 48 acrocentric chromosomes; the second by 48 chromosomes with an increase in the chromosome arms (NF); the third by a reduction in the number of chromosomes (<48 chromosomes) but with the same NF; and the fourth by reduced diploid number and NF (Alvarez et al. 1986, Sena and Molina 2007).

At the moment, Julidini is the clade with the largest amount of cytogenetic data in Labridae (Table 1). Nonetheless, they are based on conventional cytogenetic methods and very incipient yet, given its species' diversity. This tribe falls mainly into the first and second karyotypic patterns, with conserved diploid values (2n=48), mostly acrocentric chromosomes, or with variations in the NF due to pericentric inversions. Different classes of repetitive DNAs are linked to chromosome rearrangements in many fish groups (Kidwell 2002, Cioffi and Bertollo 2012, Getlekha et al. 2016). Indeed, repetitive DNAs may clarify the occurrence of particular chromosome rearrangements and evolutionary relationships among different taxa (Shapiro and Sternberg 2005, Biémont and Vieira 2006, Artoni et al. 2015). However, the chromosome organization and the evolutionary dynamics of this important fraction of the genome are still poorly understood in Labridae fishes.

Among Julidini wrasses, *Halichoeres* Rüppell, 1835 is the most diversified and polyphyletic genus, comprising distinct components in the New World and Indo–Pacific Ocean (Barber and Bellwood 2005, Westneat and Alfaro 2005, Rocha et al. 2010). *Thalassoma* Swainson, 1839, phylogenetically close to *Halichoeres*, dates from 8–13 mya and contains 27 species, with a marked increase in diversification between 5–10 mya (Bernardi et al. 2004). *Thalassoma noronhanum* (Boulenger, 1890) is one of the

Table 1. Variations in diploid values (2n) and number of chromosome arms (NF) among Labridae fishes (adapted from Sena and Molina 2007, Arai 2011).

Tribe	N	2n range/ Modal value	NF range/ Modal value	NF Average
Hypsigenyini	7	48/48	56–86/78	76
Pseudocheilini	8	34–48/34	46–84/46	65
Julidini	32	48/48	48–86/48	52
Labrini	10	38–48/48	48–86/48	46
Scarini	5	46–48/48	66–88/66	74
Cheilini	8	32–48/48	38–84/60	66
Labrichthyines	1	48	48	48
Novaculini	8	22–48/48	40–56/48	47
Pseudolabrini	1	48	52	52

smallest known species (Allen 1995), with a wide occurrence on the Brazilian coast and a number of oceanic islands in the Western Atlantic. Despite some cytogenetic data available for *Halichoeres* species (Sena and Molina 2007), there are no information for *Thalassoma* ones from the Atlantic (Arai 2011). In the present study, cytogenetic investigation on C-banding, Ag-NORs, base-specific fluorochrome staining and double-fluorescence *in situ* hybridization (FISH) with 18S rDNA and 5S rDNA probes, were realized in five Julidini species. The data were useful to clarify particular chromosomal processes and phylogenetic relationships of these marine fish species, besides evidencing an unusual co-localization of 18S and 5S rDNA clusters in all species.

Material and methods

Specimens and chromosomal preparation

The specimens of *Halichoeres poeyi* (Steindachner, 1867) (N=13) and *H. brasiliensis* (Bloch, 1791) (N=6) were collected in the coast of Rio Grande do Norte (5°42'20"S, 35°11'38"W), Northeastern Brazil. Individuals of *Halichoeres radiatus* (Linnaeus, 1758) (N=16) were obtained from the Fernando de Noronha Archipelago (3°51'20"S, 32°25'32"W), *H. penrosei* Starks, 1913 (N=3) from the Trindade Island (20°30'13"S, 29°19'50"W) and *Thalassoma noronhanum* from the Rocas Atoll (N=22) (3°51'59"S, 33°48'20"W).

The specimens were submitted to intraperitoneal mitotic stimulation with fungal and bacterial antigen complexes (Molina et al. 2010). Mitotic chromosome preparations were obtained by *in vitro* methodology, using a cell suspension of kidney tissue fragments (Gold et al. 1990). The C-positive heterochromatin and nucleolus organizer regions (NORs) were visualized using the C-banding and Ag-NOR staining (Sumner 1972, Howell and Black 1980, respectively). Chromosomes were also stained with mithramycin (MM) and 4', 6-diamidino-2-phenylindole (DAPI) fluorochromes, according to Schweizer (1976).

Obtaining probes for chromosomal hybridization

The 5S and 18S rDNA probes, containing approximately 200 pb and 1400 pb, respectively, were obtained by polymerase chain reaction (PCR) from the nuclear DNA of *Rachycentron canadum*, using the primers A 5'-TAC GCC CGA TCT CGT CCG ATC-3' and B 5'- CAG GCT GGT ATG GCC GTA AGC-3' (Pendás et al. 1994), NS1 5'-GTA GTC ATA TGC TTG TCT C-3' and NS8 5'-TCC GGT GCA TCA CCT ACG GA-3' (White et al. 1990), respectively. The 18S rDNA and 5S rDNA probes were labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany) and biotin-14-dATP (Invitrogen™), respectively, according to manufacturer's specifications.

Chromosomal hybridization

Fluorescence *in situ* hybridization (FISH) was performed according to Pinkel et al. (1986). Slides with metaphase chromosomes were first treated with RNAse (20 µg/ml in 2XSSC) at 37°C for 1 hour and with pepsin (0.005% in 10mM HCl), for 10 minutes, fixed with 1% formaldehyde for 10 minutes and dehydrated in alcohol baths (70%/85%/100%) for 5 minutes each. The chromosomes were then incubated in 70% formamide/2XSSC at 72°C, for 5 minutes and once again dehydrated in an alcohol series (70%/85%/100%). The hybridization was performed at 37°C for 16h, using a hybridization solution consisting of 50% formamide, 2XSSC, 10% dextran sulfate and the denatured probe (5 ng/µl), with a final volume of 30 µl. Post-hybridization washings were done in 15% formamide/0.2XSSC at 42°C, for 20 minutes, followed by washings in 0.1XSSC at 60°C for 15 minutes and in Tween 20 (0.5%/4XSSC) for 5 minutes, at 25°C. Next, the slides were incubated for 15 minutes in a blocking solution (5% NFDM /4xSSC) and washed with Tween 20 (0.5%/4XSSC) for 15 minutes. The hybridization signals were detected using anti-digoxigenin rhodamine (Roche, Mannheim, Germany) for the 18S rDNA probe and streptavidin-FITC (Vector Laboratories) for the 5S rDNA probe. The chromosomes were counterstained with Vectashield/DAPI (1.5 µg/ml) (Vector).

At least thirty metaphase spreads were analyzed to confirm the diploid chromosome numbers, karyotype structure and FISH results. The best metaphases were photographed using an Olympus™ BX50 epifluorescence microscope, coupled to an Olympus DP73 digital capture system. The chromosomes were classified as submetacentric (sm) and acrocentric (a), according to the arm ratio (Levan et al. 1964), and arranged in decreasing order of size in the karyotypes.

Results

All species showed a high number of acrocentric chromosomes. *Thalassoma noronhanum* has 2n=48, with 2sm+46a (NF=50) (Fig. 2a). The species *H. radiatus*, *H. poeyi*, *H.*

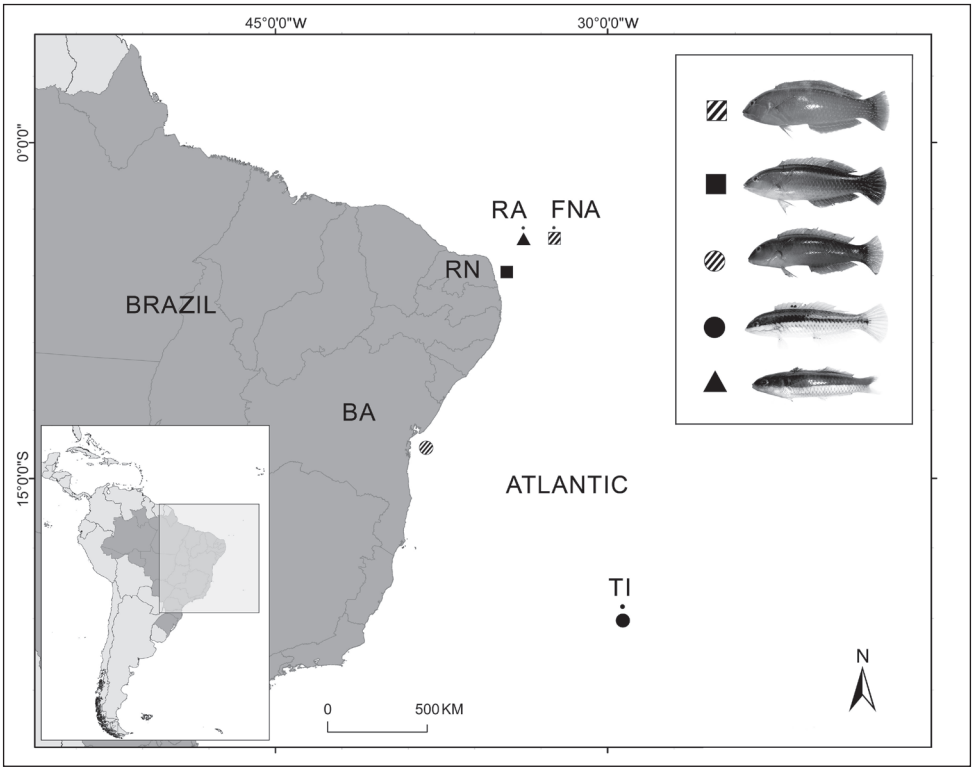

Figure 1. Collection points of the Labridae species analyzed. Fernando de Noronha Archipelago (FNA); Rocas Atoll (RA); Rio Grande do Norte coast (RN); Bahia coast (BA); and Trindade Island (TI). *Halichoeres radiatus* (FNA), *H. brasiliensis* (RN), *H. poeyi* (BA), *H. penrosei* (TI) and *Thalassoma noronhanum* (RA).

brasiliensis and *H. penrosei* have symmetric karyotypes, with 2n=48 acrocentric chromosomes (NF=48) (Fig. 2b-e). The heterochromatin occupies the centromeric and pericentromeric regions of all chromosomes, and also the telomeric regions of a few (Fig. 2a–e).

The Ag-NORs are positioned on the short arms of the single submetacentric pair of *T. noronhanum* (Fig. 2a). In *Halichoeres* species, these sites are located in two chromosome pairs, except in *H. penrosei*, where they are located on the short arms of pair 15 (Fig. 2b, highlighted). In *H. poeyi*, the Ag-NORs occupy the short arms of pairs 5 and 15 (Fig. 2c) and in *H. radiatus* and *H. brasiliensis* the short arms of pairs 5 and 24 (Fig. 2d, e).

The mapping of 18S rDNA sequences showed single sites in *T. noronhanum*, coincident with the Ag-NORs (Fig. 3a). On the contrary, all *Halichoeres* species have multiple 18S rDNA sites. They occur in the terminal position of the short arms of pairs 5, 6, 15, 19 and 22 in *H. penrosei* (Fig. 3b), of pairs 5, 6 and 15 in *H. poeyi* (Fig. 3c), and of pairs 5, 15 and 24 of *H. brasiliensis* (Fig. 3e). However, in *H. radiatus* they are found in interstitial position on pair 5 and in terminal position on pairs 15 and 24 (Fig. 3d).

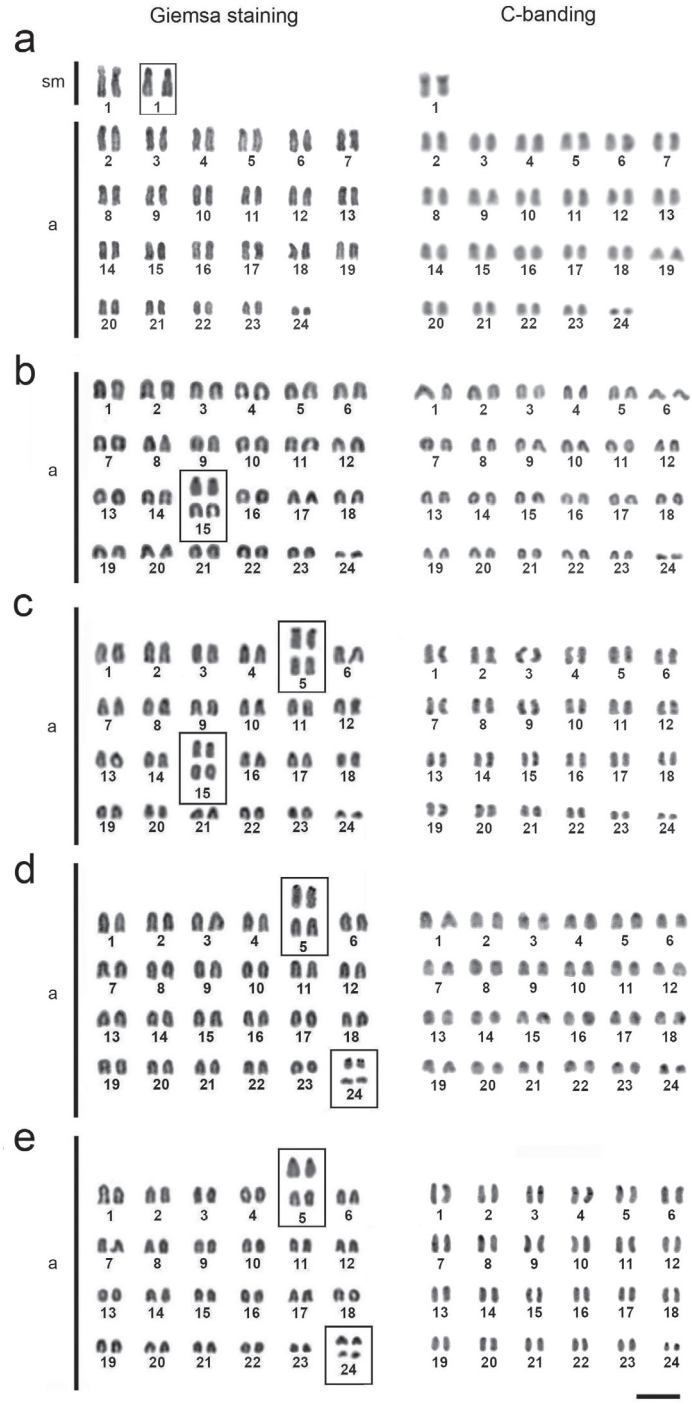

Figure 2. Karyotypes of *Thalassoma noronhanum* (**a**), *Halichoeres penrosei* (**b**), *Halichoeres poeyi* (**c**), *Halichoeres radiatus* (**d**), and *Halichoeres brasiliensis* (**e**). The chromosomal pairs bearing Ag-NORs are boxed, the silver staining in the upper row. Bar: 5 µm.

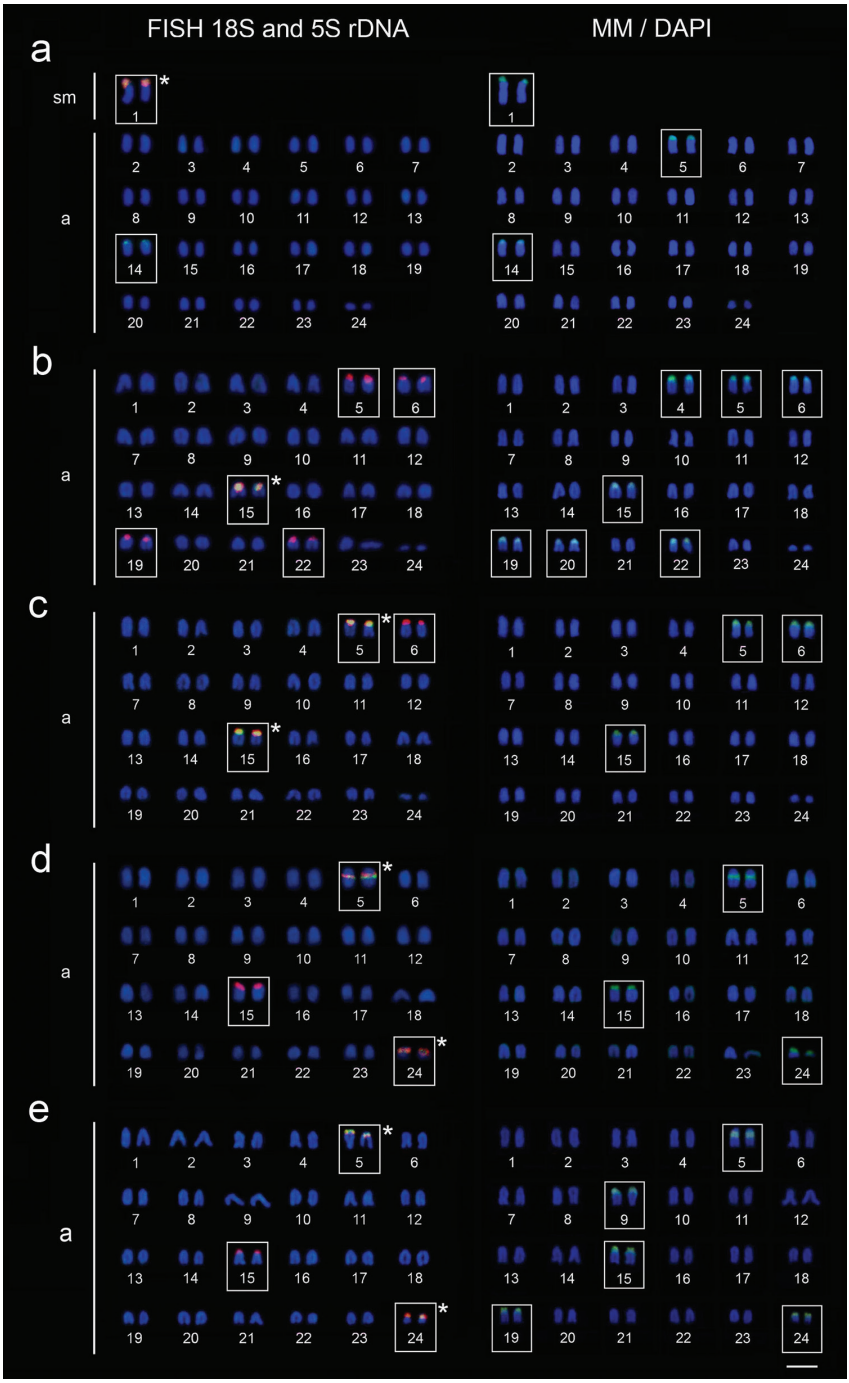

Figure 3. Double-FISH with18S rDNA (red) and 5S rDNA (green) probes and MM/DAPI fluorochromes staining in the chromosomes of *Thalassoma noronhanum* (**a**), *Halichoeres penrosei* (**b**), *Halichoeres poeyi* (**c**), *Halichoeres radiatus* (**d**) and *Halichoeres brasiliensis* (**e**). Asterisks indicate the chromosome pairs with 18S/5S rDNA arrays. Bar: 5 μm.

The 5S rDNA sites occur in an 18S/5S rDNA array in pair 1 and exclusively on the short arms of pair 14 in *T. noronhanum* (Fig. 3a). On the other hand, in all *Halichoeres* species, the 5S rDNA sites are co-located with the 18S rDNA ones. They occur in the terminal position of the short arms of pair 15 in *H. penrosei* (Fig. 3b), of pairs 5 and 15 in *H. poeyi* (Fig. 3c), of pairs 5 and 24 in *H. brasiliensis* (Fig. 3e). In *H. radiatus* they are found in interstitial position in pair 5 and terminal position in pair 24 (Fig. 3d). The Ag-NOR marks were located exclusively on the 18S/5S rDNA arrays.

The sequential staining with MM/DAPI fluorochromes showed a larger number of GC-rich regions than rDNA sites in *T. noronhanum*, *H. penrosei* and *H. brasiliensis*. However, in all the species, the 18S and 5S rDNA sites and 18S/5S rDNA arrays were coincident with GC-rich regions (Fig. 3a–e).

Discussion

The rates of chromosome diversification can vary significantly among marine fish families (Molina et al. 2014) and, in some cases, they are linked to the evolutionary dynamics of the rDNA sequences. Indeed, groups with marked karyotype conservatism (Molina 2007) usually exhibit low diversification in the frequency and organization of ribosomal sites (Motta-Neto et al. 2011, Calado et al. 2013), while those with moderate or higher rates of chromosomal diversification (Molina et al. 2014) may display marked variations in the rDNA regions (Lima-Filho et al. 2014a, b).

In contrast with several Perciformes groups, Labridae show considerable variation in the diploid values (2n=22 to 48), as well as in the number of chromosome arms (NF=38 to 92) (Sena and Molina 2007, Arai 2011). The evolutionary rates of chromosomes differ significantly among clades (Table 1), reflecting their different histories linked to a deep association with coral reefs (Wainwright et al. 2004).

The cytogenetic patterns of the five analyzed wrasses suggest a greater karyotype conservatism in Julidini than in other Labridae clades. Indeed, *Halichoeres* and *Thalassoma* species exhibit karyotypes with 2n=48 chromosomes, mostly or entirely formed by acrocentric chromosomes, small amount of heterochromatin and one or two pairs bearing Ag-NORs (Sena and Molina 2007, present paper), a characteristic recognized as basal for Perciformes (Brum and Galetti 1997, Galetti Jr et al. 2000).

The chromosomal divergences in Julidini are mainly due to a small number of pericentric inversions (Table 1). In *T. noronhanum*, the presence of an exclusive pair of biarmed chromosomes demonstrates a variant condition with respect to six other species previously described in this genus, all of them with 2n=48a. On the other hand, in *Halichoeres* species the presence of few biarmed chromosomes (1 to 3 pairs) is relatively more frequent (Sena and Molina 2007, Arai 2011), albeit not identified in the Atlantic species here investigated. However, despite the similarities in the karyotype structure of *T. noronhanum*, *H. penrosei*, *H. poeyi*, *H. radiatus* and *H. brasiliensis*, a dynamic evolutionary condition concerning the rDNA regions occurs among these species, which contribute to understanding the karyotypic evolution in Julidini. In

fact, the chromosome mapping of rDNA sequences showed a significant variation in frequency, distribution and organization, especially in the *Halichoeres* species.

Chromosomes with homogeneous and small amounts of repetitive DNAs have been found in fish species with little karyotype diversification (Molina 2007, Motta-Neto et al. 2011). On the other hand, heterogeneous and large amounts of repetitive DNAs are related in several families with notable levels of chromosomal rearrangements and differentiation (Moreira-Filho and Bertollo 1991, Souza et al. 2001, Favarato et al. 2016). Among the repetitive DNAs, rDNA has a major role in karyotype diversification. In fact, species from various fish families exhibit 18S and 5S rDNAs sequences involved in chromosome fusion points (Molina and Galetti 2002, Ziemniczak 2011, Jacobina et al. 2013, Getlekha et al. 2016), indicating their probable involvement in the chromosomal reorganization. In this sense, the presence of an 18S rDNA site in the interstitial position on pair 5 in *H. radiatus*, in contrast to its terminal position in the homeologous chromosomes of the remaining species, puts in evidence a cryptic paracentric inversion in that chromosome pair.

From the phylogenetic view, a single Ag-NOR/18S rDNA site in *T. noronhanum* likely represents an ancestral condition for Julidini species. *Halichoeres penrosei*, the most basal species analyzed in this genus (possibly belonging to the genus *Thalassoma*, according to Rocha et al. 2010), shows intermediate features, with a single Ag-NOR and multiple 18S rDNA sites. This indicates that multiple rDNA regions is an ancestral condition and that the rDNA dynamics is an ancient trait in *Thalassoma* and *Halichoeres* genera (Fig. 4). In fact, the multiple Ag-NORs present in *H. poeyi*, *H. radiatus* and *H. brasiliensis* and the large number of rDNA sites present in *Halichoeres* suggest that the dispersal process of these sequences precedes their diversification. The wide variation in distribution and organizational patterns of these sequences in Julidini are compatible with birth-and-death processes (Rooney and Ward 2005) acting in a stochastic evolutionary model.

In turn, the 5S rDNA sequences can present a conserved chromosomal distribution, even among phylogenetically non-related taxa (Perina et al. 2011). In most eukaryotes, these sequences are organized *in tandem* repetitions, in which the non transcribed spacers (NTS) present high interspecific variations, due to insertions/deletions, minisatellites and pseudogenes (Nelson and Honda 1985, Leah et al. 1990, Alves-Costa et al. 2008). However, although evolutionarily conserved, stochastic events may promote a great dispersal of the 5S rDNA sequences in a large number of chromosomes in some Perciformes species (Affonso and Galetti 2005, Lima-Filho et al. 2014b).

The location of 45S and 5S rDNA sites in different chromosomes is the most common condition in vertebrates (Lucchini et al. 1993, Suzuki et al. 1996, Gornung 2013), indicating independent evolution of these loci. The syntenic arrangement of these rDNA classes, as found in *Halichoeres* and *Thalassoma*, is not a common feature, although it has already been reported in some main fish orders, such as Perciformes (Ghigliotti et al. 2008, Merlo et al. 2013), Characiformes (Vicari et al. 2006, Bellafronte et al. 2009, Cioffi et al. 2009), Siluriformes (Mariotto et al. 2011, Ziemniczak et al. 2012), Anguilliformes (Deiana et al. 2006), Salmoniformes (Pendás et al. 1994), Nototheniformes

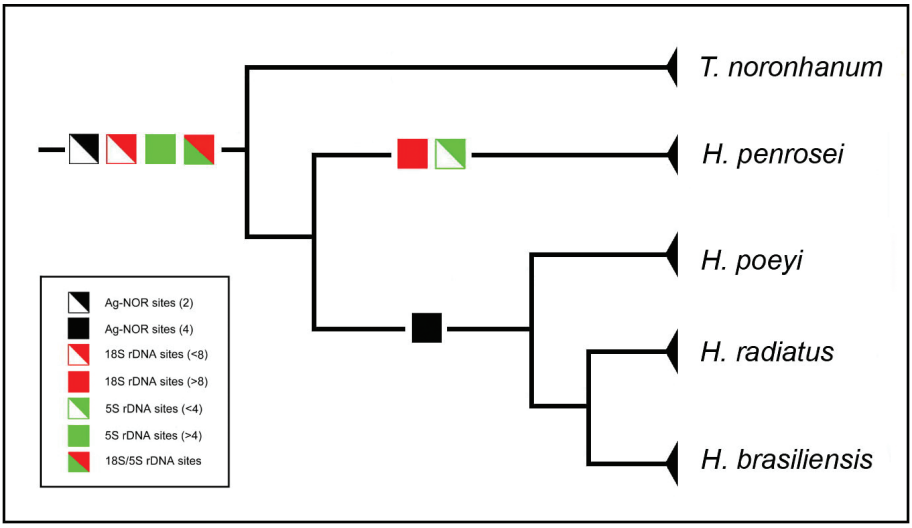

Figure 4. Evolutionary patterns of ribosomal sites in *Thalassoma noronhanum*, *Halichoeres radiatus*, *Halichoeres poeyi*, *Halichoeres brasiliensis* and *Halichoeres penrosei*, from the phylogenetic perspective (evolutionary relationships adapted from Rocha et al. 2010).

(Ghigliotti et al. 2007) and Tetraodontiformes (Martinez et al. 2010). In fishes, 45S/5S rDNA arrays are phylogenetically stochastic and limited to few species of a clade (Almeida-Toledo et al. 2002), and preferentially explained by random events in the course of the evolutionary trajectory of the genome (Calado et al. 2014). Thus, the phylogenetic spread of these arrangements in the Julidini clade indicates a noteworthy evolutionary stability. In fact, although the non-syntenic organization of these rDNA classes might be interpreted as a functional advantage (Martins and Galetti 2001), the persistent 18S/5S rDNA arrays in *Thalassoma* and *Halichoeres* indicates that they are feasible and, in this case, suggesting a probable adaptive condition for this multigene organization. In addition, syntenic rDNA genes may exhibit adjacent or interspersed arrangements (Artoni et al. 2015). In Julidini, hybridization signals are apparently superimposed, suggesting the occurrence of the latter kind of organization. Further fiber-FISH analyses will allow better understanding of the organization of these arrangements.

Final remarks

The uncommon pattern of 18S and 5S rDNA synteny presented by Julidini species indicates a shared ancestral condition, in contrast to stochastic and taxonomically restricted occurrences found in other fish groups (Drouin and Sá 1995, Calado et al. 2013). In addition to phylogenetic sharing patterns, these arrangements suggest a possible adaptive organization, given that they are all active ribosomal sites (Ag-NOR positive) in this species. The differentiated 18S/5S rDNA regions in *Halichoeres* species

are particularly useful in identifying phylogenetic homeologies (pairs 5 and 15), but also sufficiently divergent to represent effective cytotaxonomic markers for this genus. Although a conserved karyotypic pattern is maintained in some Labridae species, the present data reveal a significant dynamism of the ribosomal sequences, in accordance to the moderate/high rate of chromosomal diversification in this family.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The experimental work fulfills all ethical guidelines regarding the handling of specimens. The collection and handling of specimens followed protocols approved by the Ethics Committee on the Use of Animals of the Federal University of Rio Grande do Norte (Process 044/2015). All authors consent to participate in the publication and are in agreement with the article content.

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RESEARCH ARTICLE

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Karyotype characteristics, larval morphology and chromosomal polymorphism peculiarities of *Glyptotendipes salinus* Michailova, 1983 (Diptera, Chironomidae) from Tambukan Lake, Central Caucasus

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Abstract

Data on the karyotype characteristics, larval morphology and features of chromosomal polymorphism of a population of *Glyptotendipes salinus* Michailova, 1983 (Diptera, Chironomidae) from Tambukan Lake (on the northern macroslope of the central Caucasus) are presented. It was found that diagnostic larval characters of *G. salinus* from Caucasus in general are similar to those described in previous studies, but with some significant differences. By some morphological characteristics Caucasian larvae appeared to be closer to *G. barbipes* than to ones provided for European larvae of *G. salinus* by Contreras-Lichtenberg (1999). Obtained morphological data make possible to conclude that Caucasian population of *G. salinus* can be a markedly diverged population of the species, probably even subspecies. In the Caucasian population 12 banding sequences were found: two in arms A, B, C, E, and G, and one in arms D and F. Eight of these are already known for this species, and four, salA2, salB2, salEX, and salG3, are described for the first time. Genetic distances between all the previously studied populations of *G. salinus* were measured using Nei criteria (1972). The population of the central Caucasus occupies a distinct position on the dendrogram

compared with populations from Altai and Kazakhstan. All the obtained morphological and cytogenetic data can indicate the plausible relative isolation and complexity of the Caucasus from the viewpoint of microevolution. More researches are required in other parts of Caucasus and other geographically distant regions for more specific allegations.

Keywords

Diptera, Chironomidae, *Glyptotendipes salinus*, larval morphology, polytene chromosomes, chromosomal polymorphism, genetic distances, Tambukan Lake, central Caucasus (northern macroslope)

Introduction

Glyptotendipes salinus was first described by Michailova (1987) from Bulgaria. According to Fauna Europaea web source (http://www.faunaeur.org) the species is known in Europe from Austria, the British Isles and Bulgaria.

The karyotype of *G. salinus* has been studied from Bulgaria (Michailova 1987), Russia and Kazakhstan (Andreeva et al. 1998, Aimanova et al. 2000). In Russia this species is known from several regions (Altai Krai, Omsk Oblast, and Chelyabinsk Oblast). In Kazakhstan the two studied populations of the species are situated in the area of the Semipalatinsk Test Site (STS).

The species is a sibling species of *G. barbipes* that allows study of the earlier phases of divergence of the species in genus *Glyptotendipes* (Michailova 1987a, 1989). The species *G. salinus* differs from *G. barbipes* by chromosomal rearrangements in the chromosome arms A, C, D and E. Significant difference was also found in the amount and quality of the centromeric C- heterochromatin. Thus, some bands that were in the euchromatic state in *G. salinus* were in the heterochromatic state in *G. barbipes* (Michailova 1987b). The amount of centromeric DNA in the Ist, IInd and IIIrd chromosomes differs greatly between the two species (Michailova and Nikolov 1992). It was also found that C-heterochromatin of *G. barbipes* consists of two different types of C-bands: the dark ones at the periphery of the centromeres, which correspond to satellite II DNA, and pale C-bands corresponding to the satellite I DNA in the middle of the centromeric regions. Such heterochromatin differentiation was not expressed as prominently in the centromeric regions of *G. salinus* (Michailova 2014).

Michailova et al. (2002) also provide research on the effects of lead on the polytene chromosomes of *G. salinus*. They found that exposure to lead results in a decrease in the activity of the nucleoli (NOR) and Balbiani Rings (BRs).

Earlier it was shown that *G. salinus* occurs in brackish water, while sibling species *G. barbipes* prefers fresh water (Michailova 2014).

The aim of the work was to present the description of karyotype characteristics, larval morphology peculiarities and chromosomal polymorphism of *G. salinus* from Tambukan Lake (northern macroslope of the central Caucasus). Also it was very important to compare chromosomal polymorphism characteristics of *G. salinus* from the Caucasus with earlier studies.

Methods

The fourth instar larvae of *Glyptotendipes* were used in the karyological study. The larvae were collected from one site of the central Caucasus: 17.05.13, 43°27.30'N; 43°09.75'E, southern shore of Tambukan Lake, altitude *ca*. 550 m a.s.l. Tambukan Lake is a lake with bitter salt water (salinity varies from 30 to 100 grams per liter) located in the northern macroslope of the central Caucasus, near the border of Stavropol Krai and the Republic of Kabardino-Balkaria of Russia. The lake's surface area is 1.87 km^2, and its depth ranges between 1.5–3.1 m. With regard to vertical zonation, the collection site belongs to the steppe zone (typification of the zone variants was given according to Sokolov and Tembotov 1989).

The morphological terminology follows Sæther (1980). Head capsule and body of 20 larvae were slide mounted in Fora-Berlese solution. The specimens have been deposited in Tembotov Institute of Ecology of Mountain territories RAS, Nalchik, Russia. The karyotype and chromosomal polymorphism has been studied in 63 larvae from the Tambukan Lake.

Larvae for karyotype analysis were fixed in ethanol-glacial acetic acid (3:1). Slides of the chromosomes were prepared using the ethanol-orcein technique (see Dyomin and Ilyinskaya 1988, Dyomin and Shobanov 1990). The banding sequences were designated according to the accepted rule specifying the abbreviated name of the species, symbol of chromosome arm, and sequence number, for example salC1, salC2, etc. (Keyl 1962, Wülker 1973). The identification of chromosome banding sequences was performed with the use of the photomaps of Michailova (1983) and Andreeva et al. (1998); chromosome mapping was performed according to Martin and Porter (1973) and Kiknadze et al. (1998), with corrections of Andreeva et al. (1998).

Microscope Carl Zeiss Axio Imager.A2 was used to study the chromosome slides. The software package STATISTICA 10 was used for statistical analysis (cluster analysis).

The following parameters were used for comparison of characteristics of chromosomal polymorphism: the number of banding sequences in a population, the percentage of heterozygous larvae, and number of heterozygous inversions per specimen. Genetic distances between populations were calculated according to Nei criteria (Nei 1972) on basis of the original data and data of Andreeva et al. (1998) on inversion polymorphism of the species in Russia and Kazakhstan.

Results

The larvae of *Glyptotendipes* in the studied site were attributed to *G. salinus* by both morphological and chromosomal characteristics. Morphological characteristics of larva are presented in Fig. 1a–g.

The diagnostic larval characters of *G. salinus* from the Caucasian site in general are similar to those described previously for this species by Michailova (1987), Andreeva et al. (1998) and Contreras-Lichtenberg (1999), but there are some differences. The head

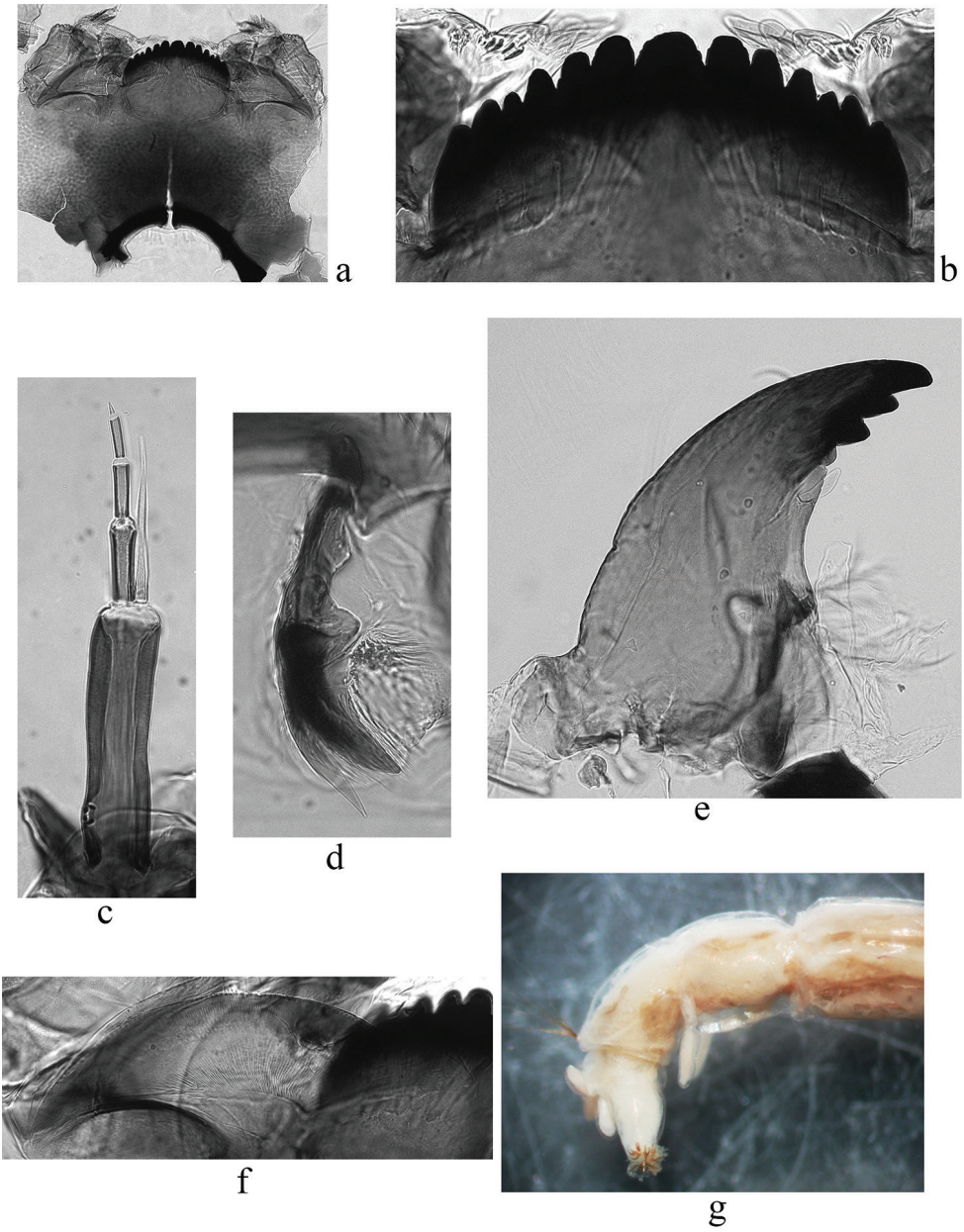

Figure 1. The larva of *G. salinus* from Tambukan Lake. **a** gular sclerite **b** mentum **c** antenna **d** premandible **e** mandible **f** ventromental plate **g** ventral tubuli on segment VIII.

capsule is lightly colored as in specimens found in other localities. Ventral tubuli are shorter than in *G. barbipes* and do not exceed the length of IX segment of larva body (Fig. 1g). The seta subdentalis of mandible is leaf-shaped (Fig. 1e), reaching the top of

the last tooth. The ratio of the width of the ventramental plate to inter-plate distance (PSR) in *G. salinus* is more than 8 (8.34 according to Contreras-Lichtenberg (1999), from 8.02 to 9.04 in Caucasian population), while PSR of *G. barbipes* is 4.2. The width of the mentum of *G. salinus* was 219 μm according to Contreras-Lichtenberg (1999), but varies from 235 to 266 μm in Caucasian population, which is closer to *G. barbipes* measurements (256 μm). The ratio of the width of the mentum to the width of the middle tooth of mentum (MR) of *G. salinus* described by Contreras-Lichtenberg (1999) is 5.33, while in Caucasian population it varies from 6.8 to 7.17 and slightly exceeds MR=6.5 of *G. barbipes*. The width of the ventramental plate of *G. salinus* in Caucasian population varies from 297 to 340 μm (307.5 μm according to Contreras-Lichtenberg 1999), and its value is much higher than 288 μm of *G. barbipes*. The inter-plate distance (IPD) of *G. salinus* from Caucasian population is similar to data of Contreras-Lichtenberg (1999) – 36.9 μm – and varies from 34.5 to 37.5 μm, IPD of *G. barbipes* is much higher - 68 μm. The most significant difference was observed for the length of the larva's body: in first description of Michailova (1987) and in paper of Contreras-Lichtenberg (1999) it is said to be 25-27 mm, while Caucasian larvae are twice shorter – 12-14 mm – which is very similar to an average larva's length of *G. barbipes* (12 mm).

Karyotype of *G. salinus* from the Central Caucasus

The diploid number of chromosomes in *G. salinus* karyotype is 2n = 8, chromosome arm combination is AB, CD, EF, and G (Fig. 2). Chromosomes AB and CD are metacentric, EF is submetacentric, and G is telocentric. Three well-developed nucleoli (N) are located on arms B, C, and E. There are two Balbiani rings (BR) in the karyotype: both are situated in arm G (Fig. 2).

The centromeric bands of long polytene chromosomes of *G. salinus* from the studied populations are large and belong to v-type according to the classification by Shobanov (2002).

Banding sequences and chromosomal polymorphism of *G. salinus* from the Tambukan Lake

Until now, eleven banding sequences have been described in the banding sequences pool of *G. salinus* (Table 1). In the studied population only eight of those banding sequences were present, and four banding sequences have been found for the first time, providing a total of 12 banding sequences in the Caucasian population (Table 2).

Arm A has two banding sequences, salA1 and salA2 (Figs 3–4, Table 2). The banding sequence salA1 was predominant in the studied population (Table 2). The banding sequence salA2 was found only in the heterozygous state with very low frequency (salA1.1 – 0.968, salA1.2 – 0.032).

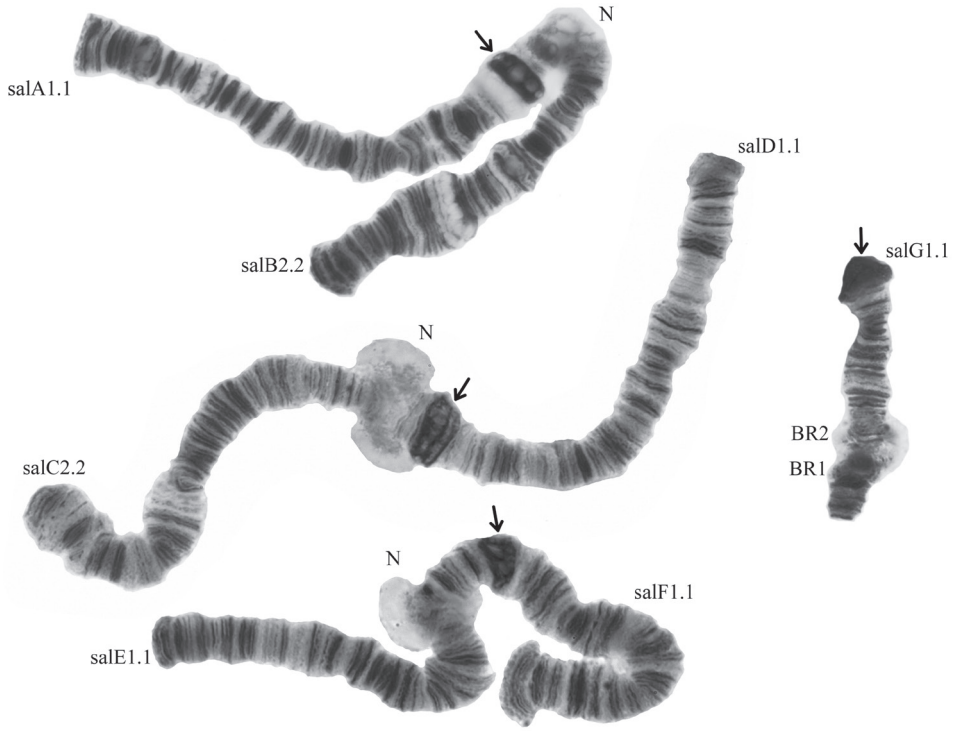

Figure 2. Karyotype of *G. salinus* from the Tambukan Lake; salA1.1, salD1.1 etc. – zygotic combinations of banding sequences; BR – Balbiani rings, N – nucleoli. Arrows indicate centromeric regions.

Table 1. Catalog of banding sequences in the banding sequences pool of *G. salinus*.

Arm	Sequence	Order of bands	Authors of mapping
A	salA1	1a-b 5n-t 6a-n 2d-h 3ba 2u-i 3c-t 4a-v 5a-m 2cba 1t-n 1c-m 6o-t 7a-s	Andreeva et al.1983
	salA2	1ab 5n-t 6a-d 5h-a 4v-a 3t-c 2i-u 3ab 2h-d 6h-e 5i-m 2c-a 1t-n 1c-m 6o-t 7a-s	Original data
B	salB1	13z-a 12t-a 11o-a 10v-a 9n-a 8p-a 7c-a	Andreeva et al.1983
	salB2	13-z-j 9d-h 10a-v 11a-o 12a-t 13a-i 9cba 8p-a 7s-a	Original data
C	salC1	1a-o 4v-k 1p-r 2a-n 3a-p 4a-m 5a-z 6a-n 7a-i	Andreeva et al.1983
	salC2	1a-o 5f-a 4m-a 3p-a 2n-a 1r-p 4n-v 5l-z 6a-n 7a-i	Andreeva et al.1983
	salC3	1a-o 5f-a 4m-a 3p-a 2n-a 4u-n 1p-r 4v 5l-z 6a-n 7a-i	Andreeva et al.1983
	salC4	1a-d 4e-m 5a-k 1o-e 4d-a 3p-a 2n-a 1r-p 4n-v 5l-z 6a-n 7a-i	Andreeva et al.1983
D	salD1	12p-a 11m-a 10w-a 8a-g 8ih 8n-j 8o-q 9a-x 7i-a	Andreeva et al.1983
E	salE1	1a-u 3a-q 4a-w 5a-t 6a-r 2l-a 1v 6s-w 7a-l	Andreeva et al.1983
	salEX		Not mapped
F	salF1	11w-a 10s-a 9t-a 8m-a 7l-a	Andreeva et al.1983
G	salG1	5t-a 4i-a 3q-a 2r-a 1g-a	Andreeva et al.1983
	salG2	5t-a 4i 2b-r 3a-g 4a-h 2a 1g-a	Andreeva et al.1983
	salG3	5t-j 2h-r 3a-g 4a-i 5a-i 2g-a 1g-a	Original data

Table 2. Frequency of banding sequences in different populations of *G. salinus*.

Banding sequence	Populations					
	Kazakhstan		Altai Krai			Central Caucasus, Tambukan Lake (original data) 63 larvae
	STS, Atomnoe Lake (Andreeva et al. 1998) 50 larvae	STS, Shagan Lake (Andreeva et al. 1998) 52 larvae	Altai, Bulatovo Lake (Andreeva et al. 1998) 50 larvae	Altai, Gorkoe Lake (Andreeva et al. 1998) 49 larvae	Altai, Bolshoe Utichie Lake (Andreeva et al. 1998) 50 larvae	
salA1	1	1	1	1	1	0.992
salA2	0	0	0	0	0	0.008
salB1	1	1	1	1	1	0.317
salB2	0	0	0	0	0	0.683
salC1	0.220	0.164	0.630	0.622	0.530	0.016
salC2	0.780	0.817	0.370	0.378	0.470	0.984
salC3	0	0.009	0	0	0	0
salC4	0	0.009	0	0	0	0
salD1	1	1	1	1	1	1
salE1	1	1	1	1	1	0.992
salEX	0	0	0	0	0	0.008
salF1	1	1	1	1	1	1
salG1	0.950	0.991	1	1	1	0.968
salG2	0.050	0.009	0	0	0	0
salG3	0	0	0	0	0	0.032
Number of banding sequences in population	9	11	8	8	9	12
Percentage of heterozygous larvae	40	29	48	57	62	51
Number of heterozygous inversions per specimen	0.34	0.30	0.60	0.61	0.60	0.60

It is new for the species and described for the first time here (Fig. 4, Table 2). It differs from salA1 by one simple inversion that involves region 6e-n 2d-h 3ba 2u-i 3c-t 4a-v 5a-h:

salA2 1ab 5n-t 6a-d 5h-a 4v-a 3t-c 2i-u 3ab 2h-d 6h-e 5i-m 2c-a 1t-n 1c-m 6o-t 7a-s

Arm B has two banding sequences, salB1 and salB2 (Figs 3–4). The banding sequence salB2 was predominant in the studied population (Table 2). The sequence salB2 is new for the species and described for the first time (Figs 3–4, Table 2). It differs from salB1 by one simple inversion that involves region 13i-a 12t-a 11o-a 10 v-a 9h-d:

salB2 13-z-j 9d-h 10a-v 11a-o 12a-t 13a-i 9cba 8p-a 7s-a

The banding sequence salB2 was found with high frequency in both homozygous (salB1.1 – 0.095, salB2.2 – 0.445) and heterozygous states (salB1.2 – 0.460).

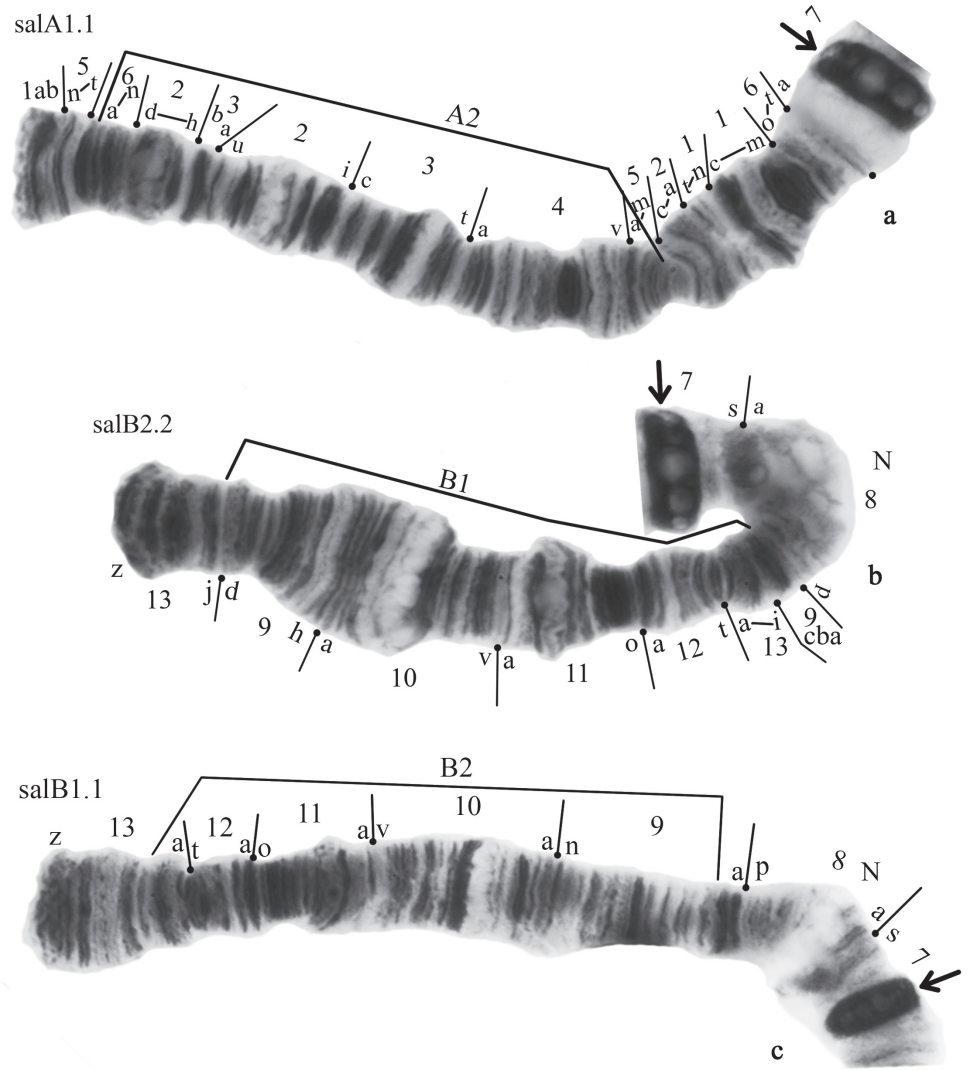

Figure 3. Banding sequences in arms A and B of *G. salinus*; **a** homozygotes salA1.1 **b** homozygotes salB2.2 **c** homozygotes salB1.1, Designations as in Fig. 2.

Arm C has two banding sequences, salC1 and salC2. The banding sequence salC2 was dominant in this population (Fig. 5, Table 2). The other banding sequence salC1 was found only in the heterozygous state (salC1.2 – 0.032, sal C2.2 – 0.968) (Fig. 4).

Arm D is monomorphic with banding sequence salD1 (Fig. 5, Table 2).

Arm E had two banding sequences, salE1 and salEX (Table 2). The banding sequence salE1 was dominant in the population (Fig. 6, Table 2). The banding sequence salEX was found only in the heterozygous state (salE1.1 – 0.968, salE1.2 – 0.032). This banding sequence is new for the species and described here for the first time

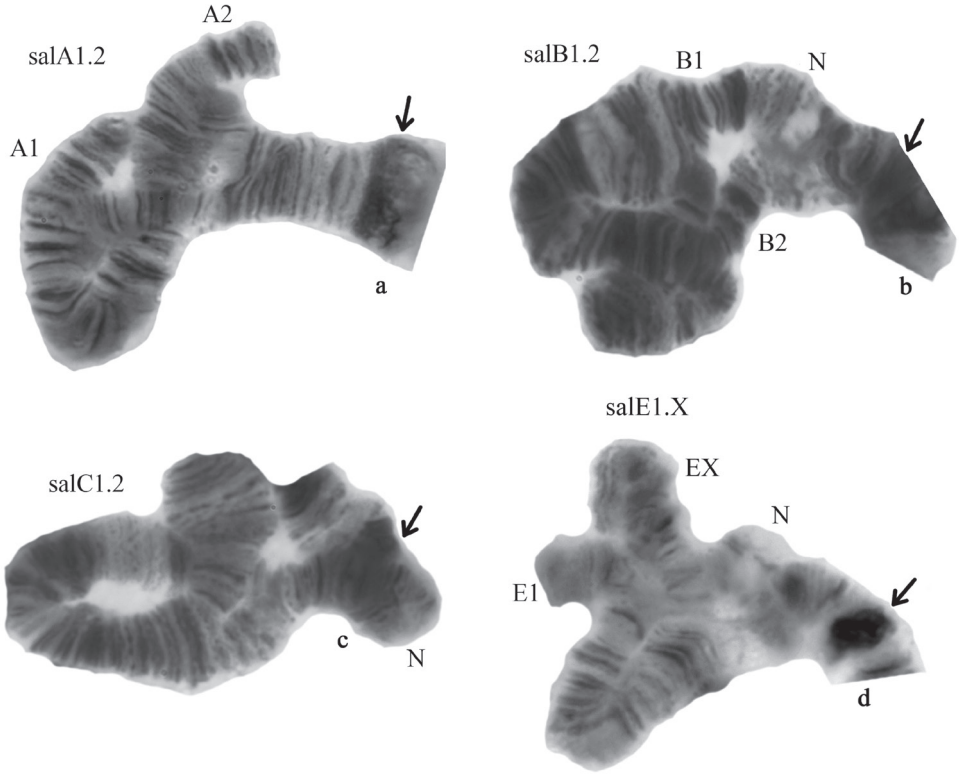

Figure 4. Chromosome inversions in different arms of *G. salinus* from Tambukan Lake. Heterozygous zygotic combination key: **a** salA1.2 **b** salB1.2 **c** salC1.2 **d** salE1.X. Designations as in Fig. 2.

(Fig. 4, Table 2). Because banding structure of salEX was unclear it was impossible to map it and so no numerical designation was assigned to it.

Arms F is monomorphic with banding sequence salF1 (Fig. 6, Table 2).

Arm G had two banding sequences, salG1 and salG3. The banding sequence salG1 was dominant in the population (Fig. 7, Table 2). The banding sequence salG3 was found only in the heterozygous state (salG1.1 – 0.937, salG1.3 – 0.063). This banding sequence is new for the species and described for the first time (Fig. 4). It differs from salG1 by one simple inversion that involves region 5i-a 4i-a 3g-a 2r-h:

salG3 5t-j 2h-r 3a-g 4a-i 5a-i 2g-a 1g-a

Comparison of chromosomal polymorphism of *G. salinus* from the Central Caucasus and other regions

Data for Russian (Altai Krai) and Kazakhstan populations are presented on the basis of publication of Andreeva et al. (1998).

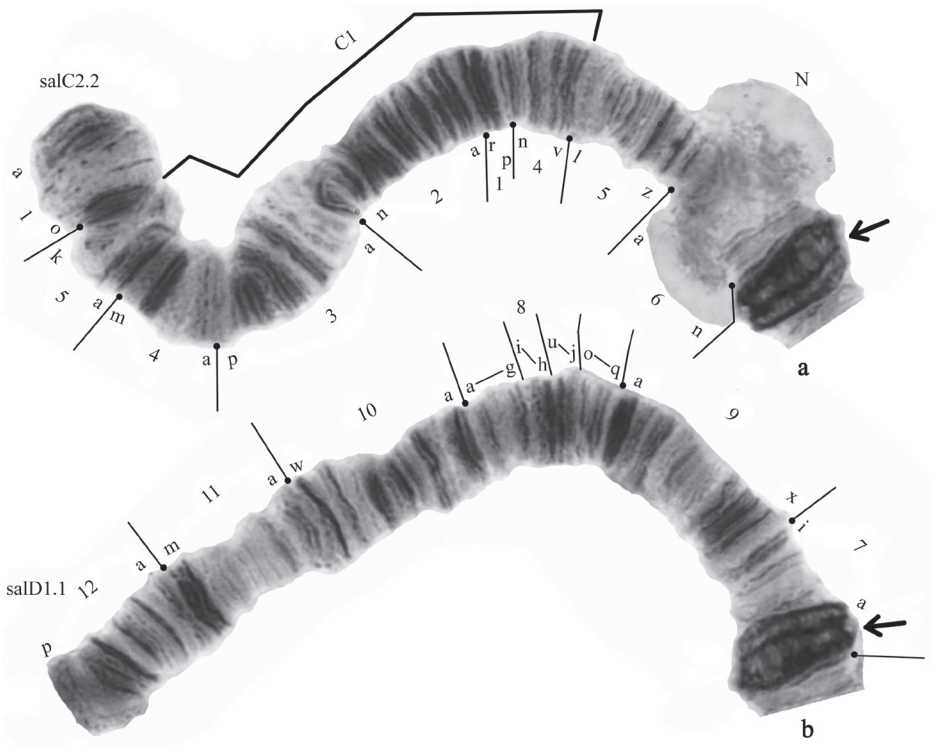

Figure 5. Banding sequences in the arms C and D of *G. salinus*. Key: **a** homozygotes salC2.2 **b** homozygotes salD1.1 Designations as in Fig. 2.

Arm A. The populations from Altai and Kazakhstan (Andreeva et al. 1998) were characterized by the presence of single banding sequence in the arm, salA1 (Table 2). Same banding sequence is dominating in population from North Caucasus but one new for the species sequence salA2 was also found with very low frequency (0.008). The new banding sequence might be endemic for this region.

Arm B was monomorphic in populations of Altai and Kazakhstan and presented only by the banding sequence salB1 (Table 2). In the Caucasian population another banding sequence new for the species – salB2, was predominant. This new banding sequence is probably endemic for this region.

Arm C of *G. salinus* in all the studied populations was polymorphic. However in Altai populations the predominant banding sequence was salC1, whereas in Kazakhstan population dominated salC2. The population of the North Caucasus is closer to populations of Kazakhstan with salC2 dominating with even higher frequency (Table 2).

Arm D of *G. salinus* was monomorphic in all the studied populations.

Arm E was monomorphic in populations of Altai and Kazakhstan and low polymorphic in Caucasian population with the same dominant banding sequence salE1. A new banding sequence salEX was found in the Caucasian population with very low frequency (0.008) and might be endemic for the region.

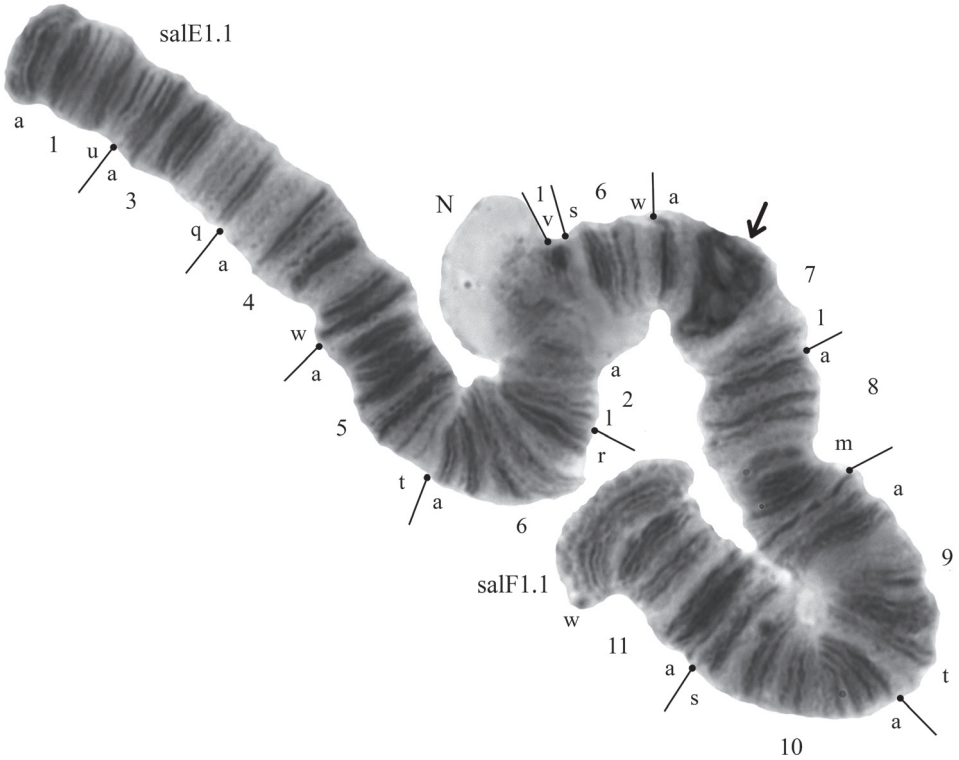

Figure 6. Banding sequences in the arms E and F of *G. salinus*. – The homozygotes salE1.1 and salF1.1. Designations as in Fig. 2.

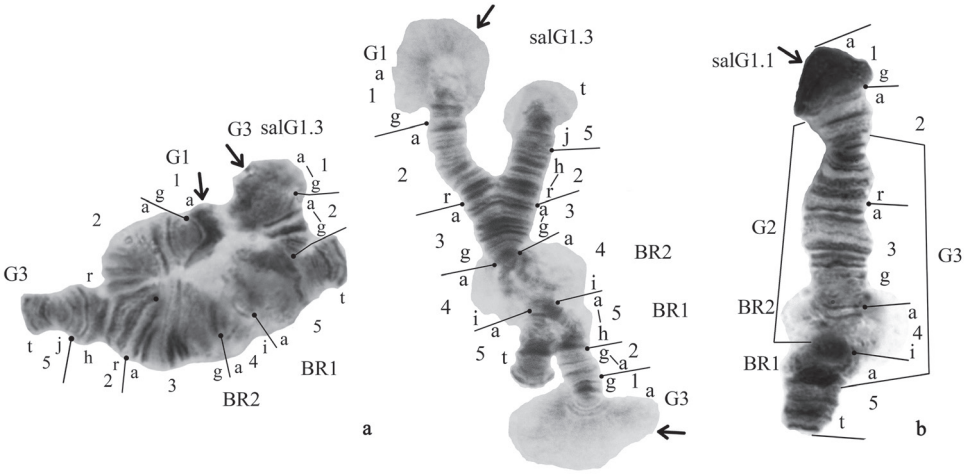

Figure 7. Banding sequences in the arm G of *G. salinus*. Key: **a** two different photo of heterozygote salG1.3 and **b** homozygote salG1.1. Designations as in Fig. 2.

Table 3. Value of genetic distances between the different populations of *G. salinus*.

Population	STS, Atomnoe Lake	STS, Shagan Lake	Altai, Bulatovo Lake	Altai, Gorkoe Lake	Altai, Bolshoe Utichie Lake	Central Caucasus, Tambukan Lake
STS, Atomnoe Lake	0					
STS, Shagan Lake	0.00057	0				
Altai, Bulatovo Lake	0.02639	0.03204	0			
Altai, Gorkoe Lake	0.02539	0.03091	0.00001	0		
Altai, Bolshoe Utichie Lake	0.01519	0.01943	0.00153	0.00129	0	
Central Caucasus, Tambukan Lake	0.08167	0.07787	0.13945	0.13777	0.11999	0

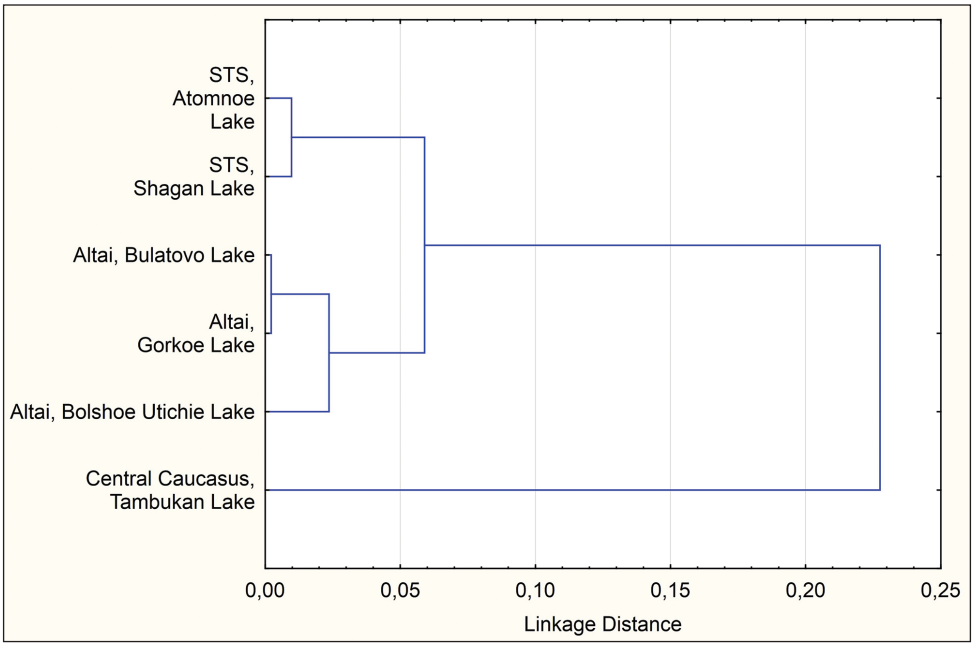

Figure 8. Tree dendrogram for six populations of *G. salinus, single linkage, Euclidean distances.*

Arm F of *G. salinus* was monomorphic in all the studied populations and presented only by the sequence salF1.

Arm G of *G. salinus* was monomorphic in populations of Altai and low polymorphic in populations of Kazakhstan and the Caucasus, although in all populations the dominant banding sequence was salG1. At the same time Kazakhstan and Caucasian populations differ by the set of rare inversions: salG2 was found in Kazakhstan while salG3 occurred in Caucasian population.

The inversion polymorphism of populations of *G. salinus* from the North Caucasus has a high level of heterozygous inversions per specimen and is similar to those of

the Altai populations (Table 2). By the number of banding sequences per population (12), the Caucasian population is closer to Kazakhstan populations, but by the percentage of heterozygous larvae (51%) the studied population is more close to that of the Altai populations (48–62%).

Genetic distances (Table 3) measured by Nei criteria (1972) on the basis of original and previous data (Andreeva et al. 1998) on inversion polymorphism of the species in Altai region and Kazakhstan (Fig. 8) indicate the significant distance and distinct position of the Caucasian population of *G. salinus* in comparison with populations of Altai and Kazakhstan. The dendrogram was constructed on the basis of Nei criteria (1972) using NJ-method.

Discussion

In the northern Caucasus (central part of the northern macroslope) as well as in European Russia, *G. salinus* has been found for the first time.

As mentioned above, the diagnostic larval characters of *G. salinus* from the Caucasus in general are similar to those described by Michailova (1987), Andreeva et al. (1998) and Contreras-Lichtenberg (1999), but there are some significant differences as by some morphological characteristics Caucasian larvae of *G. salinus* are actually closer to *G. barbipes*. The data on larval morphology from the Caucasus are close to data provided by Contreras-Lichtenberg (1999) for *G. salinus* by PSR, width of ventramental plate and inter-plate distance (IPD). However, by width of mentum, MR and total length of larva body the Caucasian material is closer to *G. barbipes*. Also the length of the body of larva of *G. salinus* from Caucasian site is strikingly different from the data provided by Michailova (1987) and Contreras-Lichtenberg (1999) where it is said to be 25–27 mm, while Caucasian larvae are twice shorter (12–14 mm) and are very similar to *G. barbipes* (12 mm). Considering the data of Contreras-Lichtenberg (1999) the length of a larva body is the most different character of *G. salinus* in comparison to other species from the subgenus *Phytotendipes* Goethjebuer, 1934. The length of a larva body of *G. salinus* is the biggest (25–27 mm) among all other species: *G. pallens* (Meigen, 1804) – 10 mm, *G. glacus* (Meigen, 1818) – 12 mm, *G. gropekoveni* (Kieffer, 1913) – 13 (11–18) mm, *G. ospeli* Contreras-Lichtenberg, 1999 – 11 mm, *G. barbipes* (Staeger, 1839) – 12 m and *G. paripes* (Edwards, 1929) - 11–13 mm. At the same time the significant difference between larval length in previously described and Caucasian population of *G. salinus* suggest that further study of these species is necessary to determine the true characteristics of its larvae.

On the basis of morphological data one can conclude that the Caucasian population of *G. salinus* can be a markedly diverged population of the species, probably even subspecies. This conclusion is also supported by comparative analysis of inversion polymorphism between the Caucasian population and populations of other regions.

At present, 15 banding sequences including four new ones – salA2, salB2, salEX, and salG3 – are known in the banding sequences pool of *G. salinus*.

By frequencies of the banding sequences Caucasian population are closer to the Kazakhstan populations than to populations from Altai, but it clearly differ from populations from both other regions by the presence of four new banding sequences. The inversion polymorphism in population of *G. salinus* from the North Caucasus has a high level of heterozygous inversions per specimen and is similar to those of the Altai populations (Table 2). By the number of banding sequences per population, the Caucasian population is close to Kazakhstan populations, but in the percentage of heterozygous larvae, the studied population is more close to that of the Altai populations.

The population of the central Caucasus on the dendrogram of genetic distances (Fig. 8) occupies a distinct position while populations of Altai and Kazakhstan form their own clusters. All the obtained morphological and cytogenetic data may indicate the plausible relative isolation and complexity of the Caucasus from the viewpoint of microevolution. Such arrangement agrees rather well with the geographic location of the studied region. One can say that the north Caucasus is a relatively isolated territory, a special place, or a kind of "island" situated in the "sea" of steppes. Considering the presence in the Ciscaucasia and Greater Caucasus of a large number of saltwater lakes and rivers (Efremov et al. 2010), one can expect a large number of new records of this species in southern Russia. More researches are required in other parts of Caucasus, i.e. Western and Eastern Caucasus and other geographically distant regions for more specific allegations.

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We are sincerely grateful to Dr NA Durnova (Saratov State Medical University named after V.I. Razumovsky) and Dr SV Zhirov (Zoological Institute RAS) for their help in the preparation of this paper. Financial support was provided by the program of the Presidium of the Russian Academy of Sciences "Live nature: modern state and problems of development".

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RESEARCH ARTICLE

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International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Cytogenetic and taxonomic studies of some legless mealybugs (Homoptera, Coccinea, Pseudococcidae)

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Abstract

A new monotypic genus and species, *Komodesia circuliplurima* **gen. et sp. n.**, from Flores Is. (Indonesia) and the new species, *Antonina diversiglandulosa* **sp. n.**, from Southern Thailand are described and illustrated. Chromosomes of these species and also the ones of *Antonina purpurea* Signoret, 1872 and *A. thaiensis* Takahashi, 1942 are studied for the first time: 2n = 30, 20, 12 and 22+Bs correspondingly; the male embryos of all four species demonstrate Lecanoid paternal heterochromatinization of one haploid set of chromosomes. The karyotypes of three widely distributed species, *Antonina pretiosa* Ferris, 1953, *A. graminis* (Maskell, 1897) and *Chaetococcus bambusae* (Maskell, 1893), are studied based on material from other regions in comparison with previously published data. Photographs of the karyotypes are provided for the first time for all seven species. The terminological problems connected with the identification and naming of the three scale insect genetic systems, Lecanoid, Comstockioid and Diaspidoid, are discussed.

Keywords

Antonina, *Chaetococcus*, *Komodesia*, morphology, chromosomes, new species

Introduction

The world fauna of mealybugs includes at least 26 genera characterized by a strong reduction or a total loss of the legs. These genera were recently combined in the informal generic morphological group *Antonina* Signoret, 1875 (Gavrilov-Zimin 2015). The species of the group share the following characters: loss or strong reduction of the

legs, strong reduction of antennae, total or partial sclerotization of the body in mature females, presence of groups of microducts or irregular discoidal pores posteriorly to hind spiracles, absence of ostioles or presence of only poorly developed hind pair of ostioles, loss (with only several exclusions) of circuli, a loss of cerarii (excluding only 1-2 posterior pairs in two monotypic genera), a complete obligate ovoviviparity, a life under the leaf sheath of bamboo grasses, more rarely of other Poaceae grasses or very rarely on some other plants.

Eight of these 26 genera are considered by most modern specialists to be closely related and placed in the separate tribe Serrolecaniini Shinji, 1935. The diagnostic characters of this tribe are caudally directed vulva and groups of microtubular ducts located on ventral cuticle in place of reduced legs or on the surface of peculiar bag-like structures (probably modified hind coxae). The other genera of legless mealybugs perhaps not closely related to each other as well as to Serrolecaniini and demonstrate convergent similarity (see more detailed discussion in Hendricks and Kosztarab 1999). In particular, Serrolecaniini do not include the largest and most widely distributed genus of legless mealybugs – *Antonina*; it includes about 30 species, known mainly from tropical and subtropical regions of the world, especially from Oriental region.

In the present paper a new monotypic genus and species from the Flores Is. (Indonesia), *Komodesia circuliplurima* gen. et sp. n., which must be undoubtedly placed in the tribe Serrolecaniini, and one new species of the genus *Antonina* from Southern Thailand, *A. diversiglandulosa* sp. n., are described and illustrated.

Chromosomal data on legless mealybugs are very scanty. Diploid numbers of chromosomes were previously reported for only 4 species of the genus *Antonina* and one species of *Chaetococcus* Maskell, 1898 (Nur et al. 1987, Gavrilov 2007, Gavrilov and Trapeznikova 2007), but the photo of karyotype was provided only for *Antonina evelynae* Gavrilov, 2003 (Gavrilov 2007). As most of other Pseudococcidae, the legless mealybugs demonstrate the so-called Lecanoid genetic system with specific paternal heterochromatinization of one haploid set of chromosomes in males (Nur 1980, Gavrilov 2007, Gavrilov-Zimin et al. 2015). This heterochromatinization is visible in male tissues during all stages of the life cycle, including embryonal cells (Figs 1c, 8b). The occurrence of heterochromatinization allows detection of the presence of males in the studied populations even when the males and male larvae are not found during the collection of the material. Males of all scale insects are always significantly smaller than females, often live outside of female colonies (even on other host plants or on other parts of the plants) and live as an active imago for a short time only, from several hours to several days. Up to now the males of mealybugs have been collected for some species only, mainly for well-known agricultural pests or for widely distributed species. For example, amongst about 500 species of Palaearctic mealybugs, males were found for no more than 30 species, i.e. about 6% of the faunistic diversity (Danzig and Gavrilov-Zimin 2014). In some species the male embryos (with heterochromatinization) are not detected at all and in these cases the obligate thelytoky is presumed; amongst legless mealybugs two such thelytokous species are known till now, *Antonina graminis* (Maskell, 1897) and *Chaetococcus bambusae* (Maskell, 1893) (Nur et al. 1987).

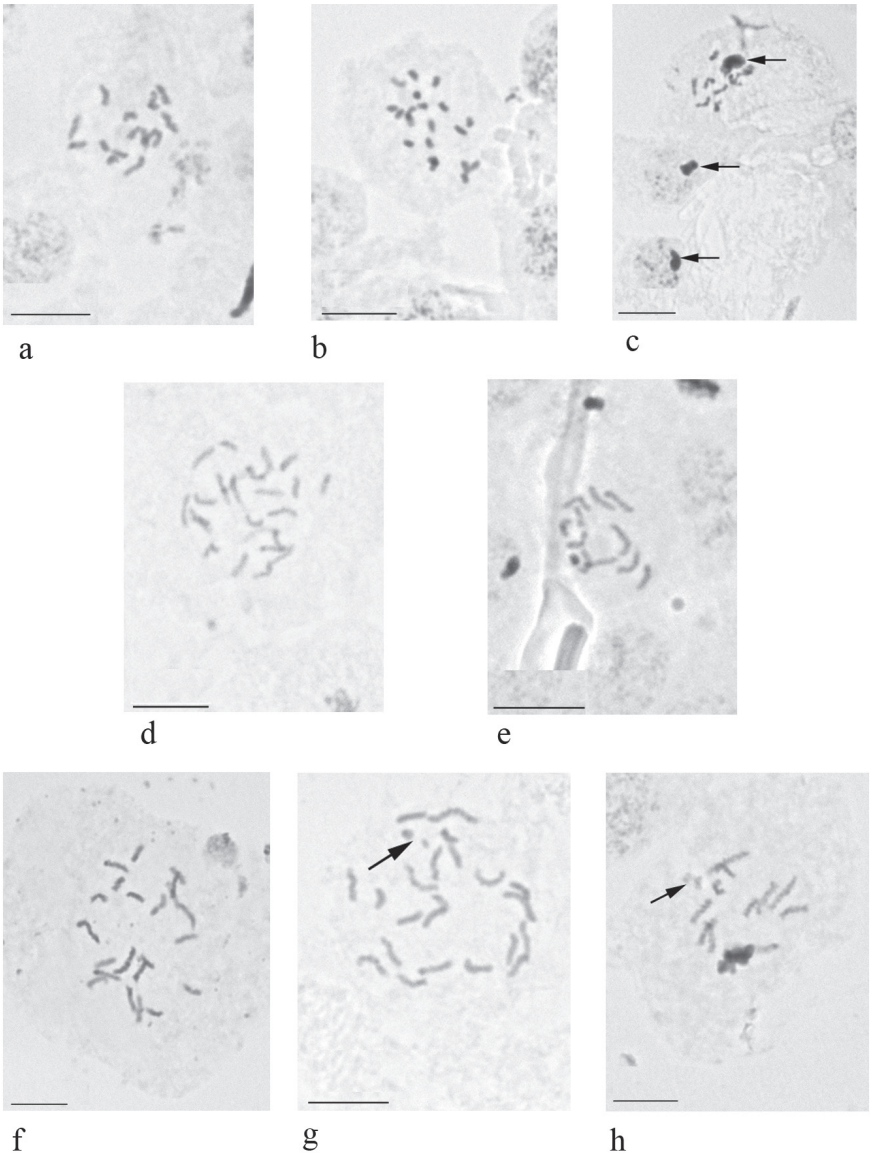

Figure 1. Embryonic cells and chromosomes of species of the genus *Antonina*. **a** *A. graminis*, 2n = 16 **b–c** *A. diversiglandulosa* sp. n. **b** karyotype, 2 n = 20 **c** cells of male embryo with heterochromatinization of paternal set of chromosomes [arrows]) **d** *A. pretiosa*, 2n = 24 **e** *A. purpurea*, 2n = 12 **f–h** *A. thaiensis* (**f** karyotype, 2n = 22 **g** karyotype with 2 B chromosomes [arrow] **h** B chromosomes in male embryo). Bar: 10 µm.

In the current study the karyotypes for both the new species and for two previously unstudied species of the genus *Antonina*, including the type species of the genus, *A. purpurea* Signoret, 1872, were investigated for the first time. Additionally, the earlier noted chromosome numbers of two other widely distributed *Antonina* species and of

the type species of *Chaetococcus*, *Ch. bambusae* (McKenzie 1967, Parida and Moharana 1982, Nur et al. 1987), are confirmed based on material collected in other regions, and the photos of the karyotypes are here published for the first time.

Material and methods

The studied material was collected by the author in different years in Southern Thailand, Indonesia (Flores, New Guinea), Malaysia (Borneo), South France and Morocco. The detailed collecting data are provided below for each species. The numbers with "K" mean unique collecting and preserving numbers for both acetic-ethanol material and Canada balsam slides. All material is deposited at the Zoological Institute, Russian Academy of Sciences (ZIN RAS), St. Petersburg, Russia.

The method of preparation of the morphological Canada balsam slides and method of squashing of the embryonic cells in lactoaceticorcein for chromosomal studies are reported, for example, in Danzig and Gavrilov-Zimin (2014).

Results and discussion

Genus *Antonina* Signoret, 1875

Antonina graminis (Maskell, 1897)

Material. K 1109, Morocco, 10 km South of Ouarzazate, oasis Fint, under leaf sheathes of undetermined Poaceae grass, 28.IX.2013, Ilya Gavrilov-Zimin.

Cytogenetic data. The chromosome number (2n = 16) of this widely distributed species and its parthenogenetic reproduction were reported for the first time in the book of McKenzie (1967) as unpublished data of "Brown, Beardsley, DeLotto, and Nur" without any other details. Many years later in the review of the chromosome numbers of mealybugs (Nur et al. 1987) these data were repeated with the designation of the collecting country "Jamaica". Moreover, Parida and Moharana (1982) reported the same chromosome number based on material from India, but without designation of the mode of reproduction. Unfortunately all the above mentioned publications did not include the karyotype photos. During the current study the gravid females of *A. graminis* from Morocco (above mentioned locality), from Indonesian part of New Guinea (vicinity of Manokwari) and Malaysian Borneo (Sabah, vicinity of Kinabalu National park) have been examined. In all these series the male embryos (with heterochromatinization) were not detected, i.e. the reproduction in the studied populations was obligate thelytokous. It worth mentioning that the females do have a spermatheca attached to the base of the unpaired oviduct, but without sperm bundles inside. The counting of the chromosomes appeared possible only in the material from Morocco, 2n = 16 (Fig. 1a).

***Antonina diversiglandulosa* sp. n.**
http://zoobank.org/B6345E97-0876-4503-BD82-1F9A2170BD63
Figs 2, 3

Material. Holotype, K 1168, Southern Thailand, about 2 km E of Ranong city, under the leaf sheathes of bamboo, 26.XI.2013, Ilya Gavrilov-Zimin, female in a black circle. Paratypes: female on the same slide and 7 females on other slides, all with the same collecting data.

Description. Female. Body broadly oval, up to 3 mm long, sclerotized in mature females (especially posterior segment of abdomen), totally enclosed in thin wax sac. Antennae 2-segmented. Legs totally absent. Anal apparatus located inside long anal tube; anal ring with 6 long setae, each longer than anal tube. Both pairs of ostioles absent. Circulus absent. Multilocular pores of two sizes: larger pores (about 10 μm in diameter) forming band along body margin except several posterior segments; smaller pores (about 6 μm in diameter) forming transverse rows on posterior abdominal sternites IV-VII. Trilocular pores (about 3 μm in diameter) and simple discoidal pores (about 2 μm in diameter) numerous and scattered all over the body surface; trilocular pores which are located inside spiracle atrium are slightly smaller than other trilocular pores. Discoidal pores of irregular structure and size (5-10 μm in diameter) forming two symmetrical groups behind posterior spiracles on abdominal sternites II-VII. Tubular ducts slightly variable in length (being more or less similar with diameter of large multilocular pores), scattered over both body sides. Short flagellate setae sparsely scattered on all segments of body.

Males and morphology of larvae unknown.

Comments. The Oriental species of *Antonina* were recently revised by Williams (2004) who provided a good identification key. The new species is similar to *A. vietnamensis* Williams, 2004 that also has multilocular pores of two sizes (in contrast to all other Oriental species). However, *A. diversiglandulosa* sp. n. differs in having many more large groups of irregular pores located behind spiracles on abdominal sternites II-VII (in contrast to compact groups in *A. vietnamensis* located on sternites II and III only) and in the absence of circulus. In the neighboring Palaearctic fauna, two-sized multilocular pores are known in *A. vera* Borchsenius, 1956 only (Danzig and Gavrilov-Zimin 2015), but this species differs from *A. diversiglandulosa* sp. n. in the total absence of irregular pores.

Etymology. The new species name is derived from two Latin words: “diversus” and “glandula”.

Cytogenetic data. Lecanoid heterochromatinization; 2n = 20 (Fig. 1b–c).

***Antonina pretiosa* Ferris, 1953**

Material. K 881, Indonesia, New Guinea (Irian Jaya), vicinity of Jayapura city, slopes of Cyclop mountains above Entrop, under the leaf sheathes of bamboo, 1.XI. 2011, Ilya Gavrilov-Zimin.

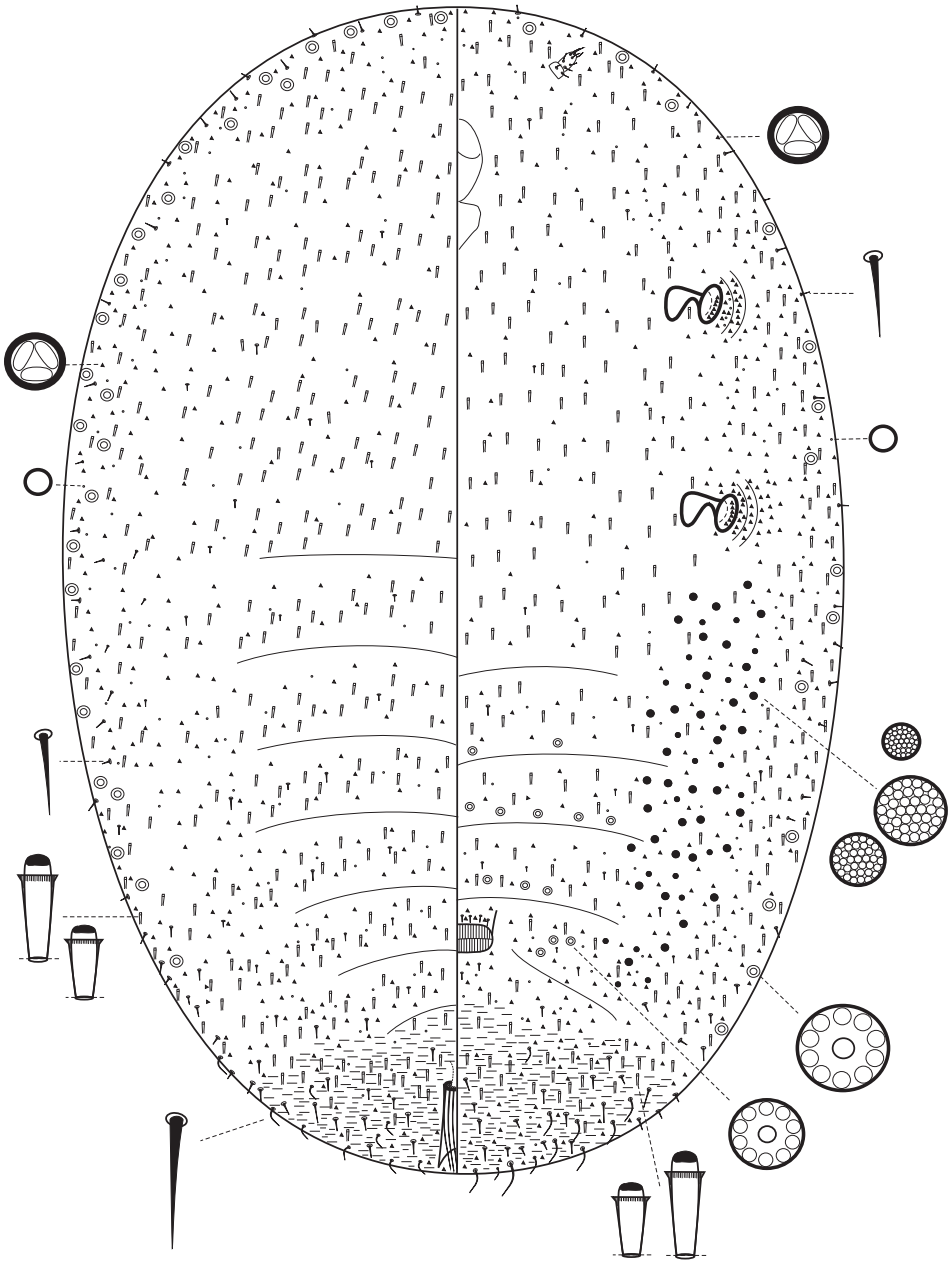

Figure 2. *Antonina diversiglandulosa* sp. n., holotype.

Cytogenetic data. Nur et al. (1987) studied this species in California (USA) and reported 2n = 24+Bs and sexual reproduction (i.e. with heterochromatinization). Our study of gravid females from New Guinea highlighted 24 chromosomes in embryonic cells (Fig. 1d) and Lecanoid heterochromatinization in the male embryos.

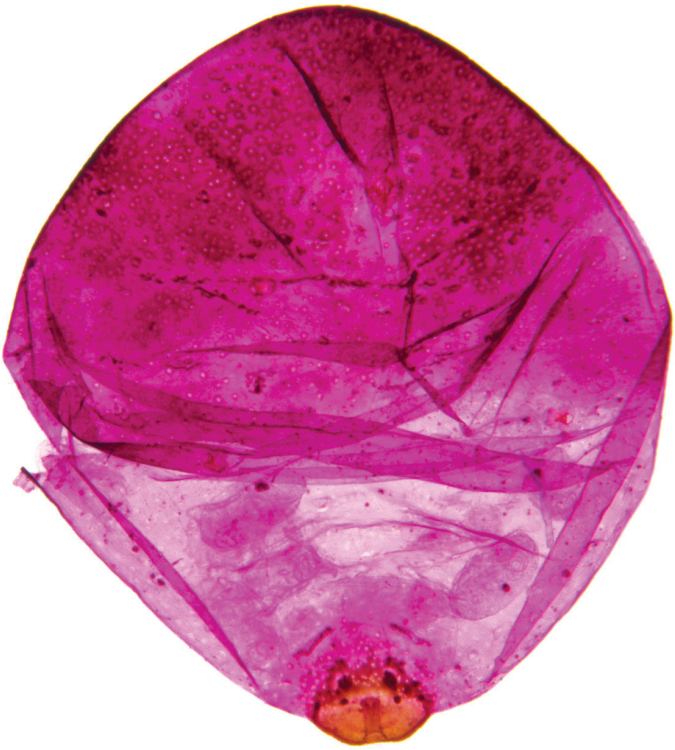

Figure 3. *Antonina diversiglandulosa* sp. n., paratype, prepared mature ovoviviparous female with several fully developed larvae inside the body.

Antonina purpurea Signoret, 1872

Material. K 1205, France, Alpes-de-Haute-Provence, Moustiers-Sainte-Marie, on underground stems of undetermined Poaceae grass (probably *Agropyron* sp.), 1.V.2014, Ilya Gavrilov-Zimin.

Cytogenetic data. Lecanoid heterochromatinization; 2n = 12 (Fig. 1e).

Antonina thaiensis Takahashi, 1942

Material. K 1167, Southern Thailand, about 2 km E of Ranong city, under the leaf sheathes of bamboo, 26.XI.2013, Ilya Gavrilov-Zimin.

Cytogenetic data. Lecanoid heterochromatinization; 2n = 22, 22+Bs (Fig. 1f–h). Cleavage cells of some embryos show 2 additional B-chromosomes which are also visible in the euchromatic haploid set in males (Fig. 1h).

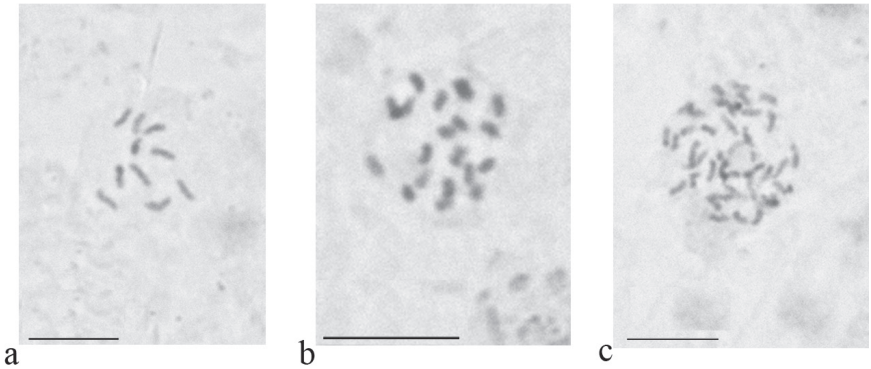

Figure 4. Embryonic cells and chromosomes of *Chaetococcus bambusae*. **a** 2n = 10 **b** 4n = 20 **c** 8n = 40. Bar: 10 μm.

Genus Chaetococcus Maskell, 1898

Chaetococcus bambusae (Maskell, 1893)

Material. K 1172, South Thailand, Phang Nga Province, vicinity of Khura Buri Greenview Resort, under the leaf sheathes of bamboo, 29.XI.2013, Ilya Gavrilov-Zimin.

Cytogenetic data. Nur et al. (1987) reported 2n = 10 and parthenogenetic reproduction in this species, based on material from Jamaica. I have studied two populations of this species from Southern Thailand (mentioned above) and from Indonesian New Guinea (vicinity of Jayapura) and also was not able to find male embryos. So, the species is clearly thelytokous. As in the other here studied thelytokous species *A. graminis*, the *Ch. bambusae* females have a spermatheca at the base of unpaired oviduct, but without sperm bundles inside. The counting of the chromosomes was possible in the material from Thailand only, 2n = 10 (Fig. 4a). Also, a lot of embryonal cells (probably cells of bacteriome) were with 20 and with 40 chromosomes (Fig. 4b–c).

Genus *Komodesia* gen. n.

http://zoobank.org/203E81CE-A5AE-4234-A695-DC68E311EBB1

Type species. *Komodesia circuliplurima* sp. n.

Description. Female. Body elongate oval, sclerotized in mature adult females. Three posterior abdominal segments with lateral lobes. Antennae 2-segmented. Legs totally absent. Clypeolabral shield with anterior projection. Vulva caudally directed. Anal apparatus located inside a short anal tube. Both pairs of ostioles absent. Circuli 5 in number, small, round, all of about the same size. Multilocular pores absent. Trilocular pores present. Simple discoidal pores sparsely scattered over all body surface. Microtubular ducts ("duct like pores") very short, button shaped, forming two sym-

Figure 5. Indonesia, Flores Is., vicinity of Labuan Bajo, the type locality of *Komodesia circuliplurima* gen. et sp. n.

metrical groups on venter from posterior spiracles to abdominal sternite VI. Tubular ducts (all with small collars) present. Dorsal and ventral surface of body covered by minute flagellate setae.

Comments. The new monotypic genus differs from all other genera of the tribe Serrolecaniini (and all legless mealybugs) in the presence of very short, button-shaped microtubular ducts in groups behind posterior spiracles and in numerous circuli (5 in number).

Etymology. The generic name is after the name of Komodo Island (and appropriate National Park) in vicinity of which the new genus was collected (Fig. 5). Gender feminine.

***Komodesia circuliplurima* sp. n.**
http://zoobank.org/510F70E0-7CBF-4554-BD55-A7FF643AE72C
Figs 6–7

Material. Holotype, female, K 979, Indonesia, Flores Is., vicinity of Labuan Bajo, under the leaf sheathes of Poaceae grass, 15.XII.2012, Ilya Gavrilov-Zimin, weakly sclerotized female on the slide. Paratypes: heavily sclerotized female on the same slide, 2 other females on other slide with the same collecting number and data; 2 females K 993, Indonesia, Flores Is., vicinity of Labuan Bajo, Wae Cicu, under the leaf sheath of Poaceae grass, 18.XII.2012, Ilya Gavrilov-Zimin.

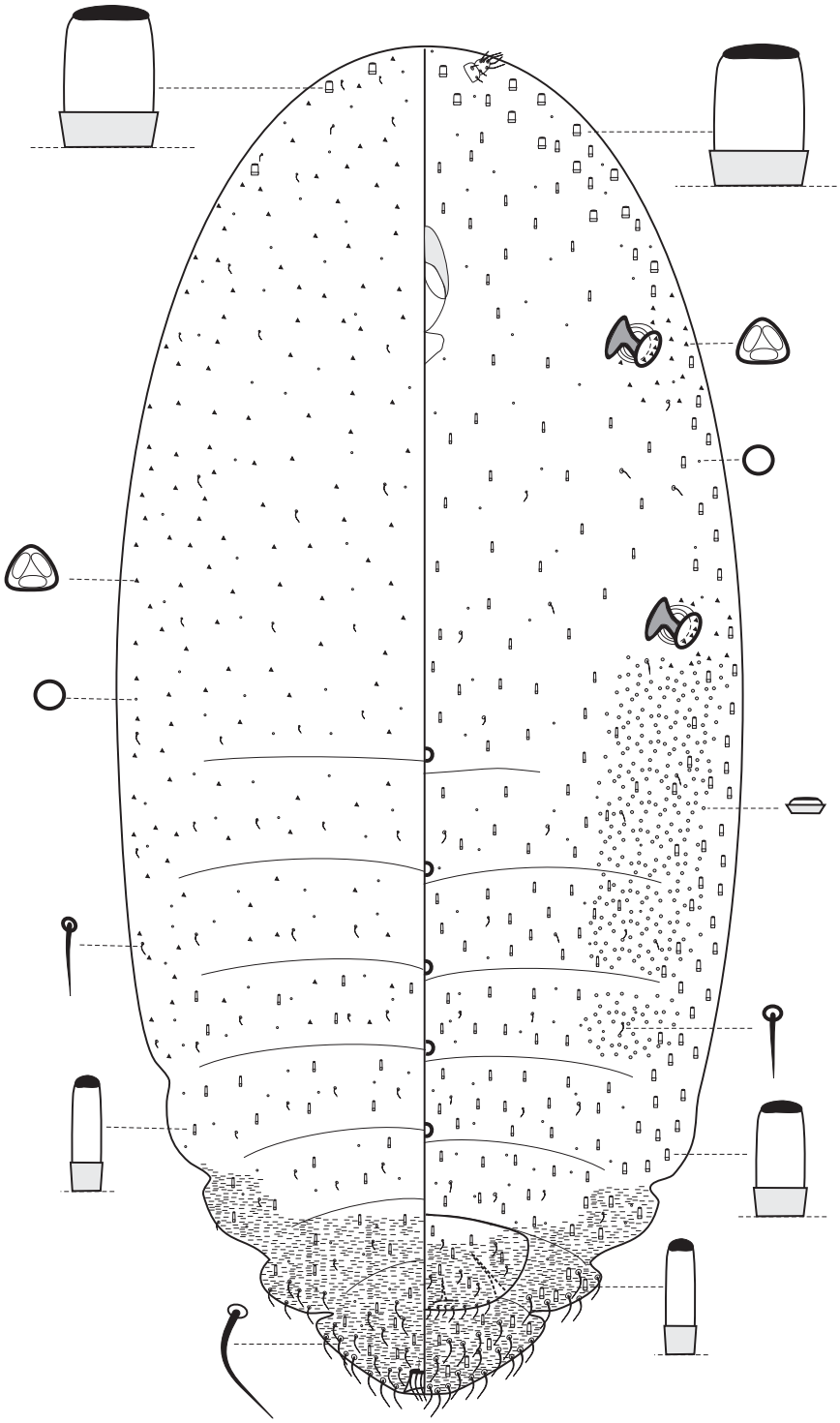

Figure 6. *Komodesia circuliplurima* gen. et sp. n., holotype.

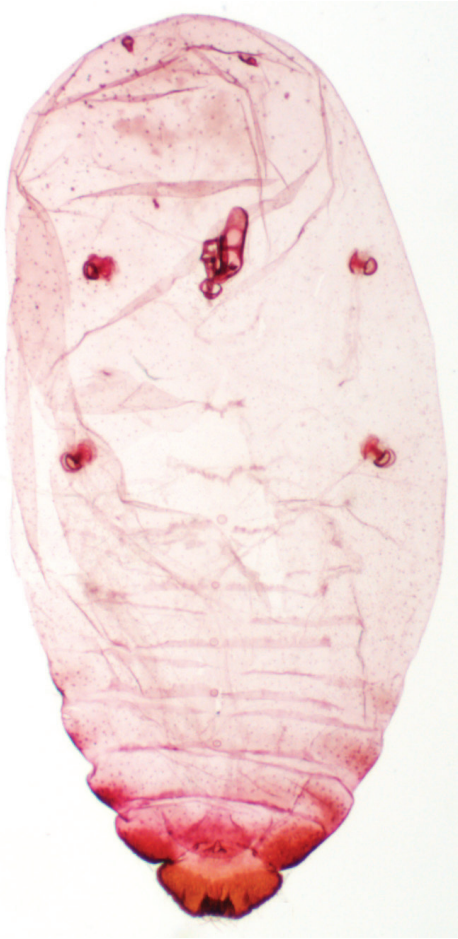

Figure 7. *Komodesia circuliplurima* gen. et sp. n., holotype, prepared female on slide.

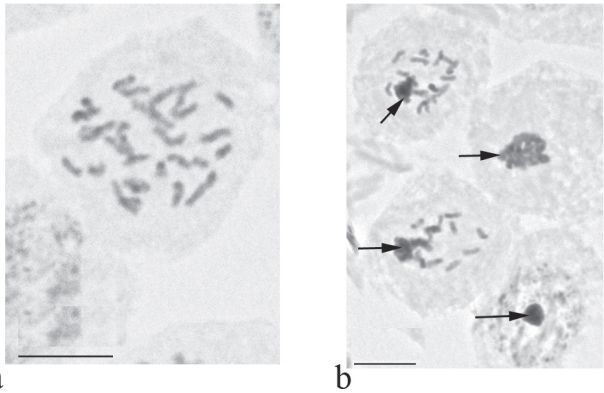

Figure 8. Embryonic cells and chromosomes of *Komodesia circuliplurima* gen. et sp. n. **a** 2n = 30 **b** cells of male embryo with heterochromatinization of paternal set of chromosomes (arrows). Bar: 10 μm.

Description. Female. Body elongate oval, up to 5 mm long, sclerotized in mature adult females. Three posterior abdominal segments with lateral lobes. Antennae 2-segmented. Legs totally absent. Clypeolabral shield with anterior projection. Vulva caudally directed. Anal apparatus located inside short anal tube; clear structure of anal ring invisible in available females, but the ring bears 6 long setae, which longer than anal tube. Both pairs of ostioles absent. Circuli 5 in number, small, round, all of about the same size. Multilocular pores absent. Trilocular pores (each about 4 μm in diameter) scattered over dorsal surface of cephalothorax and anterior abdominal tergites, forming sparse groups around spiracles on venter and totally absent on four posterior abdominal segments. Simple discoidal pores (each about 2 μm in diameter) sparsely scattered on all body surface. Microtubular ducts very short (each about 3 μm in diameter), button-shaped, forming two symmetric groups on venter from posterior spiracles to abdominal sternite VI. Tubular ducts (all with small collars) of three main sizes: largest ducts about two times wider than diameter of trilocular pore numerous on head; mid-sized ducts similar in width to trilocular pore, forming marginal band along all body venter; smallest tubular ducts about 1.5 times thinner than diameter of trilocular pore, scattered on abdominal tergites and numerous in medial and submedial zones of venter. Dorsal and ventral surface of body covered with rare minute flagellate setae; significantly longer setae present on both sides of two posterior abdominal segments.

Males and morphology of larvae unknown.

Etymology. The new species name is derived from two Latin words: "*circulus*" meaning circle and "*plurimus*" meaning many in plural and means: "with many circuli".

Cytogenetic data. Lecanoid heterochromatinization; 2n = 30 (Fig. 8). *K. circuliplurima* gen. et sp. n. demonstrates the highest number of chromosomes currently known in legless mealybugs.

Discussion of cytogenetic data

Together with the data reported in this paper, the chromosome numbers are now known in nine species of legless mealybugs from 3 genera (Table 1).

In spite of a small number of studied species, six different diploid chromosome numbers are known at present and it seems that legless mealybugs are more diverse in this character than other groups of mealybugs (see the information for all Pseudococcidae in the catalogue of Gavrilov 2007). It correlates with morphological diversity of this group, most genera of which are monotypic and distinctly differ from each other. It is also interesting that the modal number of chromosomes of all mealybugs (2n = 10) is known as of now for only one species of legless mealybugs (*Ch. bambusae*), that may be considered as an additional evidence of intensive evolution in the group.

Most of studied species (7 out of 9) of legless mealybugs possesses the Lecanoid genetic system of reproduction. Traditionally, in the works of old American

Table 1. Chromosomal numbers and genetic systems of legless mealybugs (S – sexual reproduction, P – parthenogenesis without detailing, T – thelytoky, L – Lecanoid heterochromatinization in embryos, Bs – B-chromosomes).

Species	2n	Genetic system	Reference
Antonina crawi Cockerell, 1900	12	S	Nur et al. 1987 [Hawaii, USA]
A. evelynae Gavrilov, 2003	12	L	Gavrilov 2007 [Sochi, Russia]
A. graminis (Maskell, 1897)	16	P	Nur et al. 1987 [Jamaica]
	16	?	Parida & Moharana 1982 [India]
	16	T	Present study [Morocco]
A. diversiglandulosa sp. n.	20	L	Present study [Thailand]
A. pretiosa Ferris, 1953	24+ Bs	S	Nur et al. 1987 [California, USA]
	24	L	Present study [New Guinea, Indonesia]
A. purpurea Signoret, 1872	12	L	Present study [France]
A. thaiensis Takahashi, 1942	22+Bs	L	Present study [Thailand]
Chaetococcus bambusae (Maskell,1893)	10	P	Nur et al. 1987 [Jamaica]
	10	T	Present study [Thailand]
Komodesia circuliplurima gen. et sp. n.	30	L	Present study [Flores Is., Indonesia]

cytogeneticists (Hughes-Shrader 1948, Brown 1965, Nur 1980 and others) three different genetic systems (Lecanoid, Comstockioid [Comstockiella] and Diaspidoid [Diaspidid]) with heterochromatinization and obligate bisexual reproduction were considered. In the special terminological paper (Gavrilov and Kuznetsova 2007) we suggested unifying the endings of these names and use them not only for bisexual reproduction (as earlier), but also for facultative arrhenotoky and deuterotoky when males also have the same heterochromatinization (see our argumentation in the cited paper). However, at that time we did not discuss another terminological problem: the differences between the Lecanoid system and its derivate Comstockioid system are very difficult to detect in practice being visible only just prior to prophase I of spermatogenesis; moreover, some species demonstrate Lecanoid and Comstockioid systems (and different variants of Comstockioid!) in the same male (Nur 1980). In practice, it leads to the situation when it is impossible to clearly identify the genetic system in absolute majority of karyotyped species, because the male larvae (with meiotic and premeiotic cells inside) are collected very rarely and even if collected some of them only may provide a good material for chromosome studies. Consequently, most species listed by Nur et al. (1987) are noted as having "sexual reproduction", without exact designation of the genetic system. We propose that only two systems with heterochromatinization, Lecanoid and Diaspidoid which show discrete differences from each other at all stages of the life cycle, should be accepted. On the other hand, the term "Comstockioid" is proposed to use only for the derivative variant of spermatogenesis within the Lecanoid system. Thus, for the species of legless mealybugs studied in the present paper I designate the Lecanoid system based only on the presence of paternal genome heterochromatinization in the embryos.

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The author thanks Dr Dmitry A. Gapon for consultations in the Latin grammar and Dr Boris A. Anochin for the help with the microphotographs. I am also grateful to Dr Giuseppina Pellizzari and Dr. San-an Wu for useful comments and remarks. The work was performed in the frame of the state research project no. 01201351189 in Zoological Institute, Russian Academy of Sciences and was partly supported by a grant from the government of St. Petersburg.

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RESEARCH ARTICLE

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International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

An uncommon co-localization of rDNA 5S with major rDNA clusters in Callichthyidae (Siluriformes): a report case in *Corydoras carlae* Nijssen & Isbrücker, 1983

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Abstract

Corydoras Lacepède, 1803 is the most specious genus of Corydoradinae subfamily and many of its species are still unknown in relation to molecular cytogenetic markers. However, the diploid number and karyotypic formula were recorded for many species of this group. In current study, we provided the first cytogenetic information of *Corydoras carlae* Nijssen & Isbrücker, 1983, an endemic fish species from Iguassu River basin, Paraná State, Brazil. The individuals were collected in Florido River, a tributary of Iguassu River and analysed with respect to diploid number, heterochromatin distribution pattern, Ag-NORs and mapping of 5S and 18S ribosomal genes. The karyotype of this species comprises 46 chromosomes arranged in 22m+22sm+2st. The heterochromatin is distributed in centromeric and pericentromeric positions in most of the chromosomes, and also associated with NORs. The Ag-NORs were detected in the terminal position on the long arm of the metacentric pair 6. The double-FISH technique showed that 5S rDNA and 18S rDNA were co-localized in the terminal portion on the long arm of the metacentric pair 6. This condition of co-localization of ribosomal genes in *C. carlae* seems to represent a marker for this species.

Keywords

Ag-NORs, cytogenetic markers, ribosomal DNA, heterochromatin, karyotype

Introduction

In higher eukaryotes, rDNA is organized into two distinct gene classes: major class (45S rDNA), which contains the genes that code for the 18S, 5.8S and 28S rRNAs, and the minor class (5S rDNA), which contains the genes that code for 5S rRNA. Fish species have been analyzed for 5S and 18S rDNA location in chromosomes using fluorescent *in situ* hybridization (FISH). The major rDNA sequences detected by FISH always coincided with silver-stained NORs (Ag-NORs) location, although in species with multiple Ag-NORs the number of markings was usually smaller than the regions detected by the DNA probes.

The most common condition in the karyotype of different fish groups is the positioning of ribosomal genes in different chromosome pairs (Galetti Jr. and Martins 2004). However, syntenic localization of the major rDNA clusters and the 5S sites were observed for the first time in the genus *Corydoras* Lacepède, 1803 (present study) and *Callichthys callichthys* (Linnaeus, 1758) (Konerat et al. 2014), the other integrant of the family Callichthyidae. In Loricariidae, *Kronichthys lacerta* (Nichols, 1919), *Isbrueckerichthys duseni* (Miranda Ribeiro, 1907), *Parotocinclus maculicauda* (Steindachner, 1877) and *Trichomycterus* sp. (Ziemniczak et al. 2012) also presented syntenic localization of ribosomal genes. Thus, the mapping of ribosomal genes has added important information about the chromosomal diversification in *Corydoras*, as in other groups of Siluriformes.

Callichthyidae is a family of the order Siluriformes widely distributed in Neotropical region, which has 215 valid species, divided in two subfamilies, Callichthyinae with 17 valid species and Corydoradinae with 198 valid species (Eschmeyer and Fong 2016). *Corydoras* is the most specious and cytogenetically studied genus of Corydoradinae, demonstrating different diploid numbers, which may vary from 2n = 40 chromosomes in *C. nattereri* Steindachner, 1876 (Oliveira et al. 1990, 1993) to 2n = 134 chromosomes in *C. aeneus* (Gill, 1858) (Turner et al. 1992).

Considering aspects related to number and morphology of chromosomes, as well as analysis of DNA content, Oliveira et al. (1992) and Shimabukuro-Dias et al. (2004) proposed the existence of five groups of species in *Corydoras*. However, the vast majority of studies in *Corydoras* is restricted to conventional analysis and little is known about location of the different types of rDNA, only in *C. paleatus* (Jenyns, 1842) and *C. ehrhardti* Steindachner, 1910 for 18S rDNA (Artoni et al. 2006) and *C. britskii* (Nijssen & Isbrücker, 1983) for 18S and 5S rDNA (Takagui et al. 2014), making essential the development of studies with this approach to better understand the relationships between species of *Corydoras*.

Thus, the current paper presents the first cytogenetic description of *Corydoras carlae*, focusing on karyotype characterization, heterochromatin distribution pattern and location of 5S and 18S rDNA sites. Besides the new data for the species, this study also reveal for the first time the co-localization of 5S and major rDNA in Callichthyidae.

Materials and methods

Ten individuals (four females and six males) of *Corydoras carlae* were sampled in the Florido River (26°00'32.60"S; 53°25'50.70"W), Paraná State, Brazil. A sub-tributary of left margin of Lower Iguassu River that flows into Capanema River, which flows immediately above of the Iguassu falls (Fig. 1). Voucher specimens were deposited in the fish collection of the Núcleo de Pesquisas em Limnologia Ictiologia e Aquicultura (NUPELIA), Universidade Estadual de Maringá, Paraná, Brazil, as *Corydoras carlae* (NUP 17885).

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, approved by the Committee on the Ethics of Animal Experiments of the Universidade Estadual do Oeste do Paraná (License Number: Protocol 13/09 – CEEAAP/Unioeste). Before the evisceration process, the individuals were anesthetized by an overdose of clove oil (Griffiths 2000). Metaphase chromosomes were obtained from anterior kidney cells using the air-drying technique (Bertollo et al. 1978). Analysis of the C-positive heterochromatin (C-bands) followed the basic procedure of Sumner (1972), with some minor adaptations (Lui et al. 2012). The NORs were detected by means of silver nitrate staining (Ag-NORs), according to Howell and Black (1980). The chromosomes were classified as metacentric (m), submetacentric (sm), and subtelocentric (st) according to their arm ratio (Levan et al. 1964). For the determination of the fundamental number (FN), or number of chromosome arms, the m, sm and st chromosomes were considered as bearing two arms and the acrocentric chromosomes only one arm.

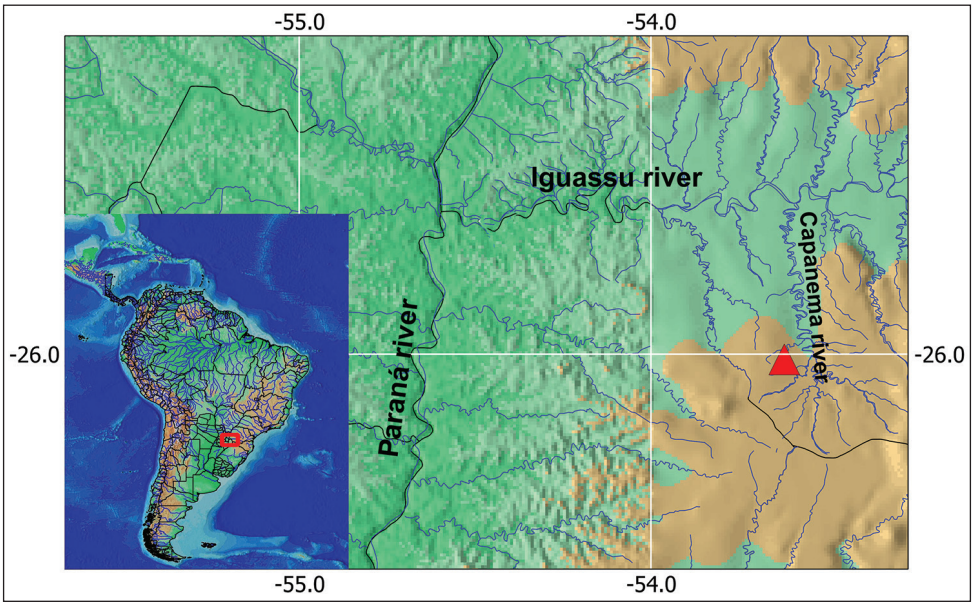

Figure 1. Localization of Florido River from the Iguassu River basin, where *Corydoras carlae* individuals were captured. Red triangle indicates the sampled point.

The localization of the 5S and 18S rDNA sites in the chromosomes was performed using the fluorescence *in situ* hybridization (FISH) method (Pinkel et al. 1986) with modifications (Margarido and Moreira-Filho 2008), with probes obtained from the fish species *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti Jr 1999) and *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka and Galetti Jr 2004), respectively. The probes were labelled through nick translation, with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA) (Roche). Detection and amplification of the hybridization signal were made using avidin-FITC and anti-avidin biotin (Sigma) for probes labelled with biotin, and anti-digoxigenin rhodamine (Roche) for probes labelled with digoxigenin. Slides were counterstained with DAPI (50 µg/mL) and analysed in epifluorescence microscope (Olympus BX61). The images were captured using the software DP controller (Media Cybernetics).

Results

Corydoras carlae presented a modal diploid number of 46 chromosomes in males and females, and the karyotype contained 22 metacentric, 22 submetacentric and 2 subtelocentric chromosomes (22m+22sm+2st), yielding a FN of 92 in both sexes (Fig. 2a). The Ag-NORs was detected in the terminal position on the long arm of metacentric pair 6 (Box Fig. 2a). Positive C-band heterochromatins were detected in the centromeric and pericentromeric regions of nine and eight pairs, respectively, and coincident with the ribosomal sites (Fig. 2b). The double-FISH technique showed 5S rDNA cluster appears interspersed with 18S cistrons in the terminal portion of the long arm of pair 6 (Fig. 2c). Thus, featuring synteny and co-location of ribosomal genes in *C. carlae*. The ideogram summarizes all markers on chromosomes of *C. carlae* (Fig 3).

Discussion

Cytogenetic studies have classified the species of the genus *Corydoras* into five groups according to their karyotype composition (Oliveira et al. 1992, Shimabukuro-Dias et al. 2004). *Corydoras carlae* has been included in group 4 (2n = 40-52 chromosomes, with many metacentric and submetacentric chromosomes). Considering our results, three species of this group occurring in the Iguassu River basin were cytogenetically analyzed: *C. carlae* (2n=46, 22m+22sm+2st), collected in the Lower Iguassu River; *C. paleatus* (2n=44, 20m+24sm) collected in the Upper Iguassu River (Oliveira et al. 1993), and *C. paleatus* and *C. ehrhardti* (2n=44, 18m+26sm), collected in the Upper Tibagi River (Artoni et al. 2006).

Individuals of *C. carlae* analyzed here probably do not co-occur with *C. paleatus* from Upper Iguassu River, since the lower portion is characterized by numerous waterfalls which gave rise to several reservoirs (Baumgartner et al. 2012). Therefore, the geographic isolation of *C. carlae* may have facilitated the establishment of this nu-

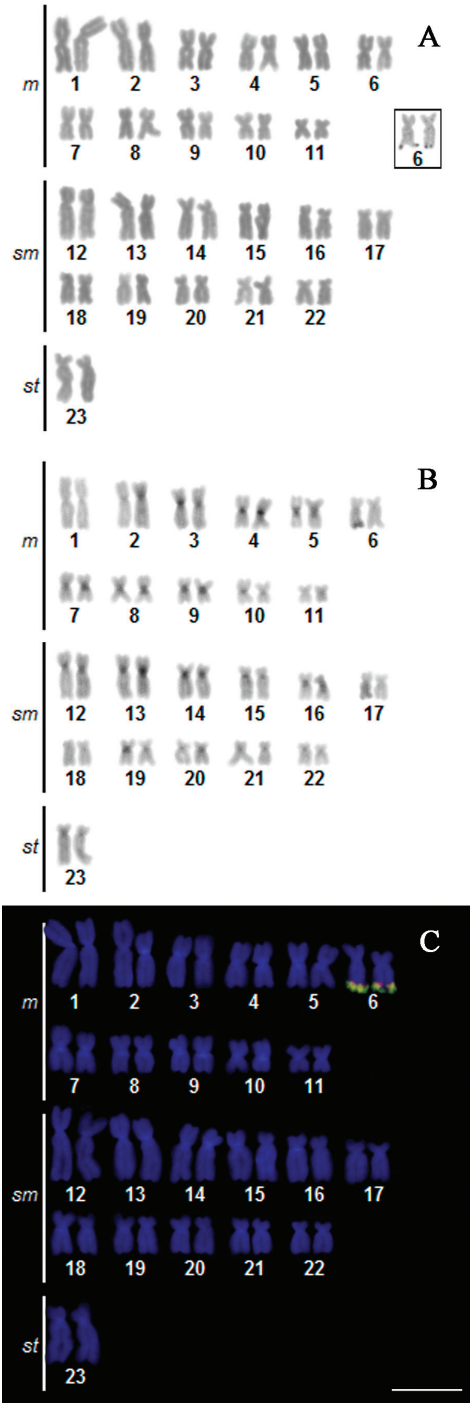

Figure 2. Karyotypes of *Corydoras carlae* stained with **a** Giemsa **b** C-banded and **c** after double FISH with 5S rDNA probes (red) and 18S rDNA (green). The NORs bearing chromosomes (pair 6) are boxed. Bar = 10 µm.

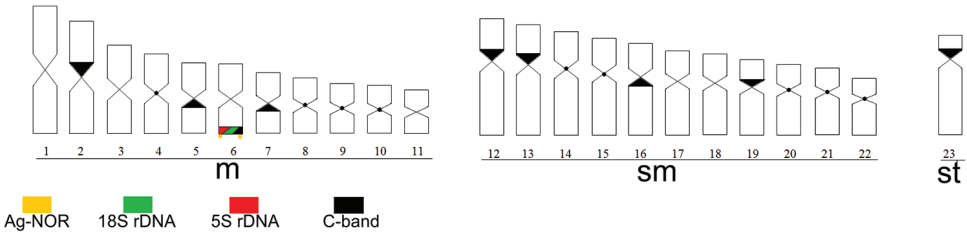

Figure 3. Ideogram of *Corydoras carlae*, showing the heterochromatin, Ag-NORs, 18S and 5S rDNA distribution pattern.

merical and structural karyotypic variation, as also observed in different populations of *Glanidium ribeiroi* Haseman, 1911 along the Iguassu River basin (Lui et al. 2015). Thus, the lack of gene flow among *Corydoras* species in the Iguassu River basin could favor different changes in each sample, supposedly resulting in speciation.

The number and position of NORs in *Corydoras* species are quite variable and almost all information pertaining to the characterization of NORs in this species is based on silver nitrate impregnation (Table 1). These data show that most species have simple NORs located in the terminal position, as in the case of *C. carlae*. However, not all species have this pattern, as in the case of *C. simulatus* Weitzman & Nijssen, 1970 with interstitial NORs (Oliveira et al. 1992), as well as *C. britskii* (Takagui et al. 2014), *C. simulatus*, *Corydoras* sp. Galheiro river, *C. flaveolus* Ihering, 1911 and *C. metae* Eigenmann, 1914 (Oliveira et al. 1992), which exhibits a systems of multiple NORs. According to Oliveira and Gosztonyi (2000), the condition of simple Ag-NORs in terminal location is the possible basal condition for Siluriformes. Thus, *C. carlae* and other species presenting simple Ag-NORs in terminal location seem to maintain this basal condition.

Despite exhibiting wide variation on the diploid number, chromosome morphology and location of NORs, *Corydoras* species share a heterochromatin distribution pattern very similar, preferably centromeric and pericentomeric, and in most cases, associated to NORs. In *C. carlae*, this pattern was also observed, with heterochromatic blocks also displayed in many chromosomes. *Corydoras britskii* from Miranda River also showed large amount of pericentromeric heterochromatin, but with terminal heterochromatic blocks (Takagui et al. 2014), which were not observed in this study.

The mapping of 18S rDNA and 5S rDNA are scarce in Callichthyidae, being known only for some species (Table 1). Concerning the genus *Corydoras*, *C. carlae* exhibited only one chromosome pair bearing 18S rDNA sites, as well as *C. ehrhardti* (Artoni et al. 2006), confirming the system of simple NORs evidenced by silver impregnation for both species. FISH with rDNA probes has helped detect the presence of inactive NORs, as in the case of *C. paleatus* (Artoni et al. 2006), which presented multiple NORs sites after 18S-FISH, while the silver impregnation had detected only simple NORs. Thus, studies with 18S-FISH can be useful for better evaluating the pattern distribution of the NORs in *Corydoras*.

Table 1. Ag-NORs, major and minor ribosomal genes sites distribution in Callichthyidae. The 45S and 5S columns report the number of chromosomes bearing the cistrons and its location.

Family Callichthyidae	Locality	2n	Ag-NOR	45S	5S	Note	References
Subfamily Corydoradinae							
Corydoras carlae	Florido River/Paraná State, Brazil	46	simple	2, q terminal	2, q terminal	Synteny, Co-localization	Present study
Corydoras britskii	Miranda River/ Mato Grosso do Sul State, Brazil	90	multiple	3, p terminal	2 p, interstitial	Non-Synteny	Takagui et al. (2014)
Corydoras paleatus	Tibagi River/Paraná State, Brazil	44	simple	3, q terminal	-------	---------	Artoni et al. (2006)
Corydoras ehrhardti	Tibagi River/Paraná State, Brazil	44	simple	2, q terminal	-------	---------	Artoni et al. (2006)
Corydoras sodalis	from aquarium	74	simple	---------	---------	---------	Shimabukuro-Dias et al. 2004
Corydoras arcuatus	Tabatinga River/frontier Brazil and Peru	46	simple	---------	---------	---------	Oliveira et al. 1992
Corydoras trilineatus	Caripi River/Pará State, Brazil	46	simple	---------	---------	---------	Oliveira et al. 1992
Corydoras schwartzi	Negro River/Amazonas State, Brazil	46	simple	---------	---------	---------	Oliveira et al. 1992
Corydoras cf. *simulatus*	Colombia	62	simple	---------	---------	---------	Oliveira et al. 1992
Corydoras sp. *Caripi River*	Caripi River/Pará State, Brazil	60	simple	---------	---------	---------	Oliveira et al. 1992
Corydoras reticulatus	Negro River/Amazonas State, Brazil	74	simple	---------	---------	---------	Oliveira et al. 1992
Corydoras aff. *punctatus* Negro River	Negro River/Amazonas State, Brazil	102	simple	---------	---------	---------	Oliveira et al. 1992
Corydoras simulatus	Colombia	62	multiple	---------	---------	---------	Oliveira et al. 1992
Corydoras sp. Galheiro River	Galheiro River/Minas Gerais State, Brazil	84	multiple	---------	---------	---------	Oliveira et al. 1992
Corydoras flaveolus	Alambari River/São Paulo State, Brazil	58	multiple	---------	---------	---------	Oliveira et al. 1992
Corydoras metae	Colombia	92	multiple	---------	---------	---------	Oliveira et al. 1992
Subfamily Callichthyinae							
Hoplosternum littorale	Contas River/Bahia State, Brazil	60	simple	2, p terminal	4, p terminal	Non-Synteny	Almeida et al. (2012)
Callichthys callichthys	Paraná River/Paraná State, Brazil	56	simple	2-3, p terminal e interstitial	7-9, p interstitial and terminal	Synteny, Adjacent regions	Konerat et al. (2014)
Hoplosternum littorale	Coastal River/São Paulo State Brazil	60	simple	2, p terminal	4 p terminal	Non-Synteny	Pazza et al. (2005)
Callichthys callichthys	Contas River/Bahia State, Brazil	54	multiple	7, p terminal, 5, q terminal, 1 p interstitial	8-12, p interstitial and terminal	Non-Synteny	Almeida et al. (2013)
Lepthoplosternum pectorale	Paraná River/Paraná State, Brazil	64	multiple	10, p terminal; 2, q terminal	6, p terminal	Non-Synteny	Konerat et al. (2014)

In *Corydoras*, data on the location and number of 5S rDNA cistrons had only been described for *C. britskii*, for which was detected interstitially in a pair of subtelocentric chromosomes (Takagui et al. 2014). In *C. carlae*, the 5S rDNA was displayed at terminal position on the long arm of the metacentric pair 6. The presence of one chromosome pair bearing 5S rDNA is a common feature in different families of Siluriformes (Swarça et al. 2009), although multiple loci of 5S rDNA have been observed in Callichthyinae (Table 1). Inter– and intra–individual numerical and position variations of 5S rDNA cistrons have been observed in Callichthyidae and seem to represent a species-specific marker.

Furthermore, 5S rDNA cluster appears interspersed with 18S cistrons, featuring synteny and co-location of ribosomal genes in *C. carlae*. The synteny is an unusual feature in fish, and such condition could influence an unwanted translocation of 5S sequences within 45S clusters, which could probably occur if these clusters were maintained linked in the same chromosome area (Martins and Galetti Jr 1999). This may explain why most vertebrates have these sequences on different chromosomes. Interestingly, all the possible syntenic conditions have been found in fishes, both sets of genes in distinct and disjoint chromosomal regions, as observed in *Parodon nasus* Kner, 1859 cited as *Parodon tortuosus* (Vicente et al. 2001) and *Astyanax paranae* Eigenmann, 1914 cited as *Astyanax scabripinnis* (Mantovani et al. 2005), or in adjacent regions, as in *Triportheus nematurus* (Kner, 1858) (Diniz et al. 2009), *Mugil incilis* Hancock, 1830 (Hett et al. 2011), *Kronichthys lacerta*, *Isbrueckerichthys duseni*, *Parotocinclus maculicauda*, *Trichomycterus* sp. (Ziemniczak et al. 2012) and *Callichthys callichthys* (Konerat et al. 2014), or the 5S rDNA interspersed along the clusters of 45S rDNA (co-localization), as in *Astyanax altiparanae* Garutti & Britski, 2000, *Astyanax lacustris* (Lütken, 1875), *Astyanax fasciatus* (Cuvier, 1819), *Astyanax schubarti* Britski, 1964 and *Astyanax paranae* cited as *Astyanax scabripinnis* (Almeida-Toledo et al. 2002), *Solea senegalensis* Kaup, 1858 (Cross et al. 2006), *Bryconamericus* cf. *iheringii* (Piscor et al. 2013) and *Corydoras carlae* (present study).

Despite little studies about mapping of rDNA genes in Callichthyidae, the majority of the species share the condition of non-synteny between the 5S rDNA and 45S rDNA. Therefore, this condition of co-localization of ribosomal genes in *C. carlae* seems to represent a marker for this species.

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RESEARCH ARTICLE

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First cytogenetic analysis of *Ichthyoelephas humeralis* (Günther, 1860) by conventional and molecular methods with comments on the karyotypic evolution in Prochilodontidae

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Abstract

We used conventional cytogenetic techniques (Giemsa, C-banding, Ag-NOR), and fluorescent *in situ* hybridization (FISH) with 5S and 18S rDNA probes to investigate the karyotype and cytogenetic characteristics of *Ichthyoelephas humeralis* (Günther, 1860) from Ecuador. The specimens studied have a karyotype with 2n=54 biarmed chromosomes (32 M + 22 SM) and C-positive heterochromatin located on the centromeric, pericentromeric, interstitial, and terminal regions of some chromosomes. The nucleolus organizer regions occurred terminally on the long arm of chromosome pair 2. FISH confirmed the presence of only one 18S rDNA cluster with nonsyntenic localization with the 5S rDNA. Cytogenetic data allow us to refute the earlier morphological hypothesis of a sister relationship between *Semaprochilodus* Fowler, 1941 and *Ichthyoelephas* Posada Arango, 1909 and support the molecular proposal that *Ichthyoelephas* is a sister group to the monophyletic clade containing *Prochilodus* Agassiz, 1829 and *Semaprochilodus*.

Keywords
Karyotype, evolution, Prochilodontidae, Fluorescent *in situ* hybridization, NORs

Introduction

The fish family Prochilodontidae includes 21 valid species, with three recognized genera: *Ichthyoelephas* Posada Arango, 1909, *Prochilodus* Agassiz, 1829 and *Semaprochilodus* Fowler, 1941 (Castro and Vari 2004, Eschmeyer and Fong 2016). These species constitute a valuable resource of commercial and subsistence freshwater fish distributed throughout the South American countries, except Chile (Lowe-McConnell 1975, Goulding 1981, Flecker 1996). *Ichthyoelephas* live in the Andean rivers west of Colombia and Ecuador. *Prochilodus* is present in all major South American river systems on both sides of the Andes, and *Semaprochilodus* is broadly distributed east of the Andes along the Amazon, Tocantins and Orinoco basins and some coastal rivers draining the Guiana Shield (Castro and Vari 2004).

Cytogenetic studies conducted thus far in Prochilodontidae are limited to *Prochilodus* (8/13 species karyotyped) and *Semaprochilodus* (4/6 species karyotyped). Those works revealed a conserved karyotype composed of 54 metacentric-submetacentric chromosomes with a fundamental number (FN)=108 (Arai, 2011), with a heteromorphic ZW pair reported only in *Semiprochilodus taeniurus* (Valenciennes, 1817) karyotype (Terencio et al. 2012a). However, no cytogenetic data are available for the two *Ichthyoelephas* species, *Ichthyoelephas longirostris* (Steindachner, 1879), and *Ichthyoelephas humeralis* (Günther, 1860)

In this research, for the first time we used the available karyotyping techniques, including Giemsa-staining, Ag-staining, C-banding, and localization of 18S rDNA and 5S rDNA to investigate the cytogenetic characteristics of *Ichthyoelephas humeralis*.

Methods

We analyzed nineteen specimens of *Ichthyoelephas humeralis* (undetermined sex) collected with seine nets in the channels fed by the Babahoyo River (2°00'41.4"S 79°47'00.1"W), which supply water to the rice plantations of Samborondon, Guayas Province, Ecuador. Voucher specimens were fixed in 10% formalin and deposited in the fish collection of the Laboratório de Biologia e Genética de Peixes, UNESP, Botucatu (São Paulo State, Brazil) (collection numbers LBP 19326), and Universidad Técnica de Machala (collection numbers UTMach-00184).

We obtained kidney cell suspensions from fish 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 (1.0 ml/100 g body weight) 50 min before sacrificing, as described by Nirchio and Oliveira (2006). Before being sacrificed, the specimens received a numbing overdose of Benzocaine (250 mg/L) until the cessation of opercular movements

(AVMA 2013). Mitotic chromosome preparations were obtained by the conventional air-drying method, as described in Nirchio and Oliveira (2006).

We analyzed a minimum of 10 metaphases per sample using all investigative techniques separately. Silver (Ag) staining revealed active nucleolus organizer regions (NORs), as described by Howell and Black (1980) sequentially after Giemsa staining (Rábová et al. 2015). We obtained C-bands following the method of Sumner (1972).

Physical mapping of major and minor ribosomal genes on the chromosomes was performed by fluorescence *in situ* hybridization (FISH) following the method described by Pinkel et al. (1986). Both major (18S rDNA) and minor (5S rDNA) ribosomal probes were isolated from DNA extracted from samples of the same species by PCR. The probe for rDNA was obtained using the primers 18S6F (5'CTCTTTCGAGGC-CCTGTAAT3') and 18S6R (5'CAGCTTTGCAACCATACTCC3') (Utsunomia et al. 2016). We accomplished the labeling of this probe with Digoxigenin-11-dUTP (Roche Applied Science), and hybridization signal detection was performed using Anti-Digoxigenin-Rhodamine (Roche Applied Science). To obtain the 5S rDNA probe, we used the primers 5SF (5'TCAACCAACCACAAAGACATTGGCAC3') and 5SR (5'TAGACTTCTGGGTGGCCAAAGGAATCA3') (Pendás et al. 1994). This probe was labeled with Biotin-16-dUTP (Roche Applied Science), and hybridization signal detection was performed using conjugated Avidin-Fluorescein (FITC).

We photographed the mitotic chromosomes using a Motic B410 microscope equipped with a Motic Moticam 5000C digital camera. The chromosomes were classified as metacentric (M) or submetacentric (SM) 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 CC 2015.

Results

The karyotype of *Ichthyoelephas humeralis*, obtained from 247 metaphases achieved from the 19 analyzed individuals, revealed a modal diploid number of 2n=54 composed of 32 M and 22 SM (Fig. 1a). Chromosomes of metacentric and submetacentric series decrease uniformly in size, making it difficult to identify homologous chromosomes. Only the metacentric chromosome pair 1, the largest in the complement, can be identified unequivocally in the metacentric series. Pair 1 consistently showed a variation in size in all the recorded metaphases of all fishes studied (Fig.1a). Chromosomal differences between sexes were not observed.

C-banding showed heterochromatic blocks located in the centromeric region of pairs number 4, 5, 9, 11, 14, 15, 16, 18. C-bands appeared in the terminal regions of pairs 2, 3, 10, 17, 19, 20, 22, 23, 24, 25, 26, 27; and, in the pericentromeric regions of pairs 1 and 9; and interstitially on pair 6. Chromosomes 7, 8, 12, 13, and 21 did not show typical constitutive heterochromatin marks (Fig. 1b). Discrete C-banding marks in the terminal regions of the long arm of chromosome pair N° 2 were coincident with the Ag-NORs (Fig. 1a).

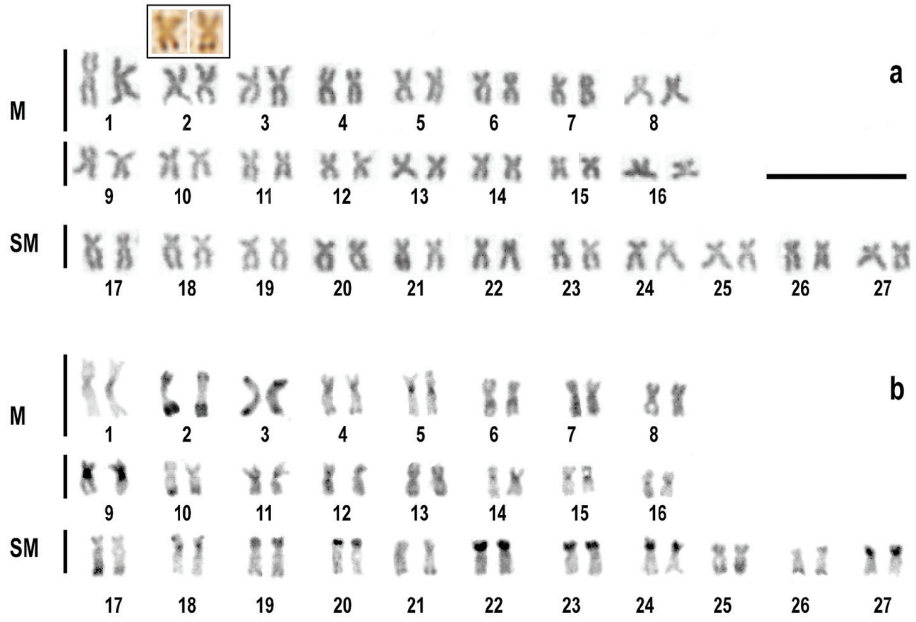

Figure 1. Karyotypes of *Ichthyoelephas humeralis* after Giemsa staining (**a**) and C-banding (**b**). Ag-NORs inbox. Bar = 10 μm

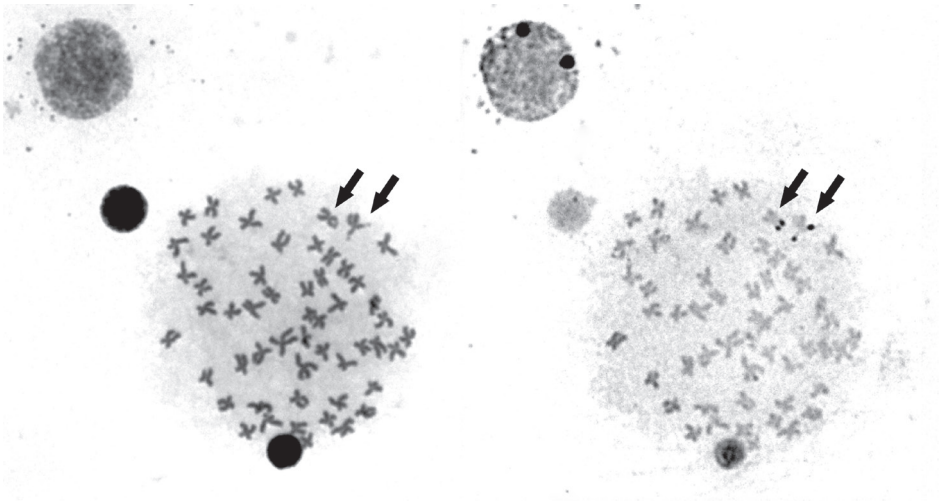

Figure 2. Ag-NOR staining on metaphase chromosomes of *Ichthyoelephas humeralis* after Giemsa staining (arrows show the NOR-bearing chromosomes).

Impregnation with $AgNO_3$ after Giemsa staining revealed only one pair of active nucleolus organizer regions (Ag-NOR), located on the tips of the long arms of a metacentric chromosome possessing an evident secondary constriction (Fig. 2). This chromosome was identified as pair 2 in the karyotype (Fig. 1a). FISH with 18S rDNA

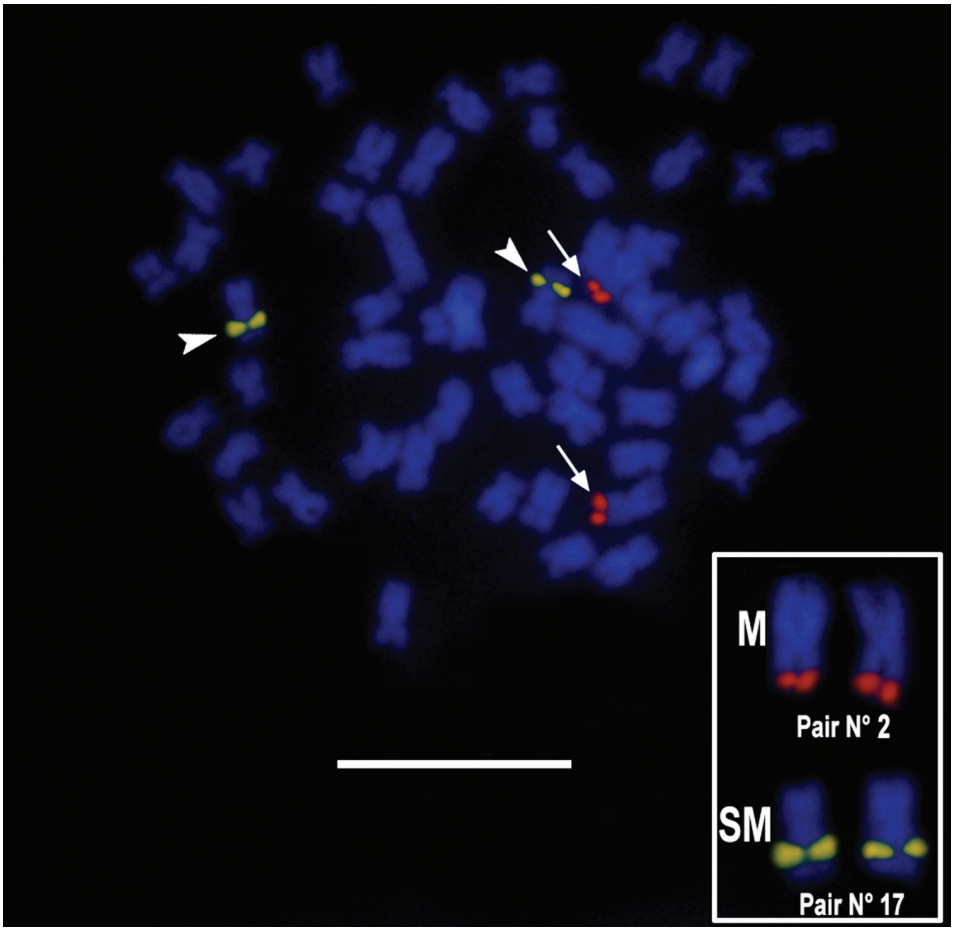

Figure 3. Double FISH staining of metaphase chromosomes of *Ichthyoelephas humeralis* (arrows show the 18S rDNA, and arrowheads show the 5S rDNA); inbox details of chromosome bearing 5S and 18S rDNA. Bar = 10 μm.

probe produced bright Avidin-Fluorescein (FITC) signals only on the tips of the long arms of chromosome pair 2, which indicates that the species does not possess additional NOR-sites (Fig. 3). FISH with 5S rDNA probe produced interstitial FITC signals on the long arm of a chromosome pair, apparently the largest of the SM series (Pair N° 17), thus demonstrating by double FISH that both ribosomal gene clusters are located on different chromosomes (Fig. 3).

Discussion

By adding the chromosome information on *Ichthyoelephas humeralis* reported herein to the Prochilodontidae database, the number of the species of the family so far cytogeneti-

cally analyzed rises to 13, out of the 21 currently recognized valid species (Eschmeyer and Fong 2016). Cytogenetic studies conducted with 12 representatives of the genera *Prochilodus* and *Semaprochilodus* show that they have an evolutionarily conserved karyotype with 2n=54 biarmed elements, composed of 40 metacentric and 14 submetacentric chromosomes with a fundamental number (FN)=108 (Arai, 2011). The exception lies in a few *Prochilodus* species or populations showing intra and interpopulation karyotype variation related to supernumerary B chromosomes (Pauls and Bertollo 1983, 1990, Oliveira et al. 2003, Gras et al. 2007, Penitente et al. 2015). The present data about *I. humeralis* confirm the occurrence of a conservative chromosome diploid complement and fundamental number in Prochilodontidae. Notwithstanding, its karyotypic formula differs in the number of metacentric and submetacentric chromosomes suggesting that pericentromeric inversions occurred in four submetacentric pairs changing the number of metacentric chromosomes from 32 to 40 or vice-versa. These events occurred after the divergence of *Ichthyoelephas* from *Prochilodus* and *Semaprochilodus* (Melo et al., 2016) since these two groups belong to different lineages described in Prochilodontidae.

C-banding in *I. humeralis* revealed constitutive heterochromatin in the centromeric, pericentromeric, interstitial, and terminal regions. These characteristics are difficult to compare quantitatively to other Prochilodontidae species. Nevertheless, this heterochromatin distribution is different regarding the particular pattern in other species of Prochilodontidae, which show heterochromatin typically restricted to the centromeric and pericentromeric regions of their chromosomes (Oliveira et al. 2003, Vicari et al. 2006, Terencio et al. 2012b, Voltolin et al. 2013).

Ribosomal sites in Prochilodontidae (5S and 18S ribosomal clusters) are syntenic, commonly located in the interstitial position on chromosome pair 2 in all species of *Prochilodus* and *Semaprochilodus* analyzed (Pauls and Bertollo 1990, Oliveira et al. 1997, 2003; Venere et al. 1999, Cavallaro et al. 2000, Maistro et al. 2000, Jesus and Moreira-Filho 2003, Hatanaka and Galetti Jr. 2004, Artoni et al. 2006, Vicari et al. 2006, Gras et al. 2007, Voltolin et al. 2009, 2013, Jorge et al. 2011, Terencio et al. 2012a, 2012b, Penitente et al. 2015).

The localization of ribosomal clusters on distinct chromosome pairs in *I. humeralis* with the18S rDNA terminally located on pair 2 and the 5S rDNA interstitially positioned on pair 17, suggests the occurrence of at least two chromosome reorganization events when *Ichthyoelephas*, *Prochilodus* and *Semaprochilodus* diverged from their common ancestor: 1) a paracentromeric inversion to explain the displacement of the 18S rDNA cluster from a terminal to an interstitial position or vice-versa, and 2) a translocation of the ribosomal 5S rDNA site from its bearing chromosome to an 18S rDNA bearing chromosome or vice-versa.

The most comprehensive molecular phylogenetic study in Prochilodontidae based on mitochondrial and nuclear loci (Melo et al. 2016) provides evidence supporting the position of *Ichthyoelephas* as a sister group to the clade of *Prochilodus* and *Semaprochilodus*. Curimatidae and Chilodontidae are sister groups to Prochilodontidae (Oliveira et al. 2011, Melo et al. 2016). Data on NORs in Chilodontidae and Curimatidae show that species in these families have only one NOR-bearing chromosome pair, usually a

large metacentric with NORs in the terminal position (Martins et al. 2000, De Rosa et al. 2006, Rodrigo et al. 2008, Venere et al. 2008, Arai 2011) as observed in *I. humeralis,* subject of this study. FISH experiments with species of Curimatidae show that the 18S rDNA sites are coincident with the Ag-NORs, and 5S rDNA are found on different chromosomes in interstitial positions in all species analyzed (De Rosa, 2006, 2007).

Castro and Vari (2004) proposed a close relationship between *Semaprochilodus* and *Ichthyoelephas* based on morphological studies. This result was refuted by Melo et al. (2016), who, based on molecular data, observed a close relationship between *Semaprochilodus* and *Prochilodus*. As described above, the present cytogenetic data show that *Prochilodus* and *Semaprochilodus* share several chromosomal characteristics, such as the syntenic location of 5S and 18S ribosomal genes, constitutive heterochromatin distribution pattern, and karyotypic formula. All these chromosomal characteristics are not observed either in *Ichthyoelephas*, Curimatidae, or Chilodontidae. Thus, cytogenetic data corroborated the hypothesis of Melo et al. (2016), whereby *Prochilodus* and *Semaprochilodus* are closely related and may be sister groups to *Ichthyoelephas humeralis*. Further studies should be performed to establish whether *I. humeralis* shares the chromosome characteristics with the only additional species in the genus: *I. longirostris*.

The results described here demonstrate the usefulness of conventional and molecular cytogenetic techniques as tools for understanding the evolutionary history in Prochilodontidae suggesting the occurrence of some micro and chromosomal macrostructural reorganization events in the ancestral karyotype wherefrom *Ichthyoelephas* arose as a clade that diverged from the ancestor of their sister group *Prochilodus-Semaprochilodus* approximately 12 million years ago (Melo et al. 2016).

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RESEARCH ARTICLE

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Cytogenetic data on *Ancistrus* sp. (Siluriformes, Loricariidae) of the Paraguay River basin (MS) sheds light on intrageneric karyotype diversification

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Abstract

Ancistrus Kner, 1854 is a diverse catfish genus, currently comprising 66 valid species, but karyotype data were recorded for 33 species, although only ten have their taxonomic status defined. Considerable karyotype diversity has been found within this genus, with 2n varying from 34 to 54 and structural variability including heteromorphic sex chromosomes. In many cases, uncertainty on the taxonomic status of the study populations hampers reliable interpretation of the complex chromosomal evolutionary history of the group. This study aims to present the first karyotype data for a population of the *Ancistrus* sp. collected in Criminoso stream (tributary of the Paraguay River Basin, Mato Grosso do Sul, Brazil) in which a combination of different chromosomal markers was used and results integrated in broad discussion on karyotype evolution in the genus. The specimens presented 2n=42 with 18m+16sm+8st and a single NOR revealed by silver nitrate and fluorescence *in situ* hybridization (FISH) with 18S rDNA probe, located in pair No. 10. Clusters of 5S rDNA were located in the pericentromeric region of three chromosomes: pair No. 1 (metacentric) and one of the homologues of the nucleolar pair No. 10. Heterogeneity in the

molecular composition of the heterochromatin was confirmed by the association of C-banding and fluorochrome CMA_3/DAPI-staining. Exploring the differential composition of constitutive heterochromatin in *Ancistrus* may provide an important perspective to understand genome organization and evolution within this group. Our data reinforce the chromosomal diversity present in *Ancistrus* genus and we discuss the potential sources these variation. The karyotype structure of *Ancistrus* sp. "Criminoso stream" appears to be consistent with the existence of a new candidate species.

Keywords

Ancistrini, cytotaxonomy, CMA_3/DAPI, heterochromatin, rDNA

Introduction

Ancistrus Kner, 1854 is the most species-rich genus of the tribe Ancistrini (Hypostominae), currently consisting of 66 valid species (Bifi et al. 2009, Froese and Pauly 2016). This genus is widespread in the Neotropical region, from Panama to Rio de La Plata in Argentina, although the greatest diversity of *Ancistrus* species is found in the basin of the Amazon River (Fisch-Muller 2003).

Up until now, the karyotypes of 33 *Ancistrus* species have been described, even though most of these species have yet to be formally identified (e.g. de Oliveira et al. 2009, Mariotto et al. 2011, 2013, Favarato et al. 2016). The karyotype data available for this genus indicate considerable chromosomal diversity, with diploid numbers ranging from 2n=34 chromosomes in *Ancistrus cuiabae* (Mariotto et al. 2009) to 2n=54 in *Ancistrus claro* (Mariotto et al. 2011). In addition to this numerical diversity, there is considerable variation in chromosome structure, including differences among populations that suggest the existence of species complexes, as observed in *Ancistrus* prope *dubius* (Mariotto and Miyazawa 2006). Another remarkable feature of *Ancistrus* is the occurrence of heteromorphic sex chromosomes in some species, including simple (Mariotto et al. 2004, Alves et al. 2006, Mariotto and Miyazawa 2006) and multiple systems (de Oliveira et al. 2007, 2008) which has also contributed to karyotype diversification within the genus.

The chromosomal mapping of the two classes of rDNA (45S and 5S genes) has contributed to the understanding of the organization and evolutionary dynamics of these genes in fish genomes. The 5S rDNA sites are commonly located in interstitial or proximal positions and separated from the 45S rDNA genes, as observed in many groups, but in Loricariidae little is known about the distribution and number of 5S ribosomal genes, being more studied for some species of *Hypostomus* Lacépède, 1803 (Kavalco et al. 2004, 2005, Mendes-Neto et al. 2011, Traldi et al. 2012, Bueno et al. 2014) and some *Ancistrus* species (Mariotto et al. 2011, Konerat et al. 2015, Favarato et al. 2016). These studies indicate that the distribution of 5S rDNA genes varies considerably and may occur on one or more chromosome pairs, with or without synteny among the 18S rDNA sites.

The complex taxonomic scenario that has been noted in the *Ancistrus* genus also contributes to the difficulty in understanding the karyotype evolution of this group. In the present study, we provide a detailed description of the karyotype of *Ancistrus* sp. "Criminoso stream" based on specimens collected in the basin of the Paraguay River

(Brazil), using classical and molecular cytogenetic techniques. We provide physical chromosome maps of the 18S and 5S rDNA clusters, and of the heterochromatin, highlighting the GC- and AT-rich composition using base-specific fluorochromes. We also compiled the cytogenetic data available for the genus *Ancistrus* to provide a more systematic overview of the karyotypic variation in this group.

Material and methods

Authorization for the collection of specimens was granted by the Brazilian Environment Ministry through its Biodiversity Information and Authorization System (SISBIO), under the license number 36575-1. The protocols used in this study were submitted and reviewed by the Ethics Committee on the use of animals (CEUA) of Universidade Estadual de Maringá under the case number 013/2009.

Cytogenetic analyses were conducted on 13 specimens (5 females, 6 males and 2 of undetermined sex) of *Ancistrus* sp. collected from the Criminoso stream (18°29.333'S, 54°45.233'W), a small tributary of the Taquari River, near the town of Coxim in the basin of the upper Paraguay River, in Mato Grosso do Sul state, Brazil. The specimens were identified as *Ancistrus* sp. "Criminoso stream" (NUP 12018) and deposited in the ichthyological collection of the Limnology, Ichthyology and Aquaculture Research Center (Nupélia) at Maringá State University, Paraná, Brazil.

Chromosome preparations were obtained from kidney cells following the technique described by Bertollo et al. (1978). The nucleolus organizer regions (NOR) were detected by impregnation with silver nitrate (Ag-NO_3), as described by Howell and Black (1980). Double staining was carried out with chromomycin A_3 (CMA_3) and DAPI, according to Schweizer (1976). The constitutive heterochromatin was identified by the C-banding technique as described in Sumner (1972) and stained with propidium iodide according to the method of Lui et al. (2012). The physical mapping of the 18S and 5S rDNA sequences was carried out by Fluorescence *in situ* Hybridization (FISH) according to Pinkel et al. (1986). The 18S probe was obtained from *Prochilodus argenteus* Spix et Agassiz, 1829 (Hatanaka and Galetti Jr. 2004), and labelled with the Nick Translation Biotin kit and 5S rDNA probes from *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti Jr. 1999) labelled with the Nick Translation Digoxigenin kit. The hybridization signals were detected using avidin-FITC (fluorescein isothiocyanate) for the 18S rDNA probe and anti-digoxigenin-rhodamine for the 5S rDNA probe. The chromosomes were counterstained with DAPI. The metaphases were photographed using an epifluorescence microscope and optimized for best contrast and brightness with Adobe Photoshop CS6 software.

The chromosomes were identified based on the modified arm ratio (AR) criteria of Levan et al. (1964), and classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a). The fundamental number (FN) was established considering the meta-submetacentric and subtelocentric chromosomes to have two arms and the acrocentric chromosomes, only one.

Results

The specimens of *Ancistrus* sp. "Criminoso stream" had a diploid number of 2n=42 with 18m+16sm+8st and a FN of 84, in both sexes (Figure 1a). The Ag-NOR sites were found in the terminal position of the long arm of a submetacentric pair (No. 10), which presented a clear size heteromorphism (Figure 1a, in box), also confirmed by 18S rDNA-FISH (Figure 1b, in box). The 5S rDNA sites were detected in the pericentromeric regions of the first pair of metacentric chromosomes and in one of the homologues of the nucleolar pair (Figure 1b).

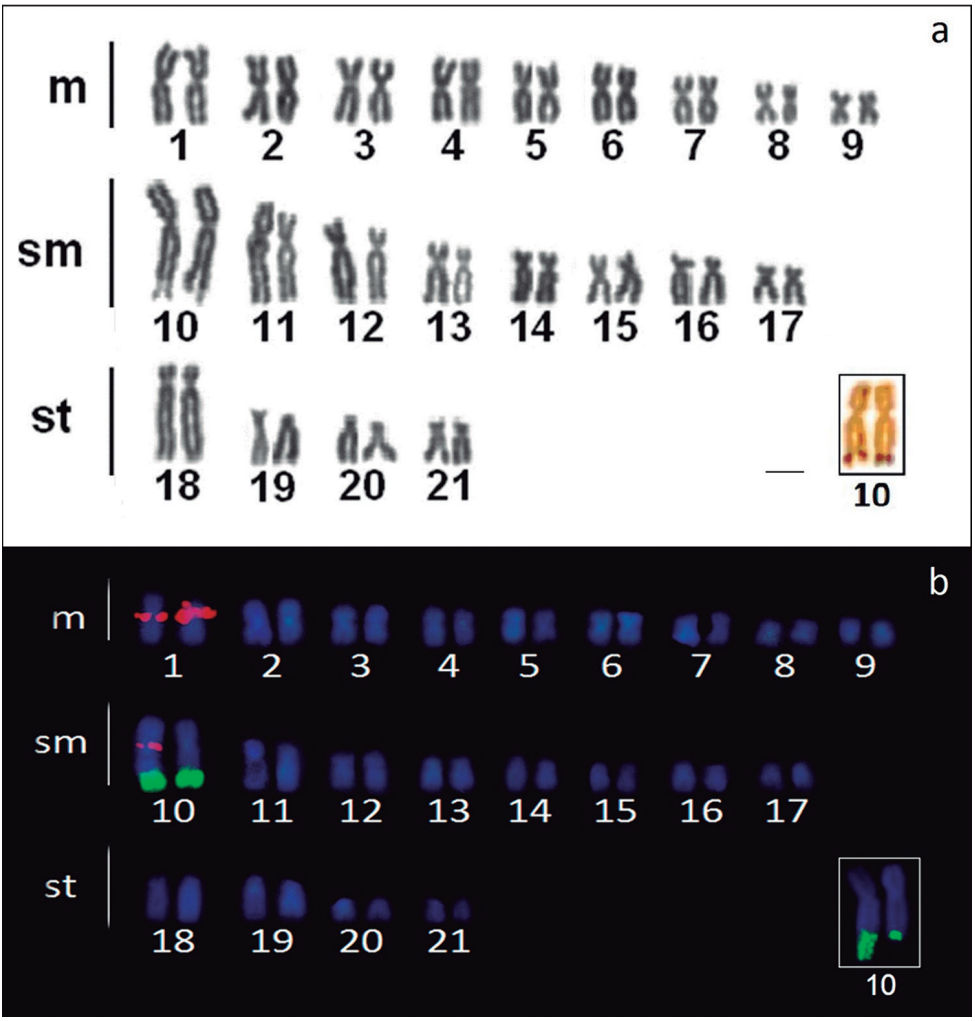

Figure 1. Karyotype of *Ancistrus* sp. "Criminoso stream" after: **a** Giemsa-staining and the NOR-bearing chromosome pair No. 10 (in box) **b** double-FISH using 18S rDNA (green) and 5S rDNA (red) probes. Note the size heteromorphism in the NOR-bearing chromosomes from a different metaphase (in box). Bar = 10 µm.

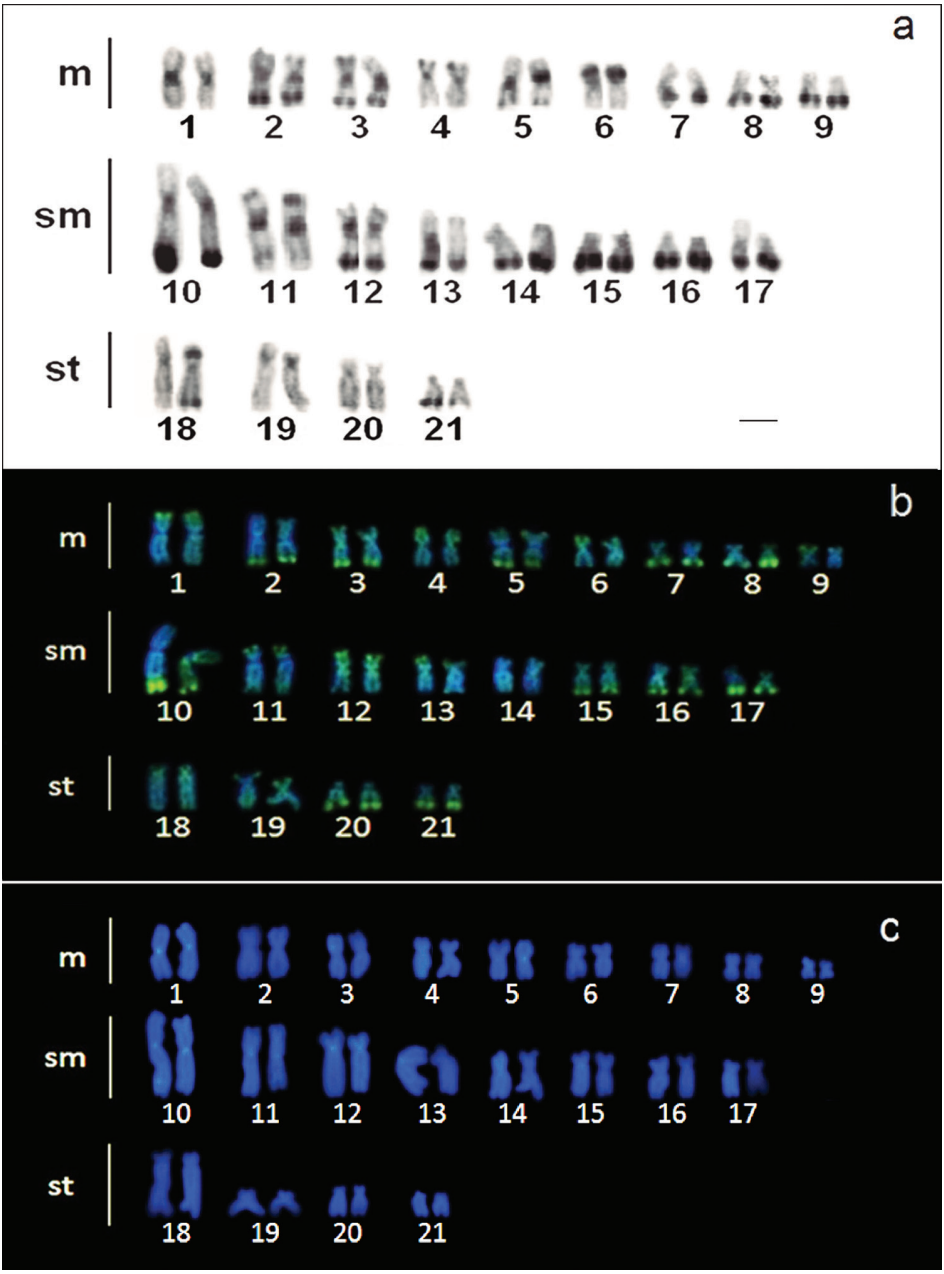

Figure 2. Karyotype of *Ancistrus* sp. "Criminoso stream" showing: **a** the heterochromatin distribution pattern after C-banding **b** CMA_3/DAPI base-specific profile and **c** DAPI staining. Bar = 10 µm.

C-banding detected telomeric and pericentromeric heterochromatic blocks in almost all chromosomes pairs of *Ancistrus* sp. "Criminoso stream" (Figure 2a). The chromosome locations corresponding to 5S (pair No. 1 and one homologue of pair No. 10)

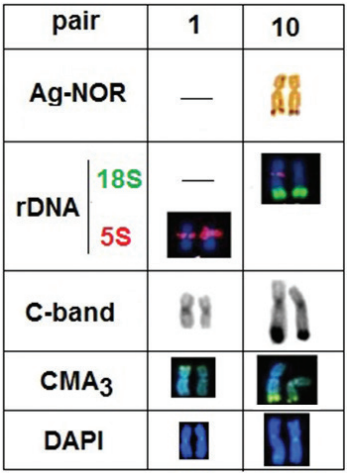

Figure 3. Chromosome pairs, 1 and 10 of *Ancistrus* sp "Criminoso steam" after different banding: pair No 1 showing the localization of the pericentromeric 5S rDNA sites (red), positive for C-band and DAPI; pair No 10 showing Ag-NOR sites coincident with 18S rDNA signals (green), positive for C-band and CMA_3; pericentromeric 5S rDNA sites (red) were shown in only one of the homologues of the pair 10, whose sequence was coincident with heterochromatic blocks (C-band) and DAPI in this pair.

and 18S rDNA (pair No. 10) were also positive for C-bands. Some of the chromosomes also presented interstitial heterochromatic blocks (pairs Nos. 2, 3, 6, 9 and 11). Chromomycin A_3 staining revealed several GC-rich regions besides the Ag-NORs sites (Figure 2b), which coincided with most of the heterochromatic blocks in the telomeric regions (Figure 2a). The DAPI fluorochrome produced a brighter signal in the centromeric regions of chromosome pairs Nos. 1, 5, 10, 11, 12 and 13 (Figure 2c). It should be noted that these $DAPI^+$ blocks in pairs Nos. 1 and 10 are interspersed with the 5S rDNA sites (Figures 1b, 2c). These heterochromatin blocks were observed in all specimens analyzed, irrespective of the sex. Figure 3 highlights the pairs 1 and 10 with all banding.

Discussion

The karyotypes described for the genus *Ancistrus* have been obtained from species found in the basins of the Paraguay and Amazon Rivers, with extensive variability observed in the genus, whose diploid numbers vary of 34, 38, 40, 42, 44, 46, 48, 50, 52 and 54 chromosomes (de Oliveira et al. 2009, Mariotto et al. 2011, 2013, Favarato et al. 2016). The available descriptions of karyotypes for unidentified *Ancistrus* species emphasize the potential diversity of the genus in these basins. The 2n=42 karyotype as observed in *Ancistrus* sp. "Criminoso stream" has also been recorded in other *Ancistrus* species albeit with distinct karyotype formulae (Table 1), indicates the presence of another species, yet to be identified, in the Pantanal region. Additional taxonomic studies are clearly needed to determine the full diversity of the genus in the region.

Table 1. Cytogenetic data available for *Ancistrus* with 2n=42.

Species	River/ Basin/ State	2n	NF	Karyotype formulae/ Sex chromosome	NOR		rDNA 5S	Ref
					Ag-NOR	rDNA18S		
Ancistrus cf. *dubius*	Pantanal/ Paraguay/ MT	44	80	18m+10sm+8st+8a / ZZ/ZW	it	sm, it (16)*	sm, it (16)*; m, it (4); sm, pc (14)	Mariotto et al. 2004, 2006, 2011
		42	84	24m+10sm+8st / XX/XY	it			
		42	84	24m+10sm+8st	it			
Ancistrus sp. 12	Santa Cruz/ Paraguay/ MT	42	84	28m+10sm+4st	it	–	–	Mariotto 2008
Ancistrus sp. 10	Vermelho/ Paraguay/ MT	42	82	22m+14sm+4st+2a / ZZ/ZW	it	–	–	Mariotto 2008
Ancistrus sp. 11	Araputanga/ Paraguay/ MT	42	84	24m+12sm+6st / XX/XY	it	–	–	Mariotto 2008
Ancistrus sp. Vermelho	Demini/ Amazon/ AM	42	78	26m+6sm+4st+6a	te	a, (20)	–	de Oiveira et al. 2009
Ancistrus sp. "Criminoso stream"	Criminoso stream/ Paraguay/ MS	42	84	18m+16sm+8st	te	sm, te (10)*	sm, it (one chromosome, 10)*; m, it (1)	Present study

Subtitles: 2n: diploid number; NF: fundamental number; m: metacentric; sm: submetacentric; st: subtelocentric; a: acrocentric; it: interstitial; te: terminal; pc: pericentromeric; – : not registered; *: synteny between 18S and 5S rDNA sites; numbers in parentheses refer to the chromosome pairs; MT: Mato Grosso; AM: Amazonas; MS: Mato Grosso do Sul; Ref: references.

The karyotype data available for *Ancistrus* indicate a relation between diploid number and chromosome types; species with a 2n=44 or more have a larger number of acrocentric chromosomes, while species with smaller diploid numbers (2n=34 to 42) have few or no acrocentric chromosomes (de Oliveira et al. 2009, Medeiros et al. 2016). Artoni and Bertollo (2001) proposed that a karyotype of 2n=54 with a predominance of meta-submetacentric pairs was the primitive condition for the family Loricariidae, which was thus far been found in *Ancistrus* sp. 01, *Ancistrus* sp. 03 (Mariotto et al. 2013) and *Ancistrus claro* (Mariotto et al. 2011) species, with karyotype formula of 14m+8sm+8st+24a. These species have a proportion karyotype of 22 meta-submetacentric (40.7%) and 32 st-acrocentric chromosomes (59.3%). From this starting point, there has been an extensive reduction in the diploid number in the genus *Ancistrus* (54 to 34) due to possible chromosomal fusions, which can be deduced from the inverse relationship between the number of acrocentric and meta-submetacentric chromosomes. This can be seen in the species with reduced diploid numbers (40, 38, 34), which show little or no acrocentric chromosomes (Mariotto et al. 2004, 2006, 2009, 2011, de Oliveira et al. 2009, Favarato et al. 2016, Medeiros et al. 2016). However, other rearrangements such as inversions and/or translocations also occurred during the evolution of the karyotype, resulting in the considerable diversity of the fundamental number observed in this genus

Chromosome banding in *Ancistrus* sp. "Criminoso stream" revealed a single nucleolar pair (Ag-NOR), a character shared with all other species of the genus analyzed to date, except *Ancistrus* sp. (Reis et al. 2012). This arrangement has been confirmed by FISH of the 18S rDNA in some species. A single NOR in an interstitial location has been considered the plesiomorphic condition in Loricariidae (Artoni and Bertollo 2001, Alves et al. 2003, de Oliveira et al. 2009, Mariotto et al. 2009, 2011, Mendes-Neto et al. 2011). However, in *Ancistrus*, the NOR has been observed in a terminal position for most species but, there is also a significant number of species with interstitial NORs (Medeiros et al. 2016). The structural diversity of NORs in *Ancistrus* related to the different types and sizes of chromosomes may be associated with extensive structural rearrangements (fusions, translocations and/or inversions) that occurred during the karyotype diversification of the genus (de Oliveira et al. 2009, Mariotto et al. 2011). A clear example of these rearrangements was provided by Mariotto et al. (2009) in *Ancistrus cuiabae*, which has a pericentric inversion in the nucleolar pair (Ag-NOR) supported by C-banding and 18S rDNA-FISH.

Assuming that the primitive Loricariidae karyotype was composed of 2n=54 chromosomes, with synteny between the 18S and 5S rDNA sites as in *Ancistrus claro*, Mariotto et al. (2011) suggested that this layout may represent the basal condition for the genus. This proposal was supported for the species *A. dolichopterus*, *Ancistrus* prope *dolichopterus*, *A. ranunculus* and *A. maximus* analyzed by Favarato et al. (2016). Thus, *Ancistrus* sp. "Criminoso stream" presented a derived condition related to synteny break between the rDNA 18S and 5S sites (pairs 10 and 1, respectively), but the presence of an interstitial 5S rDNA site in one homologues of nucleolar (pair No. 10), may be a remnant of the primitive condition in this genus.

The heterogeneity in the molecular composition of the heterochromatin of *Ancistrus* sp. "Criminoso stream" was demonstrated by the combination of C-banding, CMA_3 and DAPI-staining, representing a valuable approach in comparative cytogenetics. DAPI bands were clearly related with the pericentromeric heterochromatin observed by C-banding in most chromosomes and coincided with the 5S rDNA sites (pair No. 1 and one homologue of pair No. 10). The GC-rich heterochromatin associated with (or interspersed between) ribosomal genes, as observed, is common in most fish chromosomes; however, the presence of terminal CMA_3^+/C-band$^+$ blocks in most chromosomes is not a feature frequent in fishes karyotype, included *Ancistrus* species. In addition, to date there is no karyotypic study in *Ancistrus* demonstrating coincidence of heterochromatin blocks in several chromosomes with GC/AT rich content, as obtained in the species under study. So, it could be a good chromosomal mark to characterize the karyotype of this putative new species. All *Ancistrus* species cytogenetically described to date exhibit profiles of low constitutive heterochromatin content, with varied distribution form being found in interstitial and pericentromeric regions to occupying large portions of the long or short chromosome arms (e.g. de Oliveira et al. 2009). In some cases, the accumulation of heterochromatin could be related to the origin of heteromorphic sex chromosomes, as found in *Ancistrus* prope *dubius* (Mariotto et al. 2004, Mariotto and Miyazawa 2006) and

A. taunayi (Konerat et al. 2015), being also associated with paracentric inversions in the latter. Particularly in *Ancistrus* sp. "Criminoso stream" we hypothesized that unique constitutive heterochromatin pattern observed in their karyotype sheds light on intrageneric karyotype diversification, however such studies are needed in other species of the genus.

Conclusion

Our results further reinforce the considerable variation in karyotype macrostructure within the genus through the description of a new karyotype formula and unique constitutive heterochromatin pattern observed in the population of the *Ancistrus* sp. "Criminoso stream", contributing with information about the complex chromosomal evolution history of the catfish genus.

This result also appears to be consistent with the existence of a new candidate species. Notwithstanding, our results also emphasize the need for an integrated approach to the understanding of the taxonomic status of this population, based on morphological, ecological, and molecular data. Ultimately, ecological and behavioral traits other than reproductive strategies may be contributing to the mechanisms of isolation that underpin the chromosomal diversification in *Ancistrus*.

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RESEARCH ARTICLE

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Karyotypic features including organizations of the 5S, 45S rDNA loci and telomeres of *Scadoxus multiflorus* (Amaryllidaceae)

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Abstract

Scadoxus multiflorus Martyn, 1795 is an ornamental plant with brilliantly colored flowers. Even though its chromosomes are rather large, there is no karyotype description reported so far. Therefore, conventional and molecular cytogenetic studies including fluorescence *in situ* hybridization (FISH) with 45S and 5S rDNA, and human telomere sequence $(TTAGGG)_n$ probes (Arabidopsis-type telomere probes yielded negative results) were carried out. The chromosome number is as reported previously, 2n = 18. The nine chromosome pairs include two large submetacentric, five large acrocentric, one medium acrocentric, two small metacentric and eight small submetacentric chromosomes. Hybridization sites of the 45S rDNA signals were on the short arm ends of chromosomes #1, #3 and #8, while 5S rDNA signals appeared on the long arm of chromosome 3, in one homologue as a double signal. The telomere signals were restricted to all chromosome ends. Three chromosome pairs could be newly identified, chromosome pair 3 by 5S rDNA and chromosomes #1, #3 and #8 by 45S rDNA loci. In addition to new information about rDNA locations we show that the ends of *S. multiflorus* chromosomes harbor human instead of Arabidopsis-type telomere sequences. Overall, the *S. multiflorus* karyotype presents chromosomal heteromorphy concerning size, shape and 45S and 5S rDNA positioning. As *Scadoxus* Rafinesque, 1838 and related species are poorly studied on chromosomal level the here presented data is important for better understanding of evolution in Amaryllidaceae.

Keywords
Scadoxus multiflorus, aceto-orcein staining, fluorescence *in situ* hybridization (FISH), rDNA, telomere

Introduction

Scadoxus multiflorus Martyn, 1795 (also known as *Haemanthus multiflorus* Martyn, 1795 and *H. kalbreyeri* Baker, 1878) is a species belonging to the family Amaryllidaceae (Chase et al. 2009), which can naturally only be found in Southern and tropical Africa (Patwary and Zaman 1980). Formerly, the genus *Scadoxus* Rafinesque, 1838 was *Haemanthus* Linnaeus, 1753 and was included in the family Liliaceae (Ahirwar and Verma 2014); however, it is not included in the current circumscription of this family (Peruzzi 2016), and was indeed separated out and relocated by Angiosperm Phylogeny Group (APG IV) classification system.

Scadoxus multiflorus is economically important as it is popular as cultivated ornamental plant. Nonetheless, this species was not studied yet for its karyotype in details. There are only two previous reports based on conventional chromosome staining of *S. multiflorus*. Both studies reported 2n = 18 chromosomes, however, Ahirwar and Verma (2014) found six submetacentric and 12 acrocentric chromosomes while Patwary and Zaman (1980) reported one metacentric, nine submetacentric, six acrocentric and two telocentric chromosomes. Besides these contradictory data, molecular cytogenetic approaches like florescence *in situ* hybridization (FISH) have not been applied in this species yet. Such methods being available since the late 1980s (Li et al. 2016, Taguchi et al. 2016) enable detection, characterization and localization of rDNA regions (Chirino et al. 2015) and/or telomeres. The latter are known to be important to protect chromosomal ends of all eukaryotes against nucleolytic degradation, non-homologous end-joining and replication-mediated shortening. They usually consist of short tandem repeats, such as $(TTTAGGG)_n$ in *Arabidopsis* Heynhold, 1842 (Richards and Ausubel 1988) or $(TTAGGG)_n$ in humans (Moyzis et al. 1988). The *Arabidopsis* G-rich telomere repeat is rather conserved and has been detected at the ends of most chromosomes of higher plants examined so far. Nevertheless, in some plants the TTTAGGG-type telomere repeat is lacking and substituted by other repeat sequences (Fajkus et al. 2016).

As for *S. multiflorus* reported karyotypic details are contradictory and no FISH studies have been performed so far the present study aimed to close this gap of knowledge using conventional staining and FISH.

Material and methods

Plant material and chromosome preparation

Scadoxus multiflorus plant was collected in Khon Kaen Province, Northeastern, Thailand (A. Chaveerach et al. 903, Department of Biology, Faculty of Science, Khon Kaen Univer-

sity, Khon Kaen, Thailand). The roots were collected from bulbs placed in distilled water at room temperature. The root tips were excised and kept in cold water for 1 h at 4°C, after that transferred to 0.05% colchicine solution for 4 h at room temperature to accumulate metaphase chromosomes before fixation in ethanol:acetic acid (3:1, v/v) for at least 24 h at 4°C. The protocol for the SteamDrop method (Kirov et al. 2014) was adopted with a few modifications. Briefly, fixed root tips were washed twice with enzyme buffer (0.01 M citric acid, 0.01 M sodium citrate, pH 4.7) to remove the fixative and digested at 37°C for 4 h in enzyme solution consisting of 0.7% cellulase R10 (Duchefa C8001), 0.7% cellulase (CalBioChem 319466), 1% pectolyase (Sigma P3026) and 1% cytohelicase (Sigma C8274) in enzyme buffer. Then, the soft meristematic tissues were washed twice with distilled water and 96% ethanol to remove supernatant with centrifuge before broken with a dissecting needle in a tube in fixative. The suspension was dropped on a glass slide and air dried. Preparations with well spread metaphases were selected for further analyses.

Orcein staining and idiogram generation

Conventional staining was carried out on slides using 2% aceto-orcein for 5 min at room temperature and then covered with a coverslip. Ten well-spread metaphases were selected for photomicrography with a digital camera under oil immersion by light microscope. The length of short and long chromosome arms (p and q) were measured separately and added to calculate the total length (LT). The relative length of chromosome (RL), the centromeric index (CI) and standard deviation (SD) of RL and CI were calculated according to Chaiyasut (1989). The CI (q/p+q) between 0.500–0.599, 0.600–0.699, 0.700–0.899 and 0.900–0.999 were considered as metacentric (m), submetacentric (sm), acrocentric (a) and telocentric (t), respectively, following Turpin and Lejeune (1965) to classify the types of chromosome. These parameters were used for idiograming by computer. Comparison of different estimators of intrachromosomal asymmetry was performed, including mean centromeric asymmetry (M_{CA}) and coefficient of variation of chromosome length (CV_{CL}) based on the equations provided by Peruzzi and Eroğlu (2013). Metaphase chromosomes from overall 10 cells were included.

DNA probe generation and labeling

45S and 5S rDNAs which were isolated from *Arabidopsis thaliana* Schur, 1866 and telomere repeat sequences from *Arabidopsis* $(TTTAGGG)_n$ and human $(TTAGGG)_n$ were applied in this study. The plasmid of 45S rDNA cloned in the vector $T_{15}P_{10}IV_1$ was labelled with Alexa 488-dUTP, while the 5S rDNA probe was labelled with Cy3-dUTP by Nick translation (Roche Cat No 11745808910). The telomeric probes were generated by polymerase chain reaction (PCR) in absence of a DNA template using primers $(TTTAGGG)_5$ and $(CCCTAAA)_5$, and $(TTAGGG)_5$ and $(CCCTAA)_5$ according to Ijdo et al. (1991) and labelled with Cy3-dUTP by nick translation.

Fluorescence *in situ* hybridization

A FISH protocol according to Lysak et al. (2006) was applied with minor modifications. The slide with fixed metaphase cells was washed in 2 × SSC (300 mM Na-citrate, 30 mM NaCl, pH 7.0) for 5 min at room temperature and treated with 45% acetic acid for 3 min. Then the slides were washed twice in 2 × SSC for 5 min each at room temperature before digestion in pepsin solution (10 mg/ml) in 0.01 M HCl for 1 min at 37°C, rinsing twice in 2 × SSC for 5 min, post-fixation in 4% formaldehyde in 2 × SSC for 5 min at room temperature, and two washes in 2 × SSC, with final dehydration in an ethanol series (70%, 90%, 100%) for 2 min, each, at room temperature and air drying.

A 3 µl (60 ng) of labelled probe was dissolved in 17 µl of hybridization mixture (20% dextran sulfate, 50% formamide in 2 × SSC, pH 7.0), and pre-denatured at 95°C for 5 min. Then the solution was added to the slide, covered with a coverslip and sealed by rubber gum. Now, the slide was placed on a heating plate at 80°C for 2 min for co-denaturation of probe and target DNA and incubated in a moist chamber at 37°C for 18 h for hybridization.

After hybridization, slides were washed in 2 × SSC for 5 min at 42°C and three times with 50% formamide in 2 × SSC for 5 min at 42°C. After that, slides were washed three times with 2 × SSC for 5 min at 42°C for 5 min each. Finally, the slides were dehydrated in an ethanol series, air dried, counterstained with 4, 6-diamino-2-phenylindole (DAPI) plus Vectashield antifade mounting medium (Vector Laboratories, USA) and covered with a coverslip. Signals were detected using an epifluorescence microscope with Triple filter (UV, Texas Red and FITC) and photographed (microscope: Axioplan2, Zeiss; Camera: Hammamatsu-ORCA-ER C4742-80, Japan; Lamp: Flouarc, Zeiss).

Results

The idiogram and karyotype analyses established from the metaphases confirmed the diploid chromosome number of *S. multiflorus* to be 2n = 18. The karyotype analysis of this species is summarized in Table 1. The range of total arm length, centromeric index and relative chromosome length are 3.78–16.01 µm, 0.57–0.87 µm and 0.04–0.15 µm respectively. The karyotype comprises two large submetacentric, five large acrocentric, one medium acrocentric, two small metacentric and eight small submetacentric chromosomes (Fig. 1). The plant shows a clear tendency to have karyotypes distinct on asymmetry grounds with relatively low M_{CA} and CV_{CL} as shown in Figure 2 and Table 2.

The metaphase spreads were hybridized with 45S (Alexa, green) and 5S (Cy3, red) rDNA probes as shown in Fig. 3. The hybridization signals for the 45S rDNA probe are in terminal positions of the short arms of chromosomes #1, #3 and #8 (Fig. 3A). The 5S rDNA signals were detected on the long arms of chromosome #3 with one homologue showing two adjacent signals (Figs. 3B–C).

Table 1. Mean length of the short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI, RL from metaphase chromosomes in 10 cells of the blood lily (*Scadoxus multiflorus*), 2n = 18.

Chr.	Ls	Ll	LT	CI ± SD	RL ± SD	Type	Size
1a*	4.86	11.14	16.007	0.696 ± 0.068	0.148 ± 0.010	sm	L
1b*	4.66	9.81	14.465	0.678 ± 0.100	0.134 ± 0.025	sm	L
2a*	2.85	9.89	12.741	0.776 ± 0.085	0.118 ± 0.008	a	L
2b*	2.86	9.00	11.865	0.759 ± 0.073	0.110 ± 0.015	a	L
3	1.49	9.52	11.008	0.865 ± 0.131	0.102 ± 0.015	a	L
4a*	2.29	8.58	10.870	0.790 ± 0.087	0.101 ± 0.013	a	L
4b*	2.15	7.33	9.480	0.773 ± 0.074	0.088 ± 0.016	a	M
5	1.62	3.17	4.784	0.662 ± 0.066	0.044 ± 0.006	sm	S
6	1.95	2.53	4.485	0.565 ± 0.034	0.042 ± 0.004	m	S
7	1.50	2.90	4.402	0.659 ± 0.063	0.041 ± 0.004	sm	S
8	1.50	2.65	4.153	0.639 ± 0.065	0.038 ± 0.004	sm	S
9	1.19	2.59	3.779	0.685 ± 0.069	0.035 ± 0.005	sm	S

Remarks: Chr. = chromosome pair, a = acrocentric, m = metacentric, sm = submetacentric, L = large, M = medium, S = small, a* and b* = heteromorphic pairs 1, 2, and 4.

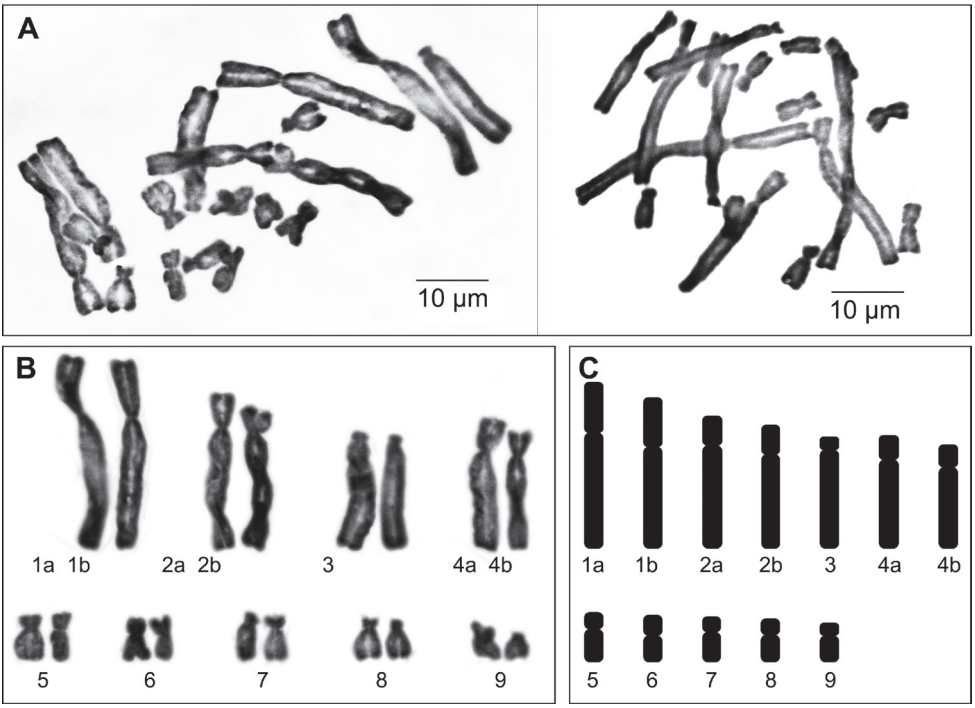

Figure 1. Mitotic metaphase chromosomes of *Scadoxus multiflorus* (2n = 18). **A** aceto-orcein staining of two cells **B** karyogram showing four large and five small pairs of chromosomes **C** idiogram.

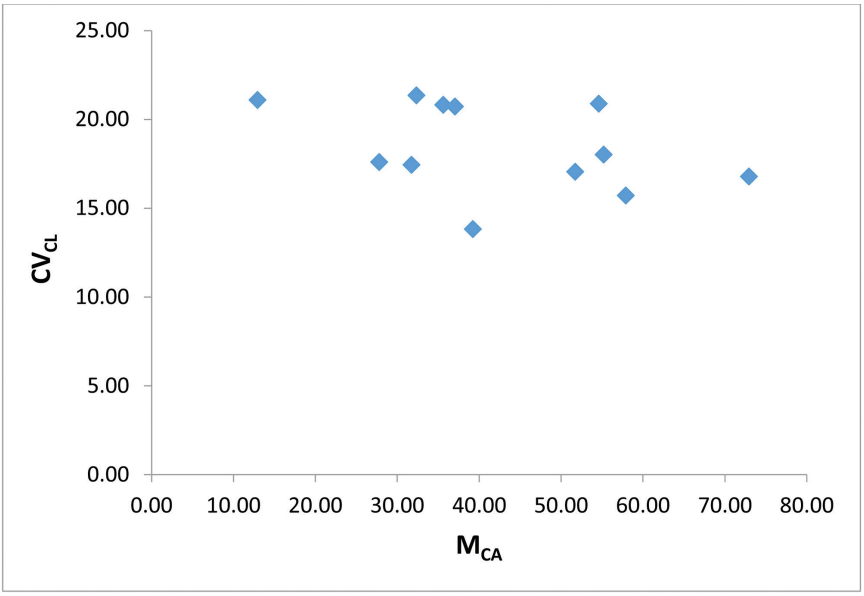

Figure 2. Scatter plot of M_{CA} against CV_{CL} of *Scadoxus multiflorus* chromosomes.

Table 2. Comparison of different estimators of intrachromosomal asymmetry including mean centromeric asymmetry (M_{CA}) and coefficient of variation of chromosome length (CV_{CL}) from metaphase chromosomes in 10 cells of the blood lily (*Scadoxus multiflorus*), 2n = 18.

Chr.	Mean from 10 metaphases						SD of chr. length	M_{CA}	CV_{CL}
	Ll-Ls	Ls / Ll	Ls / (Ll+Ls)	Ll / (Ll+Ls)	(Ll-Ls) / Ll	(Ll-Ls) / (Ll+Ls)			
1a*	6.28	0.44	0.30	0.70	0.56	0.39	2.21	39.23	13.82
1b*	5.15	0.47	0.32	0.68	0.53	0.36	3.01	35.61	20.82
2a*	7.03	0.29	0.22	0.78	0.71	0.55	2.30	55.20	18.02
2b*	6.14	0.32	0.24	0.76	0.68	0.52	2.02	51.72	17.05
3	8.03	0.16	0.14	0.86	0.84	0.73	1.85	72.94	16.78
4a*	6.29	0.27	0.21	0.79	0.73	0.58	1.71	57.90	15.71
4b*	5.18	0.29	0.23	0.77	0.71	0.55	1.98	54.60	20.89
5	1.55	0.51	0.34	0.66	0.49	0.32	1.02	32.34	21.35
6	1.40	0.52	0.34	0.66	0.48	0.32	0.77	31.74	17.44
7	0.58	0.77	0.44	0.56	0.23	0.13	0.95	12.93	21.09
8	1.15	0.57	0.36	0.64	0.43	0.28	0.73	27.79	17.60
9	1.40	0.46	0.31	0.69	0.54	0.37	0.78	37.04	20.73

Remarks: Chr. = chromosome pair, a = acrocentric, m = metacentric, sm = submetacentric, L = large, M = medium, S = small, a* and b* = heteromorphic pairs 1, 2, and 4.

The hybridization of the Arabidopsis-type telomeric probe yielded no obvious FISH signal, while the human-type (Cy3, red) revealed small signals exclusively at the end of all *S. multiflorus* chromosomes (Fig. 4).

Overall, the results show chromosomal heteromorphy in sizes and shapes.

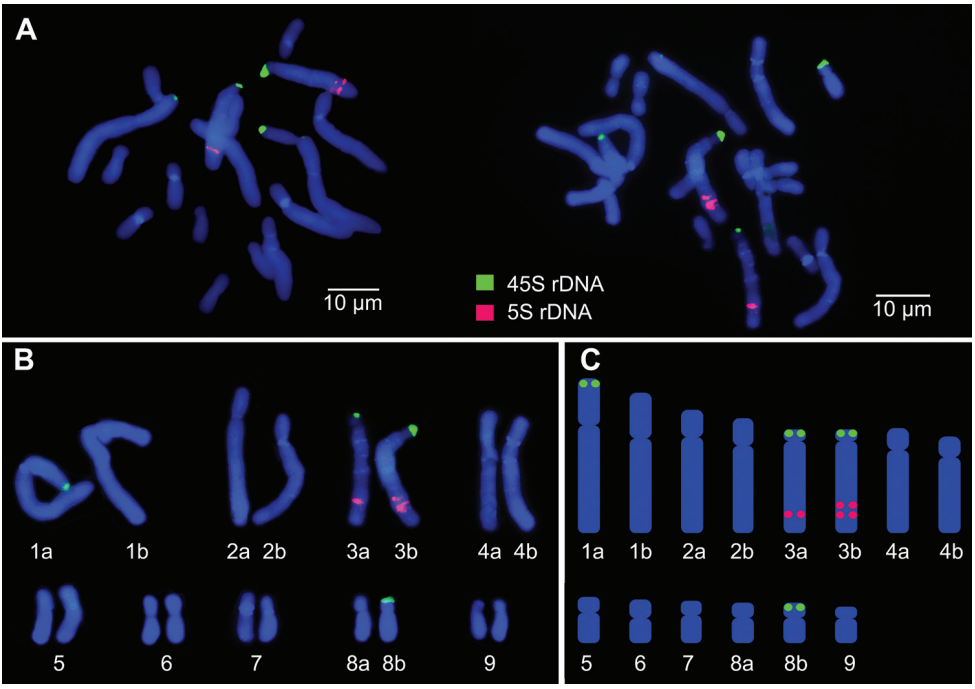

Figure 3. Organizations of 45S rDNA and 5S rDNA loci on metaphase chromosomes of *Scadoxus multiflorus* (2n = 18). **A** FISH signals of 45S (Alexa, green) and 5S (Cy3, red) rDNA probes on two cells **B** karyogram **C** idiogram.

Discussion

Here we provide the first study of *S. multiflorus* chromosomes by means of molecular cytogenetics. Furthermore, the previously reported chromosome number could be confirmed to be 2n = 18 (Patwary and Zaman 1980, Ahirwar and Verma 2014). However, here we report a karyotype variant in *S. multiflorus* as carrying two metacentric, ten submetacentric and six acrocentric chromosomes, which is in contradiction to what was previously reported by Patwary and Zaman (1980) or Ahirwar and Verma (2014). This result indicates that further studies are necessary to clarify if there are either (i) cryptic (sub-)species in *S. multiflorus*, (ii) assessment problems in any of the previous studies, or (iii) any kind of chromosomal heteromorphisms leading to the observed different chromosomal shapes.

Although the regions of 5S and 45S were not observed with conventional staining, due to several limitations, such as oil-immersion light microscopic methods, genetic processing and analysis standards, the signal intensities of rDNA probes in FISH showed clear variation in copy numbers. The copy number and distributed position on chromosome are very important as species markers (Boocock et al. 2015, Li et al 2016).

In a group of families of the monocot order Asparagales, the telomeric sequence $(TTAGGG)_n$ of the human-type was found to be maintained (Sykorova et al. 2003).

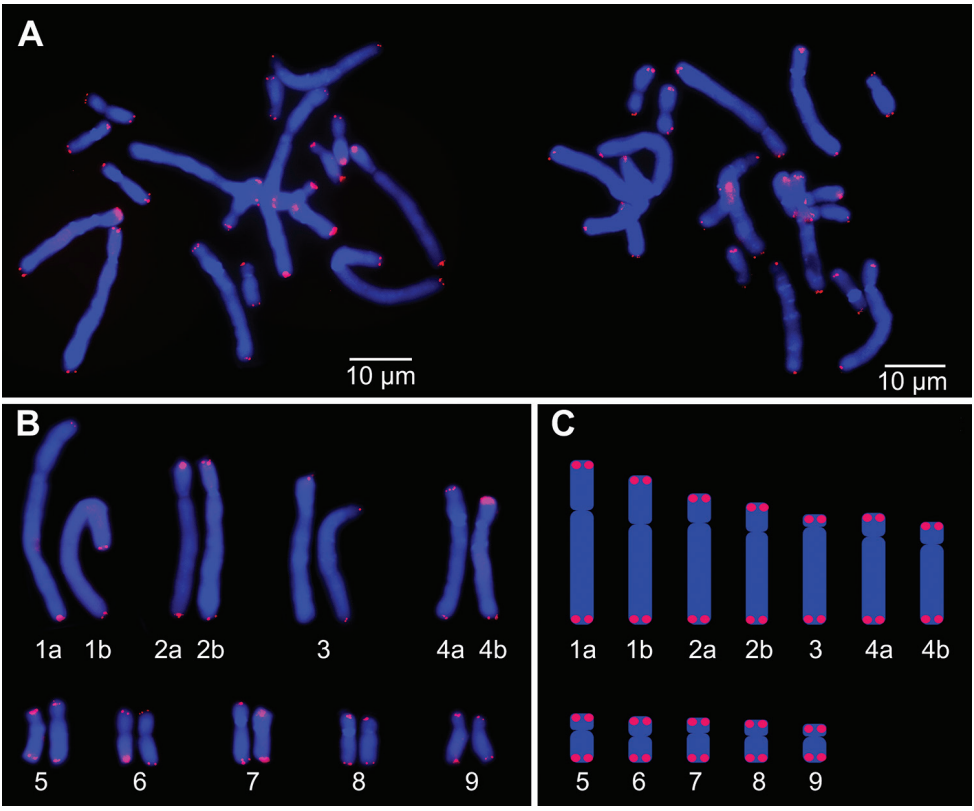

Figure 4. Localization of the human telomere repeat sequence $(TTAGGG)_n$ on metaphase chromosomes of *Scadoxus multiflorus* (2n = 18). **A** FISH signals of the $(TTAGGG)_n$ probe on two cells **B** karyogram **C** idiogram.

Thus it is in accordance with the literature that *S. multiflorus* also has this type of telomeric repeat, as the family Amaryllidaceae belongs to this branch of Asparagales which switched from *Arabidopsis*-type to human type telomere sequence. The knowledge is the one factor supports the classification theory of a common ancestor for a plant group.

Overall, our results allow now distinguishing five of the nine *S. multiflorus* chromosome pairs individually. Development of suitable genomic single-copy FISH probes might allow discrimination of all chromosome pairs and to use them for identification of homologous chromosomes in other species of genus, or even of related genera. As *Scadoxus* and related species are poorly studied on chromosomal level the here presented data is important for better understanding of evolution in Amaryllidaceae.

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RESEARCH ARTICLE

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Cytogenetic study on antlions (Neuroptera, Myrmeleontidae): first data on telomere structure and rDNA location

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Abstract

Myrmeleontidae, commonly known as "antlions", are the most diverse family of the insect order Neuroptera, with over 1700 described species (in 191 genera) of which 37 species (in 21 genera) have so far been studied in respect to standard karyotypes. In the present paper we provide first data on the occurrence of the "insect-type" telomeric repeat $(TTAGG)_n$ and location of 18S rDNA clusters in the antlion karyotypes studied using fluorescence *in situ* hybridization (FISH). We show that males of *Palpares libelluloides* (Linnaeus, 1764) (Palparinae), *Acanthaclisis occitanica* (Villers, 1789) (Acanthaclisinae) and *Distoleon tetragrammicus* (Fabricius, 1798) (Nemoleontinae) have rDNA clusters on a large bivalent, two last species having an additional rDNA cluster on one of the sex chromosomes, most probably the X. $(TTAGG)_n$ - containing telomeres are clearly characteristic of *P. libelluloides* and *A. occitanica*; the presence of this telomeric motif in *D. tetragrammicus* is questionable. In addition, we detected the presence of the $(TTAGG)_n$ telomeric repeat in *Libelloides macaronius* (Scopoli, 1763) from the family Ascalaphidae (owlflies), a sister group to the Myrmeleontidae. We presume that the "insect" motif $(TTAGG)_n$ was present in a common ancestor of the families Ascalaphidae and Myrmeleontidae within the neuropteran suborder Myrmeleontiformia.

Keywords

TTAGG, rDNA, fluorescence *in situ* hybridization, *Palpares libelluloides*, *Acanthaclisis occitanica*, *Distoleon tetragrammicus*, *Libelloides macaronius*, Myrmeleontidae, Ascalaphidae, Neuroptera

Introduction

The ends of eukaryotic chromosomes are capped with complex nucleoprotein structures, the telomeres, which preclude fusion, recombination and degradation of the chromosome ends and thus maintain the genome integrity. In a large number of organisms, telomeric DNA consists of highly repetitive short sequences. Although telomeres are diversified in their DNA sequence composition in different eukaryotic groups, several phylogenetic lineages demonstrate highly conserved motifs. In animals, three main types of telomeric repeats are known: TTAGGG, TTAGGC, and TTAGG. Motif $(TTAGGG)_n$ prevails in the multicellular animals, except roundworms and arthropods, and is probably ancestral for all Metazoa; motif $(TTAGGC)_n$ is characteristic of nematodes; motif $(TTAGG)_n$, which is a derivative of $(TTAGGG)_n$, occurs in all arthropod groups (sea spiders, chelicerates, myriapods, crustaceans, and hexapods), supporting their origin from a common ancestor (Traut et al. 2007, Gomes et al. 2010). The $(TTAGG)_n$ telomeric motif is commonly found among different higher insect taxa, and this telomere structure is supposed to be phylogenetically ancestral in the class Insecta in general (Frydrychová et al. 2004). However, the insect-type consensus telomeric sequence is known to be lost independently during insect evolution (Frydrychová et al. 2004, Lukhtanov and Kuznetsova 2010, Gokhman et al. 2014). Further on, the loss and subsequent gain of typical telomeres may occur repeatedly as it has been indicated in Coleoptera (Frydrychová and Marec 2002) and recently in Heteroptera (Pita et al. 2016). However, it is worth noting that the number of species with known telomere structure is extremely low in each insect order, including Neuroptera (Frydrychová et al. 2004).

Neuroptera, also known as Planipennia, are a highly heterogeneous insect order, with 5803 species described in 16 families (Oswald 2016). In this group, telomere structure has so far been described in only two species, *Protidricerus japonicus* (McLachlan, 1891) from the family Ascalaphidae (Okazaki et al. 1993) and *Chrysoperla carnea* (Stephens, 1836) from the family Chrysopidae (Frydrychová et al. 2004). The former species was found to have the insect-type telomeric motif $(TTAGG)_n$, whereas the latter species was reported to lack this motif. Considering the heterogeneity in the presence of the TTAGG telomeric repeats discovered in Neuroptera, it thus could be interesting to verify the presence of this motif in other members of the order.

Within Neuroptera, the family Myrmeleontidae, commonly known as "antlions", due to the fiercely predatory habits of their larvae, is the most diverse group having worldwide distribution. This family is considered monophyletic, with over 1700 extant species in 191 genera. In Myrmeleontidae, as many as 12 subfamilies, among them Palparinae, Pseudimarinae, Stilbopteryginae, Dimarinae, Echthromyrmicinae, Dendroleontinae, Nemoleontinae, Glenurinae, Myrmecaelurinae, Acanthaclisinae, Brachynemurinae, and Myrmeleontinae (Krivokhatsky 2011), but most commonly only three, Stilbopteryginae, Palparinae and Myrmeleontinae (Stange 2004, Badano et al. 2016), are recognized. Myrmeleontidae, together with the families Psychopsidae, Nemopteridae, Nymphidae and Ascalaphidae, form the monophyletic suborder Myr-

meleontiformia (= the superfamily Myrmeleontoidea) that is a derived lineage of Neuroptera diversified in the Jurassic period (Badano et al. 2016). Different phylogenetic analyses based on morphological and genetic data established a sister-group relationship between Myrmeleontidae and Ascalaphidae (Badano et al. 2016). Ascalaphidae, or owlflies, are a smaller family, with about 430 described species in 100 genera distributed in all the biogeographic regions (Tjeder 1992). The family is subdivided into two main subfamilies, Haplogleniinae and Ascalaphinae (Henry 1978).

Until now, the cytogenetic studies in the Myrmeleontidae have been carried out on 37 species from 21 genera, and were focused exclusively on the basic features of the karyotypes such as chromosome numbers and sex determination systems (reviewed in Kuznetsova et al. 2015).

The aim of the present study is to further characterize chromosomes of antlions and to study their evolution by exploring the telomere structure and chromosomal location of the major ribosomal RNA (rRNA) genes using fluorescence *in situ* hybridization (FISH). The FISH technique was applied for the first time in the family Myrmeleontidae.

We examined the presence/absence of TTAGG telomeric repeats and location of the rDNA clusters in *Palpares libelluloides* (Linnaeus, 1764), *Distoleon tetragrammicus* (Fabricius, 1798) and *Acanthaclisis occitanica* (Villers, 1789) from the family Myrmeleontidae. In addition, we studied telomere structure in *Libelloides macaronius* (Scopoli, 1763) belonging to the sister family Ascalaphidae.

Material and methods

Material

Three antlion species, involving three different genera from three subfamilies (*sensu* Krivokhatsky 2011), i.e. Palparinae (*P. libelluloides*), Nemoleontinae (*D. tetragrammicus*) and Acanthaclisinae (*A. occitanica*), as well as the only owlfly species from the subfamily Ascalaphinae (*L. macaronius*) were studied. The specimens were collected by G. Khabiev from May to October 2015 in the Republic of Dagestan (North-East Caucasus, Russia). In the field, adult individuals were fixed in a solution of 96% alcohol and glacial acetic acid (3:1) and then stored at 4°C until required. Collection localities and chromosomal traits of each species are given in Table 1.

Telomere and rDNA detection by FISH

Chromosome preparations were obtained from male gonads. Testes were dissected in a drop of 45% acetic acid and squashed. The coverslips were removed using dry ice. Prior to FISH treatment, the preparations were examined using phase contrast microscopy.

Table 1. Examined material and main karyotypic features obtained during the present study.

Taxon	Sampling locality and month and year of collection	Number of studied males	Diploid karyotype	Telomeric sequence	18S rDNA clusters location
Myrmeleontidae					
Palparinae					
Palpares libelluloides (Linnaeus, 1764)	Russia, Dagestan, near Makhachkala 42°59'59.6"N 47°13'33.0"E, VI.2015	2	24 + XY	$(TTAGG)_n$	AA*
Nemoleontinae					
Distoleon tetragrammicus (Fabricius, 1798)	Russia, Dagestan, near Makhachkala 43°00'28.7"N 47°14'51.3"E VII.2015	2	14 + XY	?**	AA + X
Acanthaclisinae					
Acanthaclisis occitanica (Villers, 1789)	Russia, Dagestan, near Makhachkala 43°00'28.7"N 47°14'51.3"E VII.2015	2	16 + XY	$(TTAGG)_n$	AA + X
Ascalaphidae					
Ascalaphinae					
Libelloides macaronius (Scopoli, 1763)	Russia, Dagestan, near Gelinbatan village 41°56"50"N, 48°10"2"E, VII.2015	1	-***	$(TTAGG)_n$	-***

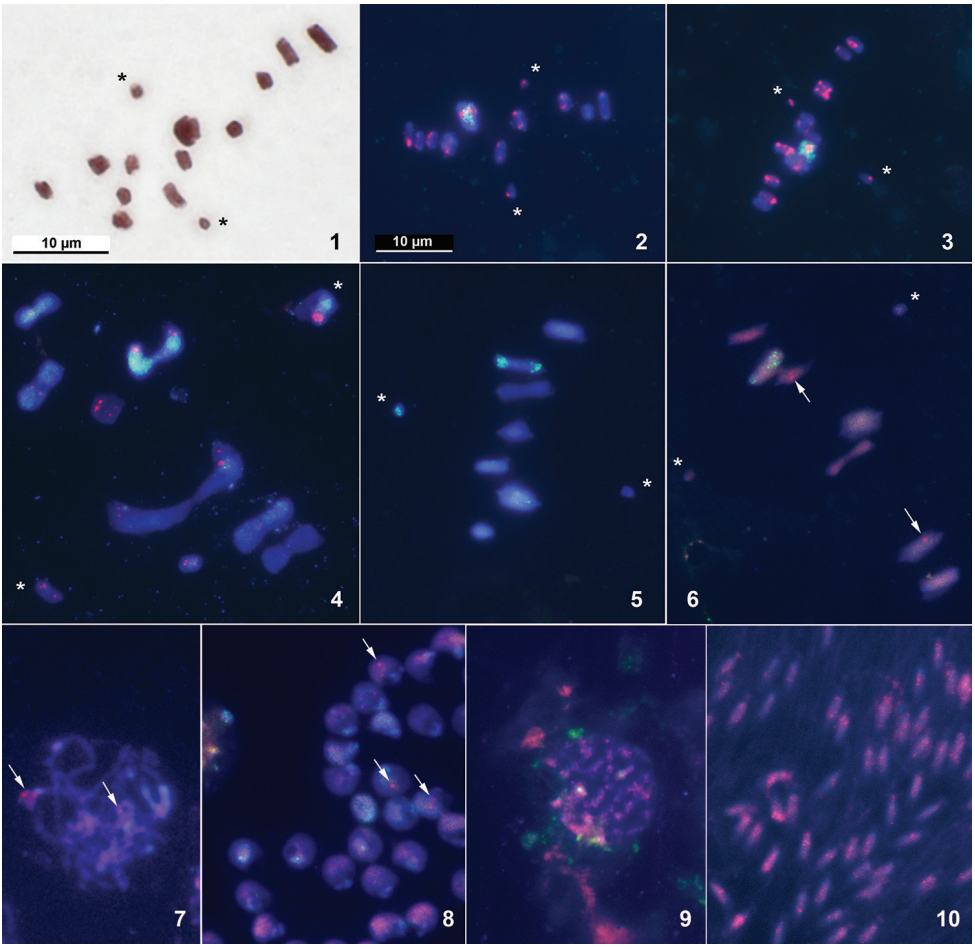
*A pair of autosomes; ** Ambiguous data; *** Missing data

FISH with $(TTAGG)_n$ and 18S rDNA probes was applied as previously reported for some other insects (Kuznetsova et al. 2015, Maryańska-Nadachowska et al. 2016, Golub et al. 2016). In brief, chromosome preparations were treated with 100 µg/ml RNase A and 5 mg/ml Pepsin solution to remove excess RNA and proteins. Chromosomes were denatured on a slide in a hybridization mixture with biotinylated 18S rDNA probe from the genomic DNA of *Pyrrhocoris apterus* (Linnaeus, 1758) and rhodaminated $(TTAGG)_n$ probe with addition of salmon sperm DNA and then hybridized for 36 h. Hybridization signals were detected with NeutrAvidin-FITC.

Chromosomes were mounted in antifade medium (ProLong Gold antifade reagent with DAPI; Invitrogen) and covered with a glass coverslip. Chromosome slides were analyzed under a Leica DM 6000 B microscope. Images were taken with a Leica DFC 345 FX camera using Leica Application Suite 3.7 software with an Image Overlay module.

Results

In male *P. libelluloides*, we found 12 autosomal bivalents and X and Y univalent chromosomes (Fig. 1), confirming the chromosome number, 2n = 26 (24 + XY), reported



Figures 1–10. Different stages of spermatogenesis in antlion species *Palpares libelluloides* (**1–3**), *Acanthaclisis occitanica* (**4**), *Distoleon tetragrammicus* (**5–8**) and owlfly species *Libelloides macaronius* (**9–10**) after standard staining (**1**) and FISH with the 18S rDNA and telomeric $(TTAGG)_n$ probes (**2–10**). **1–3** MI, n = 12 + X + Y **4** diakinesis/MI transition, n = 8 + X + Y **5, 6** MI, n = 7 + X + Y **7** pachytene **8** spermatids **9** mitotic metaphase **10** spermatids. Asterisks mark sex chromosomes; red signals identify the $(TTAGG)_n$- positive telomeres (arrowed); green signals identify rDNA clusters. Scale bar on Fig. 2 also applies to Figs 3–10.

by Kuznetsova et al. (2015). FISH with "insect" telomeric probe $(TTAGG)_n$ produced strong hybridization signals on the chromosome ends at metaphase I (Figs 2, 3) and other stages of meiosis (not shown). Some differences in hybridization intensity could be seen among different bivalents and between homologous telomeres. The rDNA probe detected 18S rDNA clusters on a large pair of autosomes (Figs 2, 3).

In male *A. occitanica*, we found 8 bivalents of various sizes and the X and Y univalent chromosomes, suggesting 2n = 18 (16 + XY). This species is cytogenetically studied for the first time. Its karyotype includes one exceptionally large pair of bi-armed autosomes, another pair of large bi-armed autosomes and the two sex chromosomes,

with the longer metacentric chromosome presumably being the X, while the other metacentric chromosome – the Y; the detailed morphology of other chromosomes remained unknown. The majority of bivalents and sex chromosomes displayed strong signals of the telomere probe. As for the heterogeneity for presence/absence and intensity of the $(TTAGG)_n$ signals, it could be explained by technical artifacts. Apart from a few scattered signals, the ribosomal probe detected a considerable accumulation of the 18S rDNA sequence on the second large pair of bi-armed autosomes and, additionally, on the putative X chromosome (Fig. 4).

In male *D. tetragrammicus*, we found 7 autosomal bivalents and X and Y univalent chromosomes (Figs 5, 6). An additional univalent, most likely a B-chromosome, which precociously segregates together with one of the sex chromosomes, was occasionally observed in first metaphase nuclei (not shown). The data obtained suggest a diploid karyotype of 2n = 16 (14 + XY) in contrast to 2n = 18 (16 + XY) reported by Kuznetsova et al. (2015). FISH with $(TTAGG)_n$ probe has detected no hybridization signals in the first metaphase nuclei (Fig. 5). However, weak and scarce $(TTAGG)_n$ hybridization signals of uncertain location could be seen in separate chromosomes of different stages as well as in the spermatids (Figs 6–8). The rDNA probe revealed 18S rDNA clusters both on a large pair of autosomes (possibly the third pair), and one of the sex chromosomes (Figs 5, 6).

In male *L. macaronius*, the karyotype remained unknown. However the $(TTAGG)_n$ -positive signals could be clearly observed in some cells including spermatids (Figs 9, 10).

Discussion

Karyotypes

As summarized recently (Kuznetsova et al. 2015), the karyotypes are currently known for 37 species of the Myrmeleontidae representing about 2.2% of the extant antlion species. The studied species belong to 21 genera and 9 subfamilies (*sensu* Krivokhatsky 2011) and were shown to have an XY-sex chromosome system as well as diploid chromosome numbers ranging from 14 to 26. The highest numbers, 2n = 22, 24 and 26, occur only in a more basal subfamily Palparinae, whereas lower numbers, 2n = 14, 16 and 18, are encountered in other subfamilies. Since the sister group to the Myrmeleontidae, i.e. the family Ascalaphidae, is characterized by higher chromosome numbers, usually 2n = 22, a higher number was suggested to be ancestral for antlions (Kuznetsova et al. 2015). The karyotypes of 2n = 16 and 2n = 18, which we recently found in *Distoleon tetragrammicus* (Nemoleontinae) and *Acanthaclisis occitanica* (Acanthaclisinae) respectively, corroborate this assumption. These karyotypes are further discussed below.

In our previous paper (Kuznetsova et al. 2015), *D. tetragrammicus* was reported to have 2n = 18, i.e., having two additional pairs of autosomes. A plausible explanation for this disagreement is that a univalent displaced from the equatorial plane was erroneously identified as a bivalent at the first metaphase in the only studied male (see

Fig. 2 in Kuznetsova et al. 2015). It is worth noting that a univalent segregating precociously with the sex chromosomes at the first metaphase was also observed in some nuclei of male *D. tetragrammicus* during the present study (see Results). We suggest that these univalent chromosomes are additional or so-called B chromosomes. One or two additional chromosomes that do not belong to the regular karyotype and are similar in their meiotic behavior to the sex chromosomes were repeatedly observed in different neuropteran species, including the antlion *Myrmeleon mexicanus* Banks, 1903 (Hughes-Schrader 1983). The effect of the supernumeraries varies in different species. For example, in *Hemerobius marginatus* Stephens, 1836 from another neuropteran family Hemerobidae, additional chromosomes influence the segregation of the sex chromosomes in meiosis (Nokkala 1986). The problem of B chromosomes in antlions needs to be further addressed in the future.

The karyotype of *A. occitanica* differs by having both very large autosomal bivalent and sex chromosome (supposedly the X). To our knowledge, this karyotype structure was never reported for the Myrmeleontidae. However, it is worth noting that antlion karyotypes were almost exclusively illustrated with drawings in the past (as opposed to photos) with no significant details of the chromosome structure and size reported.

Telomeres

The data obtained in the present study demonstrate for the first time the presence of the insect-type telomeric repeat $(TTAGG)_n$ in antlions. We have reliably shown that this motif is characteristic of *P. libelluloides* and *A. occitanica*. The third examined antlion species, *D. tetragrammicus*, in which only rare TTAGG-positive signals of uncertain location were detected at best, most likely does not have the canonical $(TTAGG)_n$ insect telomeric motif. However, the TTAGG sequence could actually be present in the telomeres but in very low copy numbers, making it difficult to detect this sequence by FISH. Consequently, we consider the data on *D. tetragrammicus* as preliminary and therefore deserving further clarification. We also showed that the $(TTAGG)_n$ repeat was present in telomeres of *L. macaronius* (Ascalaphidae). Earlier, this telomeric motif was recorded for another owlfly species, *Protidricerus japonicas*, by Frydrychová et al. (2004). Together with the results on *P. japonicus*, our data suggest that the $(TTAGG)_n$ telomere sequence found in species of Ascalaphidae and Myrmeleontidae was characteristic of the common ancestor of these sister families. The detection of this repeat in the most basal antlion subfamily examined so far, the Palparinae (*P. libelluloides*), further corroborates this suggestion.

At present, the only other neuropteran species with known telomere structure is *Chrysoperla carnea s. lato* belonging to the large worldwide family Chrysopidae (green lacewings). Based on the Southern hybridization results, Frydrychová et al. (2004) have shown that *Ch. carnea* is $(TTAGG)_n$ -negative. Despite the relatively small size of the order Neuroptera including only 5803 extant species (Oswald 2016), the data on telomere structure are still highly insufficient, and further studies are needed to fully understand the organization of telomeres in different families of this insect order.

Ribosomal clusters

Ribosomal gene markers have provided useful information regarding chromosome evolution in different groups of insects. In some groups, the number and chromosomal localization of rDNA clusters, usually located in the nucleolus organizing regions (NORs), serve as additional markers to characterize species and higher taxa (Nguyen et al. 2010, Gokhman et al. 2014), whereas in other groups they currently are the only available cytogenetic markers to differentiate species with similar karyotypes (Golub et al. 2016). Our data represent the first mapping experiments for the major rRNA genes (i.e. genes for 18S, 5.8S and 28S rRNA) not only in Myrmeleontidae but also in Neuroptera in general. The three species studied, *Palpares libelulloides*, *Acanthaclisis occitanica* and *Distoleon tetragrammicus*, showed the occurrence of one (in the first species) or two (in the two last species) rDNA clusters in their haploid karyotypes. In each species, these clusters are located on both autosomes of a particular large pair. In *A. occitanica* and *D. tetragrammicus*, another rDNA site is present on one of the sex chromosomes. Although it is at present impossible to identify homeologous chromosomes between different neuropteran species, we can suggest a single chromosome pair carrying major rDNA clusters as an ancestral state in antlions.

Conclusions

Our study contributes to the current knowledge of cytogenetics of the neuropteran family Myrmeleontidae. The principal outcomes of this study are: (1) the discovery of one or two major rDNA clusters per haploid karyotype; the clusters are located either only on a pair of autosomes in a particular species or, additionally, on one of the sex chromosomes in another two studied species and (2) the discovery of the insect-type $(TTAGG)_n$ telomeric sequence at least in two of the three studied species. Because the $(TTAGG)_n$ sequence is likewise found in the two studied owlfly species, we suggest that this telomere structure was inherent in the last common ancestor of the phylogenetic lineage Myrmeleontidae + Ascalaphidae.

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SHORT COMMUNICATIONS

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Unraveling the karyotype structure of the spurges *Euphorbia hirta* Linnaeus, 1753 and *E. hyssopifolia* Linnaeus, 1753 (Euphorbiaceae) using genome size estimation and heterochromatin differentiation

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Abstract

Euphorbia Linnaeus, 1753 (Euphorbiaceae) is one of the most diverse and complex genera among the angiosperms, showing a huge diversity in morphologic traits and ecologic patterns. In order to improve the knowledge of the karyotype organization of *Euphorbia hirta* (2n = 18) and *E. hyssopifolia* (2n = 12), cytogenetic studies were performed by means of conventional staining with Giemsa, genome size estimations with flow cytometry, heterochromatin differentiation with chromomycin A_3 (CMA) and 4',6-diamidino-2-phenylindole (DAPI) and Giemsa C-banding, fluorescent *in situ* hybridization (FISH) with 45S and 5S rDNA probes, and impregnation with silver nitrate ($AgNO_3$). Our results revealed small metacentric chromosomes, $CMA^+/DAPI^0$ heterochromatin in the pericentromeric regions of all chromosomes and $CMA^+/DAPI^-$ in the distal part of chromosome arms carriers of nucleolar organizing regions (NORs). The DNA content measurements revealed small genomes for both species: *E. hirta* with 2C = 0.77 pg and *E. hyssopifolia* with 2C = 1.41 pg. After FISH procedures, *E. hirta*, and *E. hyssopifolia* presented three

and four pairs of terminal 45S rDNA sites, respectively, colocalizing with CMA$^+$ heterochromatic blocks, besides only one interstitial pair of 5S rDNA signals. Additionally, the maximum number of active NORs agreed with the total number of observed 45S rDNA sites. This work represents the first analysis using FISH in the subfamily Euphorbioideae, revealing a significant number of chromosomal markers, which may be very helpful to understand evolutionary patterns among *Euphorbia* species.

Keywords

Cytotaxonomy, CMA/DAPI, FISH, genome size, rDNA, RONs

Introduction

The giant genus *Euphorbia* (spurges), a member of the family Euphorbiaceae, is one of the largest and most diverse groups of the plant kingdom, consisting of more than 2000 species with a very wide geographic distribution (Bruyns et al. 2006, The Plant List 2013, Webster 2014). The species of the family are used mainly for ornamental and/or medicinal purposes (e.g. Shi et al. 2008, Mwine and Van Damme 2011), although some caution must be required due to the toxic potential of these plants (e.g. Araújo et al. 2015). *Euphorbia hirta* and *E. hyssopifolia* are cosmopolitan spurges with widely known medicinal properties, standing out mainly due to their diuretic and antimicrobial activities (e.g. Ayyannar and Ignacimuthu 2009, Alisi and Abanobi 2012, Huang et al. 2012, Kuta et al. 2014, Santana et al. 2015). Both species are sub-spontaneous and ruderal, native to the New World, tolerant to drought and high temperatures (Steinmann and Porter 2002). They are broadly distributed in subtropical and tropical regions, from the sea level up to 1500 m (Amorozo 2002, Schneider 2007). In Brazil, they are often sympatric (Santana et al. 2015) and occur in all regions and biomes, where they inhabit degraded areas, roadsides, cultivated fields and gardens (Steinmann and Porter 2002).

Similarly to the family as a whole, the genus *Euphorbia* is an extremely diversified group, not only taking into account morphology and habit (Webster 1994) but also regarding karyotypic characters (Hans 1973). Therefore, the vast complexity of the genus may explain the controversies among the few analyses of the phylogenetic relationships within the group (see Bruyns et al. 2006, Horn et al. 2012, Dorsey et al. 2013). According to Bruyns et al. (2006), the development of a natural classification for *Euphorbia* has been hampered by several factors, such as the high number of species, the wide geographic distribution of the genus and a high degree of convergence in various vegetative characters.

In groups with such a complex classification, the knowledge about chromosome features, such as the organization of interphase nuclei, diploid number, nuclear DNA content and physical mapping of repetitive DNA, may be critical to support studies on systematics and understanding evolutionary pathways (Benko-Iseppon and Morawetz 2000, Guerra 2012). However, to date, just a few studies have provided some help towards elucidating the karyotypic patterns for both the genus and the family. As mentioned by D'Emerico et al. (2003), the available karyotype data for *Euphorbia* species

are minimal and, in most cases, there are only descriptions of chromosome numbers. Thus, aiming to increase the cytogenetic data and to identify chromosome markers for this important genus, cytogenetic analyses were performed with conventional staining, genome size estimations through flow cytometry, Giemsa C-banding, CMA/DAPI banding, impregnation with silver nitrate, FISH with 45S and 5S rDNA probes in the species *E. hirta* and *E. hyssopifolia*.

Material and methods

Fruits of specimens of *E. hirta* (vouchers: K.C.B. Santana 04, 05 and 06 – UFP) and *E. hyssopifolia* (vouchers: K.C.B. Santana 01, 02 and 03 – UFP) were collected in urban fragments of the Atlantic Forest in Recife (Pernambuco, Brazil). Subsequently, they were incubated at 50 °C for 5 h and then transferred to room temperature (ca. 25 °C) for three to four days to release the seeds, which were germinated in Petri dishes under an artificial system of circadian lighting (≥ 1,500 lux) at ~35 °C. Root tips were pre-treated with 2 mM 8-hydroxyquinoline for 90 min at room temperature and 23 h at 8 °C. For the conventional staining, fluorochromes and FISH procedures, the roots were fixated in ethanol:acetic acid (3:1, v:v), for 4–6 h at room temperature and stored at –20 °C.

The preparation of slides followed the methodology used by Benko-Iseppon and Morawetz (2000). Root tips were hydrolyzed in 5N HCl for 20 min at room temperature and squashed in 45% acetic acid. Slides were stained with 2% Giemsa for 10 min, washed with distilled water and mounted with Entellan (Merck).

To estimate the DNA C-values, approximately 20-30 mg of fresh leaves from *E. hirta* and *E. hyssopifolia* were chopped on ice with 1 mL of GPB buffer (Loureiro et al. 2007), with the addition of 3% PVP and 4% Triton X-100, to release the nuclei, according to Dolezel et al. (1989), using *Solanum lycopersicum* Linnaeus, 1753 (2C = 2.06 pg) as the internal reference standard. For each species, three different samples were prepared, and at least 5,000 nuclei were analyzed for each species using a BD FACSAria II (BD Biosciences, San Jose, CA, USA) cytometer. Each output flow cytometry histogram from BD FACSDiVa software v. 6.1 was analyzed using Flowing Software v. 2.5 by Perttu Terho (Turku Centre for Biotechnology, University of Turku, Turku, Finland), with all peaks presenting a coefficient of variation smaller than 4%. The DNA 2C-values of each sample were calculated by the relative fluorescence intensity of the sample and the internal reference standard.

The C-banding methodology followed the procedures described by Guerra and Souza (2002), with some modifications. Slides were immersed in preheated 45% acetic acid at 60 °C for 30 min, followed by washing in preheated distilled water (at 60 °C) that was gradually changed by water at room temperature. After drying, the slides were incubated in 5% $Ba(OH)_2$ for 30 min at room temperature and then washed with distilled water. Afterward, slides were immersed in a 2×SSC solution (300 mM NaCl and 30 mM $Na_3C_6H_5O_7.2H_2O$) for 2 h at 60 °C, being subsequently washed with distilled water. The fluorochrome staining was performed as described below.

The impregnation with silver nitrate followed the protocol described by Vieira et al. (1990), with modifications implemented by Vasconcelos et al. (2010). After pretreatment procedures, roots were fixated in 50% ethanol, acetic acid and 37% formaldehyde (18:1:1, v/v/v) for 4 h at room temperature. Fixated roots were washed with distilled water and then incubated in an aqueous solution of 20% silver nitrate at 60 °C for at least 12 h. After removal of silver residues, the staining was revealed in a solution of 1% hydroquinone in 10% formaldehyde (1:1, v/v) for 2 min, followed by washing with distilled water. The meristematic tissue was squashed between slide and coverslip in 45% acetic acid with a drop of 1% acetic carmine. Then, the slides were frozen in liquid nitrogen, immersed in absolute ethanol for 4 min, dried and mounted with Entellan.

The CMA/DAPI banding followed Schweizer and Ambros (1994), with some modifications. Root tips were washed three times (5 min each) in distilled water and digested for 2 h at 37 °C in an enzymatic solution of 2% cellulase (Onozuka) and 20% pectinase (Sigma). Meristems were washed, placed on slides and then squashed in 45% acetic acid. Chromosome preparations were aged for three days at room temperature, stained with CMA (0.5 mg/mL) for 1 h and DAPI (2 μg/mL) for 30 min, mounted in McIlvaine-glycerol buffer (1:1) and stored for three days. Two probes were used in the FISH procedures: (1) pTa71 clone, containing the repeating unit of the 18S-5.8S-26S rDNA from *Triticum aestivum* Linnaeus, 1753 (Gerlach and Bedbrook 1979), and (2) pTa794 clone, which corresponds to the unit of the 5S rDNA gene isolated from *T. aestivum* (Gerlach and Dyer 1980). Both probes were labeled with digoxigenin-11-dUTP (Roche) by nick translation and hybridized sequentially, according to Heslop-Harrison et al. (1992). Chromosome preparations previously used in the CMA/DAPI banding were pretreated as described by Pedrosa et al. (2001). Denaturation of chromosomes and probes, post-hybridization baths and the detection of the probes were carried out as described by Heslop-Harrison et al. (1991), except for the stringency wash, which was conducted in 0.1×SSC (15 mM NaCl and 1.5 mM $Na_3C_6H_5O_7.2H_2O$) at 42 °C. The hybridization mixtures consisted of 50% formamide (v/v), 10% dextran sulfate (w/v), 2×SSC and 2-5 ng/μL of the probe. The slides were denatured for 7 min at 85 °C and hybridized for at least 18 h at 37 °C. The probes were detected with a primary antibody against digoxigenin grown in sheep (DAKO) in combination with anti-sheep secondary antibody conjugated to FITC (DAKO). Slides were mounted in 2 mg/mL DAPI in Vectashield (Vector) (1:1, v/v).

Images of the best cells were captured with a Leica DMLB epifluorescence microscope with a Leica DFC 340FX camera, using the software Leica CW4000. Images were optimized for best contrast and brightness and the photos of FISH with 5S and 45 rDNA probe were pseudocolored in red and green, respectively (to allow the superposition of images), using Adobe Photoshop CS4 (Adobe Systems Incorporated). Additionally, chromosomes of 10 cells stained with DAPI of each species were measured to obtain the chromosome sizes and the relationship between the chromosome arms according to Guerra (1986), using the software UTHSCSA ImageTool, for further elaboration of the mitotic idiogram through the software Adobe Illustrator CS4 (Adobe Systems Incorporated).

Results and discussion

The interphase nuclei of both species were predominantly semi-reticulated with a proximal pattern of condensation (Figures 1A, 2A). The chromosome counts showed diploid numbers of 2n = 18 for *E. hirta* and 2n = 12 for *E. hyssopifolia* (Figures 1–3), confirming previous results for both species (e.g. Wang et al. 1999, Bolaji et al. 2014). In general, the species of *Euphorbia*, in comparison with the entire family, are relatively well represented in the chromosome count lists of Euphorbiaceae members, with approximately 310 species of the genus (15.4%) with available chromosome numbers, which ranges from 2n = 12 (in *E. akenocarpa* Gussone, 1821 and several other species) to 2n = 120 (in *E. royleana* Gussone, 1821) (see Hans 1973, Rice et al. 2015). This becomes more evident when we take into account the available data for *Croton* Linnaeus, 1753 (Euphorbiaceae), with less than 3% of the species with chromosome numbers described (34 out of ca. 1,200 species; The Plant List 2013, Rice et al. 2015). In addition, the existence of several base numbers for *Euphorbia* (x = 6, 7, 8, 9, 10, etc.) indicates a great complexity of the processes of karyotype evolution and diversification within the group (Hans 1973, Rice et al. 2015). Therefore, one may notice the importance of the data published for *Euphorbia* species in improving the knowledge of the patterns of karyotype evolution within the family.

The karyotype presented metacentric and submetacentric chromosomes with gradual decreasing sizes, ranging in average from 1.21 µm to 2.58 µm, for *E. hirta*, and from 1.43 µm to 2.04 µm, for *E. hyssopifolia*. In general, Euphorbiaceae species exhibit small chromosomes (see Hans 1973, Vanzela et al. 1997, Carvalho and Guerra 2002), such as those found in the two species analyzed, although there is also a wide variation in chromosome sizes within the genus *Euphorbia*, which ranges from 1-15 µm (Hans 1973). Additionally, as observed for *E. hirta* and *E. hyssopifolia*, the occurrence of only metacentric and submetacentric chromosomes was already reported for other species of the family, such as *Manihot* spp. (Carvalho and Guerra 2002), *Jatropha curcas* Linnaeus, 1753 (Carvalho et al. 2008) and castor (*Ricinus communis* Linnaeus, 1753) (Vasconcelos et al. 2010). On the other hand, acrocentrics and subtelocentrics were described for other *Euphorbia* species, such as *E. characias* Linnaeus, 1753 and *E. meloformis* Aiton, 1789 (Vosa and Bassi 1991, D'Emerico et al. 2003).

Euphorbia hirta presented a smaller genome size (2C = 0.77 ± 0.02 pg) than *E. hyssopifolia* (2C = 1.41 ± 0.04 pg). These results fit in the known range of DNA content of species of the genus, which varies from 2C = 0.70 pg to 2C = 18.80 pg (see Bennett and Leitch 2012). According to the most comprehensive phylogenetic reconstruction based on nuclear and plastid sequences for *Euphorbia* subgenus *Chamaesyce* Gray, 1821, provided by Yang and Berry (2011), three major clades (Acuta, Hypercifolia and Peplis) were strongly supported. Thus, despite the inclusion of both *E. hirta* and *E. hyssopifolia* in the clade Hypericifolia, they were not recovered as closely related species. Therefore, a plausible explanation for the discrepancy regarding the genome size and chromosomes numbers between these two species could be their particular evolutionary histories.

However, despite accounting for more than half of the known genome sizes of members of Euphorbiaceae (19 out of 33 analyzed species), the proportion of *Euphorbia* analyzed

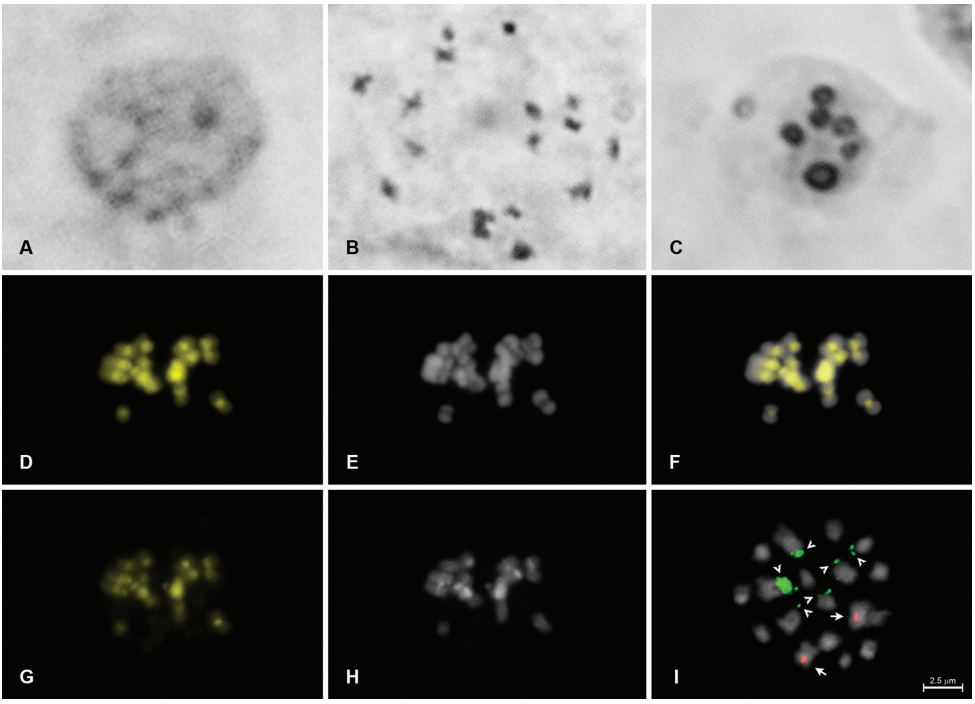

Figure 1. Karyotype analysis of *Euphorbia hirta* (2n = 18). Standard staining of mitotic interphase nucleus (**A**); standard staining of mitotic metaphase (**B**); silver impregnation of mitotic interphase nucleus (**C**); fluorochrome banding of metaphase chromosomes stained with CMA (**D**) and DAPI (**E**) and superposed images (**F**); C-banding of chromosomes stained with CMA/DAPI (C-CMA/DAPI; **G–H**); and metaphase chromosomes hybridized with 5S (red) and 45S (green) rDNA probes (**I**). Arrows and arrowheads indicate 5S and 45S rDNA sites, respectively.

species is considerably low, being less than 1% of the genus. Thus, the noteworthy range of variation of 41× among the species of the genus analyzed so far, between the diploid species *E. peplus* Linnaeus, 1758 (2n = 22; 2C = 0.70 pg) and *E. polygona* Haworth, 1803 (2*n* = 20; 2C = 28.70), although quite high, may be an underestimation (see Bennet and Leitch 2016). Also, while this is the first report of the genome size of *E. hyssopifolia*, Bennett et al. (1998) observed 2C = 1.30 pg for *E. hirta*, which is almost twice the value obtained here. Similarly, divergent genome sizes have been estimated for other *Euphorbia* species, such as *E. amygdaloides* L. with 2C = 5.48 pg (Vidic et al. 2009) and 2C = 7.02 pg (Temsch et al. 2010) and *E. pulcherrima* Willdenow ex Klotzsch, 1834 with 2C = 2.60 pg (Galbraith et al. 1983) and 2C = 3.30 pg (Bennet et al. 2000). In this regard, these differences in the genome size within the same species may be associated with intraspecific variation in the abundance and distribution of genomic repeat classes, such as transposable elements (Kidwell 2002, Heslop-Harrison 2012).

In the CMA/DAPI banding, the pericentromeric region of all chromosomes showed positive bands for CMA and were negative for DAPI ($CMA^+/DAPI^-$) for both species (Figures 1D–F, 2D–F), as previously observed in castor (Vasconcelos et

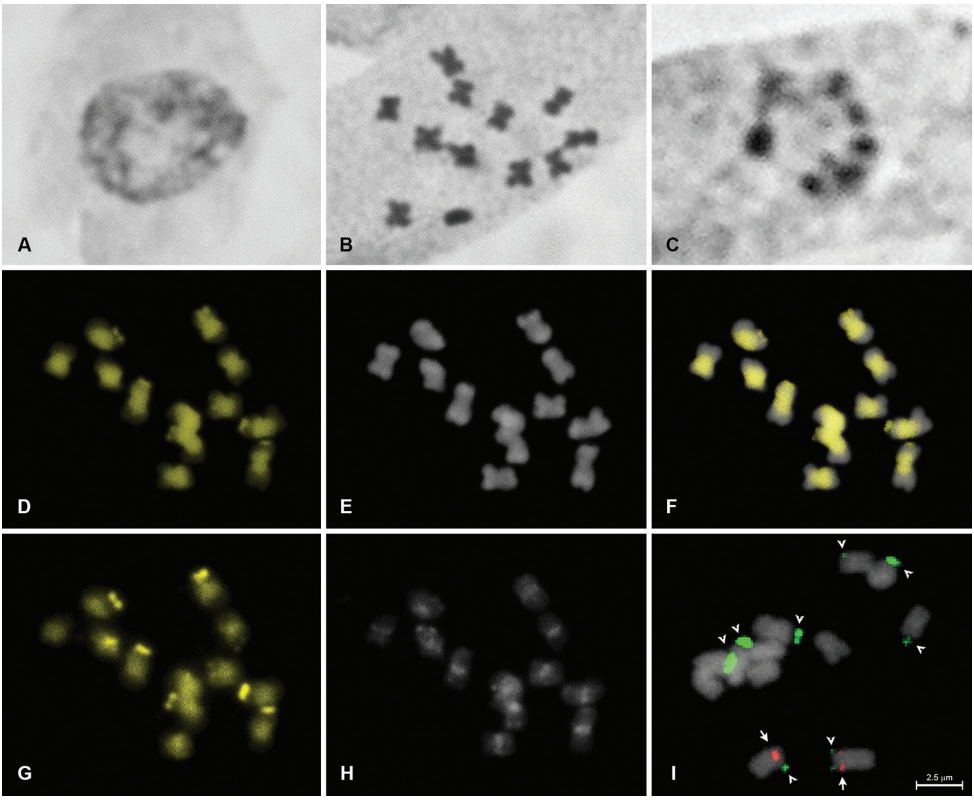

Figure 2. Karyotype analysis of *Euphorbia hyssopifolia* (2n = 12). Standard staining of mitotic interphase nucleus (**A**); standard staining of mitotic metaphase (**B**); silver impregnation of mitotic interphase nucleus (**C**); fluorochrome banding of metaphase chromosomes stained with CMA (**D**) and DAPI (**E**) and superposed images (**F**); C-banding of chromosomes stained with CMA/DAPI (C-CMA/DAPI; **G–H**); and metaphase chromosomes hybridized with 5S (red) and 45S (green) rDNA probes (**I**). Arrows and arrowheads indicate 5S and 45S rDNA sites, respectively.

al. 2010) and frequently reported for angiosperms with small chromosomes (Guerra 2000), such as cowpea (*Vigna unguiculata* Linnaeus, 1753) (Bortoleti et al. 2012). On the other hand, all chromosomes of both species presented neutral regions for both fluorochromes in the remaining portions of all chromosomes, except for the satellites, which were $CMA^+/DAPI^-$ (Figure 3). The Giemsa C-banding revealed the same heterochromatic regions evidenced by CMA/DAPI, which were sensibly enhanced by the CMA/DAPI/C-banding approach (Figures 1G–H, 2G–H, 3). Furthermore, after the CMA/DAPI/C-banding, DAPI bands could be observed in the pericentromeric region of all chromosomes of *E. hirta* and *E. hyssopifolia*, which may not necessarily be related to AT-rich heterochromatin, but to heterochromatin in general (Barros e Silva and Guerra 2010).

The FISH procedures revealed 45S rDNA terminal sites for both *E. hirta* (short arm of chromosome pairs 1, 3 and 5) and *E. hyssopifolia* (short arm of chromosome

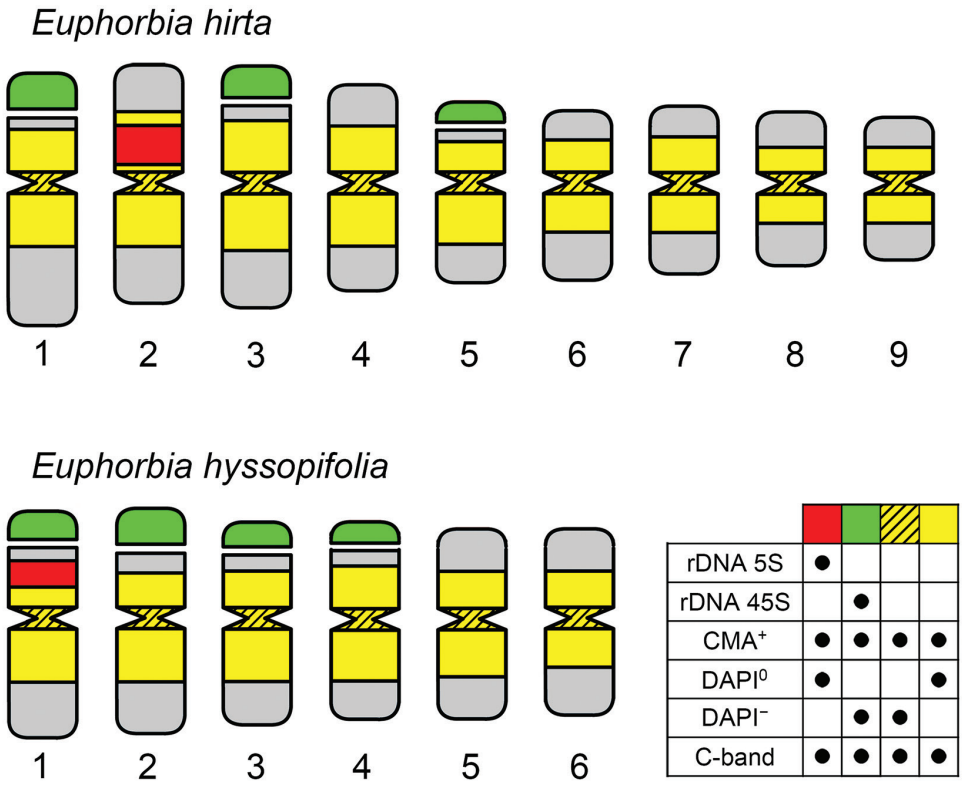

Figure 3. Representative idiograms of *Euphorbia hirta* and *E. hyssopifolia* chromosomes. The black dots in the chart in the inferior right corner associate the chromosome marks (rows) with their respective colors (columns) in the chromosomes.

pairs 1, 2, 3 and 4) (Figures 1I, 2I and 3), which were always associated with CMA$^+$/DAPI$^-$ bands (Figure 3), such as in all previously analyzed Euphorbiaceae species (see Leitch et al. 1998, Carvalho and Guerra 2002, Vasconcelos et al. 2010). On the other hand, only one site of 5S rDNA was observed in an interstitial position of the short arm of chromosome pair 2 of *E. hirta* (Figure 1I) and pair 1 of *E. hyssopifolia* (Figure 2I), as described for all other karyotypes within the family (Leitch et al. 1998, Carvalho and Guerra 2002, Witkowska et al. 2009, Vasconcelos et al. 2010). In addition, both species presented 5S rDNA sites associated with CMA$^+$ bands (Figure 3), as described for castor, in which there was also a chromosome pair bearing both 5S rDNA and 45S rDNA (Vasconcelos et al. 2010), and other few angiosperms (e.g. Cabral et al. 2006, Bernardes et al. 2013). As discussed by Roa and Guerra (2015), the occurrence of 5S and 45S rDNA sites in the same chromosome (as observed for *E. hyssopifolia*) has been reported in several angiosperm species, possibly as a consequence of random transpositions of both sequences. Additionally, the authors observed that this association is more likely to be observed when there are multiple rDNA sites, as found in the present work.

Table 1. Nucleolar frequency by interphase nucleus in mitotic cells of *Euphorbia hirta* and *E. hyssopifolia*.

Species	Nucleoli per cell						Number of cells
	1 (%)	2 (%)	3 (%)	4 (%)	5–6 (%)	7–8 (%)	
Euphorbia hirta	54.04	34.25	9.89	1.31	0.52	-	5054
Euphorbia hyssopifolia	34.72	40.19	19.35	4.91	0.72	0.12	4280

The maximum number of nucleoli per interphase nuclei visualized through impregnation with silver nitrate in *E. hirta* and *E. hyssopifolia* were six and eight, respectively (Figure 1C, 2C). However, the vast majority of cells of *E. hirta* and *E. hyssopifolia* exhibited, respectively, one (54.04%) and two (40.19%) nucleoli, with only 0.24% and 0.12% showing the maximum number of active NORs (Table 1). The low frequency of cells displaying the maximum number of evident nucleoli in both species is quite common for species with more than one NOR (see Vasconcelos et al. 2010 and references within), which probably occurred due to fusion of nucleoli and/or absence of activation of certain NORs in the previous interphase (Pikaard 2000, Preuss and Pikaard 2007).

It is interesting to note that despite similarities in the morphology, habit and occurrence of both here studied species in the sampled area, no evidence of hybridization was detected during field work. This was confirmed by a recent report using phytochemical profiling and ISSR (Inter-Simple Sequence Repeat) markers, positioning both taxa in distinct branches (Santana et al. 2015). Both species are sometimes confounded by the local herbal sellers, being sold under identical common names (e.g. *erva-de-santa-luzia*; Santana et al. 2015). Thus, the here observed cytogenetic features reassure their position as completely distinct taxonomic entities.

Conclusions

The present analysis characterized the chromosomes of two *Euphorbia* species, being a pioneer in the application of the FISH methodology with members of the subfamily Euphorbioideae. The physical mapping of repetitive DNA played a complementary role between the different methodologies employed, generating markers that showed a relatively high conservation of the distribution pattern of heterochromatin between *E. hirta* and *E. hyssopifolia*. These findings indicated the high potential of the employed approaches in describing chromosome markers that may be very helpful differentiate species and understand karyotype evolution within such a diverse genus.

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SHORT COMMUNICATION

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Mitochondrial chromosome as a marker of animal migratory routes: DNA barcoding revealed Asian (non-African) origin of a tropical migrant butterfly *Junonia orithya* in south Israel

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Abstract

The blue pansy *Junonia orithya* Linnaeus, 1758 (Lepidoptera, Nymphalidae) is widely distributed along the tropical areas of Africa, Asia and Australia. It is also known as a migrant species in the Levant. Here we record *J. orithya* in south Israel and provide a DNA-barcode-based evidence for its Asian (non-African) origin.

Keywords

barcode libraries, *COI*, Iran, Jordan, migration, Nymphalidae

Introduction

Despite its small size, the mitochondrial chromosome is a functionally important portion of the eukaryotic DNA (Taanman 1999). It is also an extremely useful genetic marker broadly used in genetic, phylogenetic, phylogeographic, biogeographic and taxonomic studies (Avise 2004, Talavera and Vila 2011, Lukhtanov et al. 2016). A relatively fast mutation rate and rapid sorting of mtDNA gene lineages, as well as absence (or at least rarity) of recombination usually result in high divergence of mitochondrial genomes among species and a comparatively small variance within species. For this reason, mtDNA-based species identification (so called DNA barcoding) has become a popular tool for identifying unknown specimens in terms of pre-existing classifications (Hebert et al. 2003, Chambers and Hebert 2016). This prompted DNA barcode databases (http://www.boldsystems.org/) and DNA reference barcode libraries (e.g. Dincă et al. 2011, 2015, Wilson et al. 2013).

Here we demonstrate how DNA barcode libraries result in an opportunity to study migration routes. Migration is a common phenomenon in animals (Dingle 2014, Chapman et al. 2015), but it is poorly studied in some groups, especially in insects. Within butterflies, with the exception of the relatively well studied monarch *Danaus plexippus* Linnaeus, 1758 (Brower 1995, Oberhauser et al. 2015) and the painted lady *Vanessa cardui* Linnaeus, 1758 (Talavera and Vila 2016), little is known about other species' migratory routes.

The blue pansy *Junonia orithya* Linnaeus, 1758 belongs to a group of butterflies able to perform long-range migrations (Larsen 1984, Benyamini 2010). This species is widely distributed within the tropical areas of the Old World: in sub-Saharan Africa, Arabia, South and South-East Asia and Australia. It is known to be adapted to tropical environments and, according to Larsen (1984), could not normally survive winter even in the hottest spots of the Palaearctic region, such as the Jordan Valley in the Middle East. In south Iraq and south Iran, butterflies can be regularly observed in most months of the year, but in the central and northern parts of these countries they are less regular (Wiltshire 1957, Tshikolovets et al. 2014). Migrant individuals occur in Jordan (Larsen 1984, Katbeh-Bader et al. 2003, Benyamini 2010) and have been recently recorded in East Turkey (Biricik 2011). In Israel, records of *J. orithya* are known from the Jordan River valley in the north-east (Benyamini 2010). This distributional pattern fits well the hypothesis that specimens from Israel (as well as from the entire Middle East) might be connected to South Asia (and not to Africa, despite its geographical proximity), moving from south-east to north-west and finally reaching the most eastern parts of Israel.

On April 28, 2016 we collected a male of *J. orithya* in the southern tip of Israel in a small cultivated green patch near kibbutz Neot Smadar (30°02'40"N, 35°01'01"E, 409 m above sea level), enclosed by the Negev desert. Israel forms a biogeographic land bridge between Asia, Africa and Europe (Chipman et al. 2013); therefore the discovery of the blue pansy in the Negev, close to the African continent, raises a possibility of an African origin of *J. orithya* in Israel.

The "African" hypothesis seems to be plausible because animals and plants of African origin comprise a large group in the ecosystems of Israel, especially in the south where African elements are predominant (Goren and Ortal 1999). For example, butterfly fauna of south Israel includes such species of African origin as *Papilio saharae* Oberthür, 1879, *Euchloe aegyptiaca* Verity, 1911, *Euchloe falloui* Allard, 1867, *Epamera claucus* Butler, 1885, *Anthene amarah* (Guérin, 1849), *Azanus ubaldus* (Cramer, 1782), *Pseudophilotes abencerragus* (Pierret, 1837) and *Gomalia elma* (Trimen, 1862) (Benyamini 2010).

Here we tested the Asian versus African hypotheses by analyzing the barcode *COI* region of the mitochondrial genome. First we inspected all *COI* barcodes available from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and BOLD (http://www.boldsystems.org/index.php/Public_BarcodeIndexNumber_Home) and revealed that *J. orithya* comprised two genetically differentiated clusters of individuals. One cluster was represented by butterflies from Africa (Kenya, South Africa, Zimbabwe), and another cluster was represented by butterflies from Asia and Australia. Despite huge territory occupied by each of these clusters and despite the substantial divergence between the clusters, each group was found to be very homogenous with respect to the *COI* gene.

Then we obtained DNA barcodes for the sample from Neot Smadar, Israel (BPALB098-16, GenBank accession number: KY118822) and in three additional samples from Iran (from the collection of the Zoological Institute, St. Petersburg: BPAL2941-15 KY118824, BPAL2942-15 KY118825, BPAL2943-15 KY118823). These samples were processed as previously described (Lukhtanov et al. 2014, 2015). We used Bayesian Inference (MrBayes 3.1.2) as described previously (Vershinina and Lukhtanov 2010) to reconstruct a phylogenetic tree (Fig. 1). We used the published sequences of *J. neildi* Brévignon, 2004 (Gemmell et al. 2014) to root the tree (Fig. 1). Uncorrected *p*-distances were calculated manually based on direct comparison of sequences. In addition to our own sequences, only published data (one sample from Zimbabwe, Africa, one sample from Australia and 31 samples from Asia) (Kodandaramaiah and Wahlberg 2007, Zhang et al. 2008, Gaikwad et al. 2012, Ashfaq et al. 2013, Wilson et al. 2013, Hebert et al. 2013, Srirama et al. 2014, Yamada et al. 2014) were used for the phylogenetic inference (Fig. 1) and *p*-distance comparison.

The analysis revealed that the collected sample from south Israel was identical with eight samples from South Asia: with three samples from Malaysia (KF226505.1, KF226504.1, KF226503.1) and with five samples from Pakistan (GU681856.1, HQ990373.1, HQ990374.1, HQ990375.1, KC755868.1) (*p*-distance = 0%). It was also very close to other samples from Oriental and Australian regions as well as to the samples from Iran (Fig. 1) (*p*-distance from 0.2 to 1.3%). At the same time it differed by 19 nucleotide substitutions from the Zimbabwean sample (*p*-distance = 2.9%).

Thus, the genetic connectivity identified in our study supports the hypothesis that *J. orithya* may colonize Israel and Iran from tropical regions in Asia. Given that the species seem not to overwinter in the north of the Middle East, its sporadic presence suggests that these are immigrants, but precise origins of the specimens studied here could not be traced because of lack of phylogeographic structure within the analysed Asian

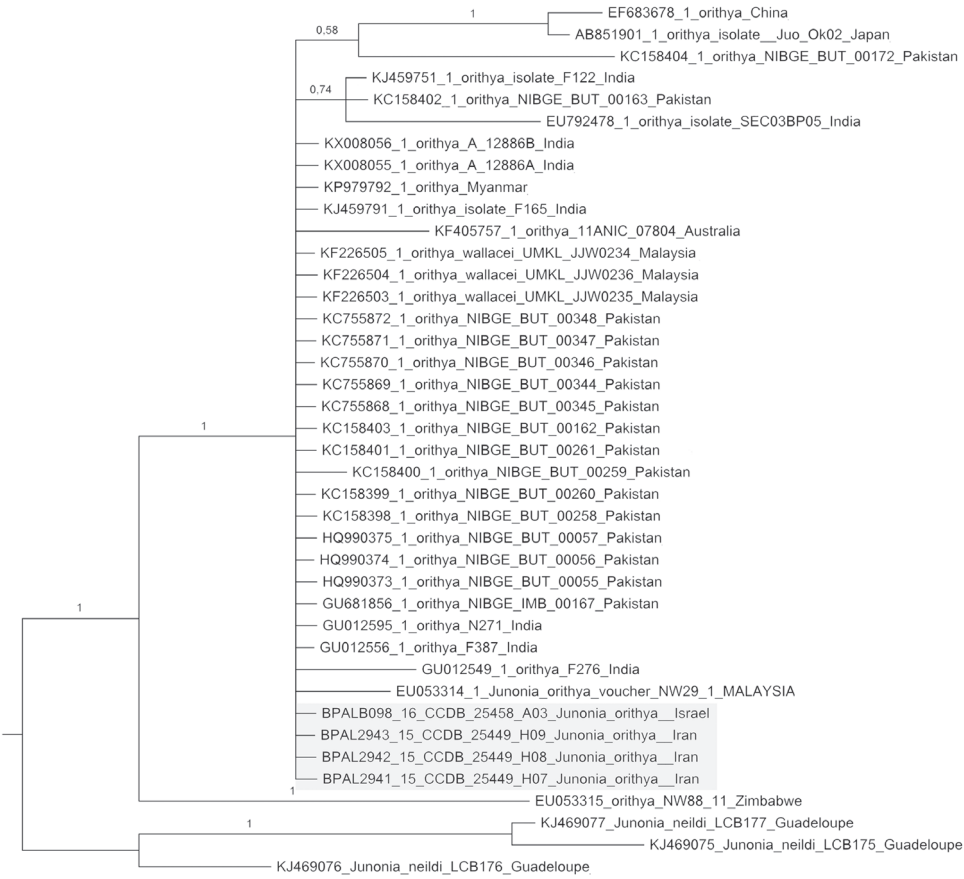

Figure 1. Bayesian tree of *Junonia orithya* samples based on analysis of the *cytochrome oxidase subunit I* (*COI*) gene (barcode region, 658 bp). Numbers at nodes indicate Bayesian posterior probability.

populations. Since the species is common in certain areas in Arabia (Wiltshire 1957), that might be one of the most likely origin for these specimens. An African origin is not supported for these specimens and whether the Asian/Arabian origins are annually recurrent should be studied in detail with temporal series of immigrants. A wider African sampling, particularly from the northern hemisphere and especially from the nearest areas to Israel is also required for further testing the African hypothesis.

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RESEARCH ARTICLE

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Karyomorphometric analysis of *Fritillaria montana* group in Greece

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Abstract

Fritillaria Linnaeus, 1753 (Liliaceae) is a genus of geophytes, represented in Greece by 29 taxa. Most of the Greek species are endemic to the country and/or threatened. Although their classical cytotaxonomic studies have already been presented, no karyomorphometric analysis has ever been given. In the present study, the cytological results of *Fritillaria montana* Hoppe ex W.D.J. Koch, 1832 group, which includes *F. epirotica* Turrill ex Rix, 1975 and *F. montana* are statistically evaluated for the first time. Further indices about interchromosomal and intrachromosomal asymmetry are given. A new population of *F. epirotica* is also investigated, while for *F. montana*, a diploid individual was found in a known as triploid population. Paired t-tests and PCoA analysis have been applied to compare the two species.

Keywords

Fritillaria epirotica, *Fritillaria montana*, karyotype analysis, PCoA, endemics, Greek flora, karyograms

Introduction

The genus *Fritillaria* Linnaeus, 1753 (Liliaceae) comprises approximately 150 taxa of geophytes, found in the temperate zones of the Northern Hemisphere (Kamari and Phitos 2006). Most of them are distributed across Eurasia while about 20 species occur in

California. Only one species, *F. camschatcensis* (L.) Ker Gawler, 1809 links both groups by its distribution in both North America and eastern Asia (Fay and Chase 2000, Ambrozova et al. 2011).

According to its latest revision (Rix 2001), the genus is divided into eight subgenera, *Fritillaria* Rix, 2001 (including two sections, *Olostyleae* Rix, 2001 and *Fritillaria* Rix, 2001); *Davidii* Rix, 2001; *Liliorhiza* (Kellogg) Bentham & Hooker, 1883; *Japonica* Rix, 2001; *Rhinopetalum* Fischer, 1835; *Petilium* Baker, 1874; the monotypic *Theresia* K. Koch, 1849 and *Korolkowia* Rix, 2001. Although Iran (and more precisely its northern part as well as the neighbouring countries) is relatively poor in species (17 species and 4 subspecies), it is considered to be the centre of *Fritillaria* diversity above species level (Rix 1977), because those taxa belong to four out of five main subgenera (Jafari et al. 2014).

In Greece, the genus is also characterized by high diversity and is represented by a multitude of taxa (24 species and 5 subspecies), all belonging to the subgenus *Fritillaria* (Kamari and Phitos 2006).

Out of the 29 taxa found or described in Greece so far, 18 taxa (14 species and 4 subspecies) are endemic to the country and no less than 17 species and 2 subspecies occur in the Aegean archipelago and the surrounding continental region (Kamari and Phitos 2000). Moreover, Turkey is the richest country concerning the number of *Fritillaria* with 35 species and 6 subspecies, 19 of which are considered endemic (Tekşen and Aytaç 2011, Advay et al. 2015, Özhatay et al. 2015). Eighteen of those species and 4 subspecies are distributed in the Mediterranean, 12 of which are endemic. Taking into consideration the total number of *Fritillaria* taxa as well as the number of the endemic ones, Greece, along with W Turkey (Rix 1984, Özhatay 2000, Tekşen 2012), constitutes a secondary evolutionary center at least for this subgenus, if not for the whole genus. As a result, the Aegean archipelago can be considered as the heart of the secondary biodiversity center for the subgenus *Fritillaria* (Kamari and Phitos 2000).

Among the *Fritillaria* taxa occurring in continental Greece two species constitute the *Fritillaria montana* group (Kamari 1991a): *Fritillaria epirotica* Turrill ex Rix, 1975, which is endemic to NW Greece and *Fritillaria montana* Hoppe ex W.D.J.Koch, 1832, which has a wide distribution in S and SE Europe. Both species of the above group are characterized by their long (2/3 of the tepal length) nectaries, as well as by their obscurely tessellated tepals.

Fritillaria epirotica is a very short plant (up to 15 cm) with dark purplish, obscurely tessellated flowers, which almost touch the ground and it grows on ophiolithic substrates, usually at high altitudes (up to 2600 m). On the contrary, *F. montana* is tall (up to 60 cm), characterized by alternate or subopposite linear, slightly canaliculated leaves, with dark purplish distinctly tessellated flowers, and it grows usually on limestone substrate at an altitude up to 1600 m.

Fritillaria epirotica is included in the Red Data Book of Rare and Threatened Plants of Greece (Phitos et al. 1995 & 2009), in the IUCN Red List of Threatened Species, Version 2014.2. and also in the Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora. It is protected by the Presidential Decree

67/81, characterized as Endangered (EN) by IUCN and as Vulnerable (VU) in the Red Data Book of Rare and Threatened Plants of Greece (Kamari 1995, Kamari and Phitos 2009). *Fritillaria montana* is characterized, according to IUCN Red List of Threatened Species, Version 2014.2., as a Data Deficient (DD) species. In Greece, some of its populations are included in Natura 2000 sites. Despite its wide distribution, the species is Rare (R) in Italy (Peruzzi et al. 2008) and included in the regional Red Lists of Italian threatened species (Conti et al. 1997). As already known, the misapplied nomenclature of the *F. montana* complicates botanical literature (Lozina-Lozinskaja 1935, Zahariadi 1966, Kamari 1991a, b, Tomovic et al. 2007). Several locations in Italy have recently been further studied and *Fritillaria montana* populations are getting distinguished, while more biometric details for the species are provided (Peruzzi and Bartolucci 2009, Bartolucci et al. 2009, Mancuso et al. 2012, Peruzzi et al. 2012). An indicative example of the situation is the very low production of fruits during fruiting season in 2008 observed by Mancuso et al. (2012). Moreover, *Fritillaria montana* is characterized as an Endangered (EN) species listed in the third edition of the Red Book of Ukraine (Chorney et al. 2009), as a Rare (R) one in Bosnia and Herzegovina (Šilić 1996), Vulnerable (VU) in Serbia (IUCN Red List of Threatened Species) and protected at a national level in France. Tomovic et al. (2007) referred that the species was listed as Rare in the Red Data Book of the PR Bulgaria (Velchev 1984 sub *F. orientalis* Adam), but the latest version does not include it anymore (Petrova and Vladimirov 2009).

Concerning the cytology of the genus, *Fritillaria* has been studied for many years due to the interest of its large chromosomes and vast genome size (Darlington 1935, 1937, Frankel 1940). Indeed, 1C values (DNA content of the unreplicated haploid chromosome complement) in *Fritillaria* are among the largest reported for all angiosperms (Bennett and Smith 1976, Sharma and Raina 2005). The karyotype is quite stable, asymmetrical and usually diploid, with a basic chromosome number of $x = 12$. Only a few species are an exception to this, with $x = 9$ (3 species), $x = 11$ (2 species) and $x = 13$ (2 species), but without a special pattern (Darlington 1937, Noda 1964, Li and Shang 1989, Jafari et al. 2014). However, the presence and the morphology of satellited chromosomes vary among the species or even among populations of the same taxon (Runemark 1970, Bentzer et al. 1971, Mehra and Sachdeva 1976, Koul and Wafai 1980, Kamari 1984a, 1991a, 1996, Zaharof 1987, Kamari and Phitos 2006). In addition, secondary constrictions and supernumerary B-chromosomes are observed very often (La Cour 1978b, 1978c, Kamari 1984a, 1991a, 1991b, Zaharof 1987, 1989, Kamari and Phitos 2006). As a result, that type of differentiations is always emphasized and specific chromosome pairs are studied as markers, in order to spot the differences among the generally stable and similar karyotypes (Kamari 1984b, Zaharof 1989, Kamari and Phitos 2000, 2006). Finally, a few triploid karyotypes have been reported with $2n = 3x = 36$ (Fedorov 1969, La Cour 1978a, Moore 1982, Marchant and Macfarlane 1980, Zaharof 1987, Peruzzi et al. 2009) or with $2n = 3x = 27$ chromosomes (Cesca 1986, Kamari 1991a).

Recently many questions have arisen, regarding the classification and phylogeny of the genus, especially for the species appearing in Greece. Although several molecular phylogenetic studies have been published (Fay and Chase 2000, Rønsted et al. 2005,

Turktas et al. 2012, Metin et al. 2013) none of them refer to the total of Greek taxa. Even though classical cytotaxonomic studies of the genus in Greece have already been published (Kamari 1984a, 1984b, 1991a, 1991b, 1996, Zaharof 1987, 1989, Kamari and Phitos 2000, 2006), neither karyomorphometric analysis, nor statistical evaluation of the cytological results, have ever been given so far. In the present study, an attempt for further karyomorphometric analysis of chromosome features has been made, concerning the two members of *Fritillaria montana* group.

Material and methods

Living plants of the *Fritillaria montana* and *F. epirotica* populations were collected (Table 1) and cultivated in the Experimental Gardens of the University of Patras and Agricultural University of Athens. Vouchers are deposited in UPA and ACA.

The cytological study is based on the squash technique and the chromosome counts were made from root tip metaphases (Östergren and Heneen 1962, Kamari 1976). The root tips were pretreated in a mixture of 1:1 8-hydroxyquinoline (0,002% w/v):colchicine (0.3 w/v) for 3 hrs (Kamari 1984a) and fixed in 3:1 (v/v) absolute ethanol:glacial acetic acid for 24 hours at 4 °C. Fixed root tips were stored at -20 °C at 75% ethanol.

Before staining, the root tips were hydrolyzed in 1N HCl 60 °C for 15 min and stained in Feulgen for 3 hrs (Darlington and La Cour 1969). Prior to squashing, the stained root tips were put on a slide with a drop of 45% (v/v) acetic acid. The slides were observed with AXIOLAB Zeiss microscope and photos were taken with Canon EOS 600D digital camera.

At least five metaphase plates of each species were analysed and indices were calculated with Microsoft Office Excel 2007, IBM SPSS Statistics version 22 and Past 3.03. Chromosome terminology follows Levan et al. (1965), Stebbins (1971) and Kamari (1976), taking into consideration comments and suggestions by Sybenga (1959), Bentzer et al. (1971) and Favarger (1978). For each taxon there is a presentation of the karyotype formula, maximum and minimum length of the chromosomes, total and average chromosome length and total haploid length of the chromosome set, along with their standard deviation. The interchromosomal asymmetry (CV_{CL}), is estimated according to Paszko (2006) and the intrachromosomal asymmetry (M_{CA}) according to Watanabe et al. (1999), Peruzzi and Eroğlu (2013) and Peruzzi and Altinordu (2014). Additionally the coefficient of variation of centromeric index (CV_{CI}) measuring the centromere position heterogeneity is estimated following Paszko (2006) and Peruzzi and Altinordu (2014). A multivariate analysis (Principal Coordinate Analysis - PCoA) was made concerning six karyological parameters: 2n, x, THL, CV_{CL}, CV_{CI} and M_{CA} (Peruzzi and Altinordu 2014). When marker chromosomes are observed (metacentric, submetacentric, SAT-chromosomes and secondary constrictions) r-index, R-length, Centromeric index and Arm difference ratio are also given. Finally, t-tests are given, regarding the indices of TCL, ACL, CV_{CL}, $M_{CA,}$ in order to check statistically significant differences between the two species.

Table 1. Origin, chromosome numbers (2n) and voucher number of *Fritillaria* material.

Taxon	Origin	2n	Voucher number, Herbarium
F. montana	Mt. Vourinos (W Macedonia)	18	16765, UPA
	Mt. Kato Olympos (Thessalia)	18	SF1089, ACA cult. no. 253, UPA
	Mt. Boutsi (NW Macedonia)	27 and 18 (1 individual)	SF1092, ACA 19865, UPA
F. epirotica	Katara Pass (Epirus)	24	21348, UPA 7919, UPA
	Mt. Vasilitsa (N Pindos)	24	SF1076, ACA
	Mt. Smolikas (N Pindos)	24	SF1097, ACA
	Mt. Kratsovo (W Thessalia)	24	cult. no. 255, UPA

Results

Fritillaria montana Hoppe — 2n = 2x = 18 + 0-3B (Figs 1, 2).

Populations karyologically studied:

Greece: Macedonia: Nomos Kozanis: mons Vourinos, in declivibus orientalibus cacuminis, alt. 1300-1350 m, in apertis ad viam et in silva Abietis et Pinetis, solo ophiolithico, 9 Jul 1981, T.R. Dudley, D. Phitos, D. Tzanoudakis, Gr. Iatrou & D. Christodoulakis 16765 (UPA); **Thessalia: Nomos Larissis:** Mt. Kato Olympos, Livadaki, north of Kallipefki, alt. ca. 1407 m, 39°57'N; 22°29'E, 30 May 2015, S. Samaropoulou, I. Patrikios & K. Tamvakas SF1089 (ACA); Mt. Kato Olympos, Livadaki, alt. 1400 m, May 2006, K. Tamvakas 253 (UPA).

Fritillaria montana is the only Greek species with a basic chromosome number of x = 9, having 2n = 18 chromosomes (Fig. 1). Its karyotype includes two metacentric (m) chromosome pairs that can be characterized as markers, the longer and the shorter ones (Table 3, chromosome pairs no. 1 and no. 5, numbered according to their chromosome length), because they bear characteristic secondary constrictions close to the end of the short arm (Fig. 2). Secondary constrictions are also observed to the rest of the metacetric chromosomes, however, they are not always visible. For this reason, the other three metacentric chromosome pairs cannot be characterized as markers.

The karyotype formula of the studied populations is given as 2n = 10m + 2st + 6t = 18 (Fig. 2). The chromosome size ranges between 24.41 μm and 11.26 μm and the total chromosome length is 316.34 μm. The karyotype is more symmetric (Table 2) concerning the variation in chromosome length (CV_{CL} = 25.2) rather than the centromere position (M_{CA} = 41.42), while the parameter CV_{CI} is even higher (CV_{CI} = 56.21). Up to three B-chromosomes were found, all of them acrocentric (st) in the studied material.

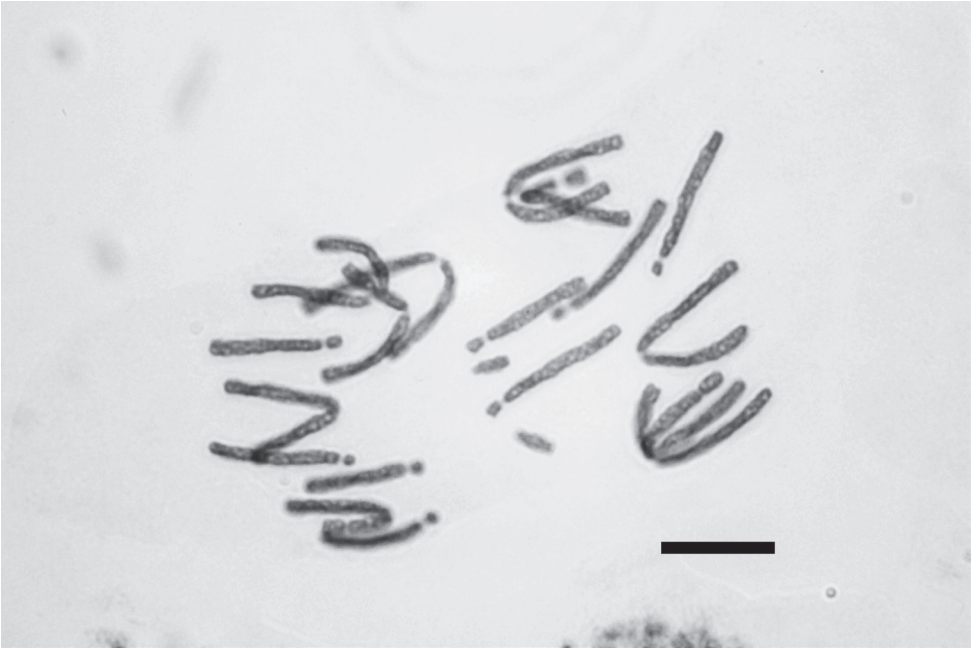

Figure 1. Photomicrograph of mitotic metaphase plate of *Fritillaria montana* from Mt. Vourinos, 2n = 2x = 18. Bar = 10 μm.

Table 2. Studied species with karyomorphometric indices. Chromosome number (2n), total (TCL) and average (ACL) chromosome length, total haploid chromosome length (THL), maximum (max l + s) and minimum (min l + s) chromosome length, karyotype asymmetry indices (CV_{CL}, CV_{CI} and M_{CA}).

Species	*F. montana*		*F. epirotica*
Chromosome number	2n = 2x = 18	2n = 3x = 27	2n = 2x = 24
Karyotype formula	10m + 2st + 6t	15m + 3st + 9t (10m + 4st + 4t, 1 individual)	2m + 2sm + 14st + 6t
TCL (μm) (SD)	316.34 (30.22)	363.23 (53.47)	324.39 (51.12)
THL (μm) (SD)	158.17 (15.11)	121.08 (17.82)	162.2 (25.56)
ACL (μm) (SD)	17.57 (1.68)	13.45 (1.98)	13.52 (2.13)
max l + s (μm)	24.41	22.86	18.44
min l + s (μm)	11.26	8.00	10.00
CV_{CL} (SD)	25.26 (1.12)	31.07 (3.61)	16.85 (2.43)
CV_{CI} (SD)	56.21 (0.99)	54.79 (3.57)	51.66 (2.99)
M_{CA} (SD)	41.42 (0.35)	40.41 (1.18)	63.33 (1.25)

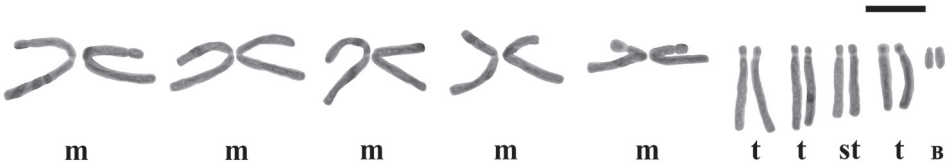

Figure 2. Karyogram of *Fritillaria montana* from Mt. Vourinos, 2n = 2x = 18. Bar = 10 μm.

Table 3. Karyomorphometric indices of marker chromosomes for each species, marker chromosome pairs (numbered according to their chromosome length), long arm's length (l), short arm's length (s), chromosome length (l + s) with minimum and maximum prices, r- index, Centromeric index, Arm difference ratio, R-length.

Species	***Fritillaria montana***		***Fritillaria epirotica***			
Chromosome number	2n = 18		2n = 24			
marker chromosomes	Pair no. 1	Pair no. 5	Pair no. 1	Pair no. 2	Pair no. 3	Pair no. 5
l (μm) **(SD)**	12.84 (1.00)	10.71 (1.09)	9.68 (1.05)	10.51 (1.04)	12.19 (1.07)	10.87 (1.23)
s (μm) **(SD)**	11.15 (0.79)	6.93 (0.67)	6.86 (0.82)	5,27 (0.63)	1.60 (0.44)	1,82 (0.36)
l + s (μm) **(SD)**	23.99 (1.71)	17.65 (1.66)	16.53 (1.73)	15.77 (1.49)	13.79 (1.18)	12.42 (1.51)
min l + s (μm)	20.88	15	13.53	12.94	11.47	8.40
max l + s (μm)	26.47	20	19.12	18.24	15.59	15
r-index **l/s**	1.15	1.54	1.40	2.03	8.06	6.24
Centromeric index **l/l + s**	0.54	0.61	0.58	0.67	0.88	0.85
Arm difference ratio **l - s/l + s**	0.70	0.21	0.17	0.34	0.77	0.71
R-length **l + s/Sn(l + s)**	0.08	0.06	0.05	0.05	0.04	0.04

Fritillaria montana Hoppe — 2n = 3x = 27 and 2n = 2x = 18 (1 individual) (Figs 3, 4).

Populations karyologically studied:

Greece: Macedonia: Nomos Florinas: Montes Triklarion, in declivibus boreo-occidentalibus cacuminis Boutsi, in apertis saxosis calc., alt. 1450-1550 m, 19 May 1987, D. Phitos & G. Kamari 19865 (UPA); Mt. Boutsi, alpine meadow, calcareous substrate, alt. ca. 1549 m, 40°38'33"N; 21°09'25"E, 2 Jun 2015, S. Samaropoulou, I. Patrikios & A. Ioannou, sub Samaropoulou SF1092 (ACA).

The triploid population previously reported for the first time by Kamari (1991a), is now further examined. The karyotype formula is given as 2n = 15m + 3st + 9t = 27 (Figs 3, 4) and the chromosome length ranges from 22.86 μm to 8 μm, while the TCL equals to 363.23 μm (Table 2). The interchromosomal asymmetry of the triploid karyotype (CV_{CL}

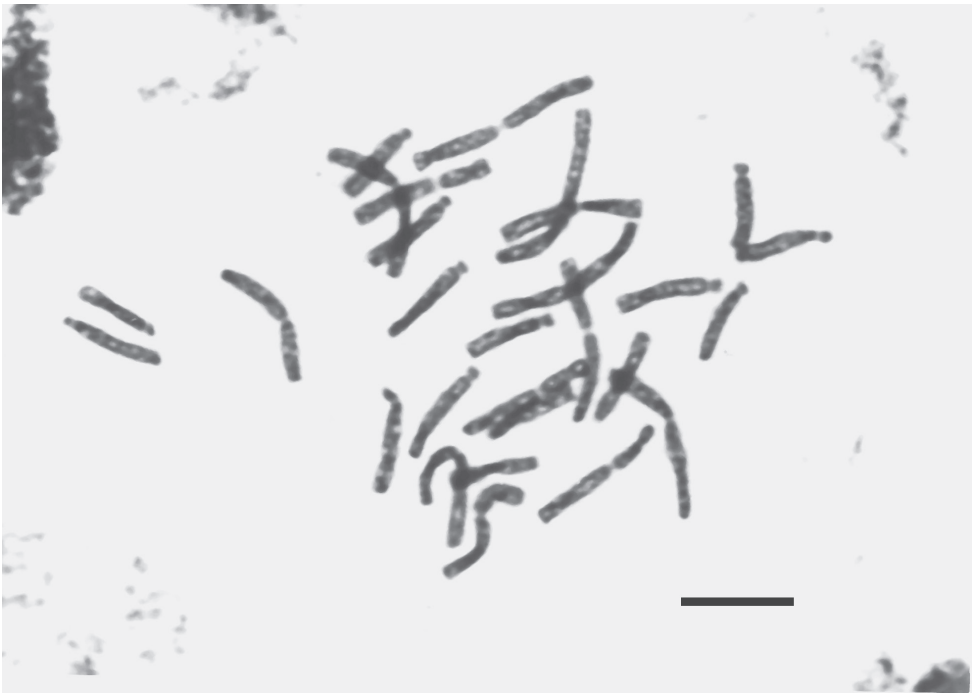

Figure 3. Photomicrograph of mitotic metaphase plate of *Fritillaria montana* from Mt. Boutsi, 2n = 3x = 27. Bar = 10 μm.

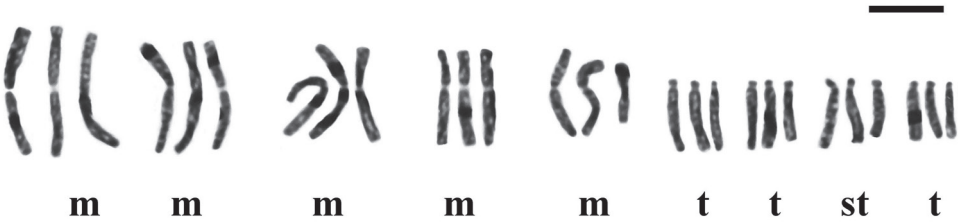

Figure 4. Karyogram of *Fritillaria montana* from Mt. Boutsi, 2n = 3x = 27. Bar = 10 μm.

= 31.07) is higher than the diploid, but the intrachromosomal is lower (M_{CA} = 40.41). The heterogeneity of the centromere position is lower than the diploid (CV_{CI} = 54.79). Even though secondary constrictions were observed again, their number and position varies in several plates, making the distinction of marker chromosomes very difficult.

It is noteworthy that a diploid individual was found for the first time at the studied triploid population. The karyotype of this individual comprises 2n = 10m + 4st + 4t = 18 chromosomes, with an additional pair of acrocentric (st) chromosomes compared with the other diploid populations studied and without B-chromosomes in contrast with the population of Mt. Vourinos. The secondary constrictions were also unclear as in the triploid individuals.

Fritillaria epirotica Turrill ex Rix — 2n = 2x = 24 (Figs 5, 6).

Populations karyologically studied:

Greece: Epirus: Nomos Ioanninon: Katara Pass, prope ekchionistikos stathmos, alt. 1750 m, in apertis (*Pinus* Linnaeus, 1753; *Buxus* Linnaeus, 1753 etc), solo serpentinico, 4 May 1990, D. Phitos & G. Kamari 21348 (UPA); Eparchia Metsovou, Katara Pass, close to the second snowplow station, c. 13.5 km of Metsovon along the road to Trikala, slopes with *Pinus nigra* Arnold, 1785 and *Buxus sempervirens* Linnaeus,1753; ophiolithic substrate, alt. c. 1640 m, 39°47'N; 21°13'E, 24 Jun 1998, Th. Constantinidis 7919 (UPA); **Macedonia: Nomos Grevenon:** Mt. Vasilitsa, alt.

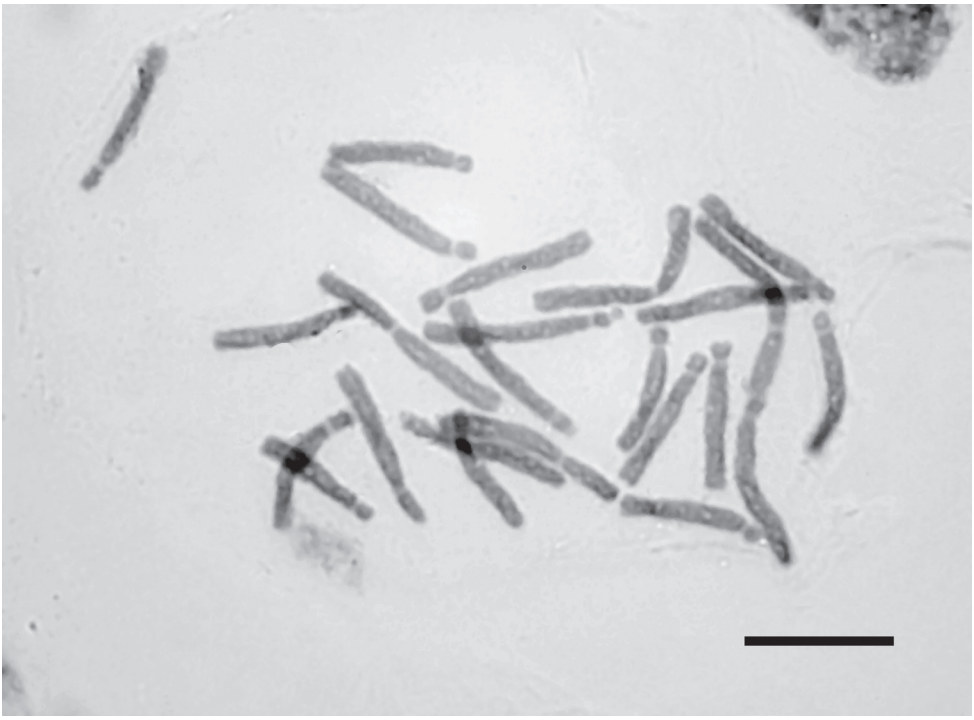

Figure 5. Photomicrograph of mitotic metaphase plate of *Fritillaria epirotica* from Mt. Smolikas, 2n = 2x = 24. Bar = 10 μm.

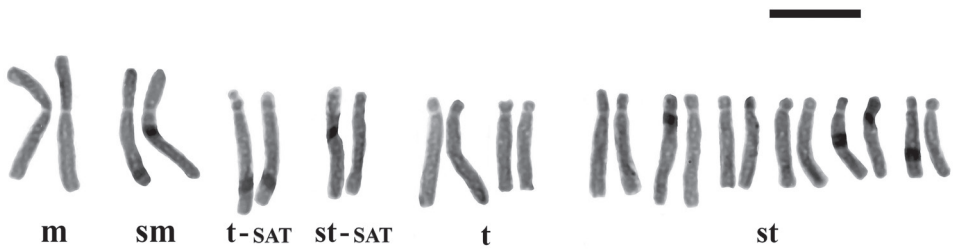

Figure 6. Karyogram of *Fritillaria epirotica* from Mt. Smolikas, 2n = 2x = 24. Bar =10 μm.

1764 m, 17 May 2015, G. Kofinas s.n. (cult. no. SF76, ACA); Mt. Smolikas, alt. 2200 m, Aug 2015, G. Kofinas s.n. (cult. no. SF97, ACA). **Thessalia: Nomos Trikalon:** Ep. Kalampakas, Mt. Chasia (Kratsovo), stony slopes close to a forest road, c. 3.0–3.5 km from Kakoplevri village, serpentine, alt. c. 1100–1180 m, 39°48'N; 21°24'E, 15 Jun 2000, D. Phitos, G. Kamari & Th. Constantinidis s.n. (cult. no. 235, UPA); Ep. Kalampakas, Mt. Chasia (Mt. Kratsovon), c. 3.1 km WNW of Kakoplevri village on the foothills of the mountain, hills with low *Buxus sempervirens* and *Juniperus oxycendrus* Linnaeus,1753; serpentine substrate, alt. 1120–1160 m, 39°49'N; 21°24'E, 24 Jul 2006, Th. Constantinidis s.n. (cult. no. 235, UPA).

Unlike *Fritillaria montana*, *F. epirotica* has the same basic somatic number as the rest of the Greek *Fritillaria* taxa, x = 12. The karyotype consists of 2n = 2m + 2sm + 14st + 6t = 24 chromosomes (Figs 5, 6), which range in size between 18.44 and 10 μm, while the TCL is 324.39 μm (Table 2). The index for interchromosomal asymmetry is small (CV_{CL} = 16.85) contradicting the big intrachromosomal one (M_{CA} = 63.33), while the centromere position heterogeneity is 51.66. Satellites on the short arms of one telocentric (t) and one acrocentric (st) pair of chromosomes (Table 3, chromosome pairs no. 3 and no. 5) are observed. However, in most metaphase plates, three of them are usually visible.

According to paired t-tests made (Table 4), the two species display an interesting similarity regarding their total chromosome length, but as far as the interchromosomal and intrachromosomal asymmetries are concerned (Table 5), the species seem to be

Table 4. Paired t-tests between the three species regarding the TCL and ACL along with degrees of freedom (df) and Significance (Sig) for every parameter. Bold characters are used for P values (Sig 2-tailed) under 0.01, which reveal significant statistical difference.

Species in comparison		TCL			ACL		
		t	df	Sig (2-tailed)	t	df	Sig (2-tailed)
F. epirotica 2n = 2x = 24	*F. montana* 2n = 2x =18	0.379	18	0.709	-4.347	18	**0.000**
F. epirotica 2n = 2x = 24	*F. montana* 2n = 3x = 27	-1.427	16	0.173	0.057	16	0.955
F. montana 2n = 2x = 18	*F. montana* 2n = 3x = 27	-1.947	10	0.080	3.898	10	**0.003**

Table 5. Paired t-tests between the three species regarding the CV_{CL} and M_{CA}, along with degrees of freedom (df) and Significance (Sig) for every parameter. Bold characters are used for P (Sig 2-tailed) under 0.01, which reveal significant statistical difference.

Species in comparison		CV_{CL}			M_{CA}		
		t	df	Sig (2-tailed)	t	df	Sig (2-tailed)
F. epirotica 2n = 2x = 24	*F. montana* 2n = 2x = 18	-8.598	18	**0.000**	44.847	18	**0.000**
F. epirotica 2n = 2x = 24	*F. montana* 2n = 3x = 27	-9.754	16	**0.000**	34.473	16	**0.000**
F. montana 2n = 2x = 18	*F. montana* 2n = 3x = 27	-4.066	10	**0.002**	1.995	10	0.074

clearly distinct. The only insignificant difference was revealed between the two cytotypes of *Fritillaria montana*, 2n = 18 and 2n = 27, as expected, since they both bear a lot of metacentric chromosomes (by Robersonian fusions).

Discussion

In the present study a detailed karyomorphological analysis of *Fritillaria montana* and *Fritillaria epirotica*, in material from Greece, was implemented focusing specifically to the study of the inter- and intrachromosomal asymmetry, as well as the detailed analysis of the marker chromosomes.

The study of marker chromosomes (Table 3) is always important since it can provide further information concerning genome organization and the differentiation of the karyotype between related species. Moreover, especially in the case of the genus *Fritillaria*, marker chromosomes are helpful for the distinction of the chromosome homologues, which is very difficult since the karyotype usually consists of mostly acrocentric and subtelocentric chromosomes with similar size.

Marker chromosomes were observed in both *F. epirotica* with 2n = 2x = 24 and *F. montana* with 2n = 2x = 18 chromosomes. However, when it comes to triploid karyotypes of the same species, the secondary constrictions are not stable in number and position.

Fritillaria epirotica (2n = 24) has four marker chromosome pairs (Fig. 6). The first two chromosome pairs, which are the longest ones of the complement, have a different morphology than all the other chromosomes of the karyotype, which are acrocentric (st) and subtelocentric (t). The longest chromosome pair is metacentric (m) (no. 1), while the second one is the second in range of length and a submetacentric (sm) one (no. 2). The third marker chromosome pair (no. 3) is telocentric and bears small spherical satellite on the short arm of the homologues. Finally, the last marker chromosome pair is the fifth in length, comprising of two acrocentric satellited (st-SAT) chromosomes. The results are in agreement with previous studies by Kamari (1991a). Zaharof (1989) reported the heteromorphism of satellites' length in one out of two SAT-chromosome pairs.

Fritillaria montana (2n = 18) has two marker chromosome pairs with secondary constrictions. The karyotype formula given here (2n = 10m + 2st + 6t = 18) differs from the previously reported karyotype of 2n = 10m + 8t = 18 chromosomes given by Zaharof (1989). This is the only one species in Greece with 18 chromosomes and this chromosomal reduction has already been claimed as the result of successive chromosomal reconstructions and Robersonian-fusion of six acrocentric chromosomes into three metacentric ones (Darlington 1936, La Cour 1978a, 1978b, 1978c, Kamari 1991a). Zaharof (1989) explained the secondary constrictions, which are also observed in the present study, with the above hypothesis. Recently, Peruzzi et al. (2016) studied an Italian population with 2n = 2x = 18 chromosomes, further confirming the chromosome number of *F. montana*, while the presence of up to three B-chromosomes is already referred by Kamari (1991a, 1991b).

The triploid chromosome number of *F. montana* (2n = 3x = 27) is known in Greece from only one population, but it has also been reported from Italy by Cesca (1986, under the name *F. tenella* Marschall von Bieberstein, 1808), for a Calabrian population (S Italy).

Paired t-tests have revealed similarities among the three karyotypes. Especially the similarity between TCL of the diploid *F. epirotica* 2n = 24 and *F. montana* 2*n* = 18 reinforces the hypothesis, apart from the secondary constrictions, that the second species has derived after successive chromosomal reconstructions and Robersonian-fusions. Less similar indices of TCL between *F. montana* 2n = 2x = 18 and *F. montana* 2n = 3x = 27 can also be explained since it is known that polyploidy usually comes with gene loss and genome amount reduction (Kamari 1992, Leitch and Bennett 2004, Adams and Wendel 2005, Buggs et al. 2009). Another proof for gene loss, is the fact that the triploid cytotype of *F. montana* has the lower price of THL.

The results concerning the heterogeneity of centromere position CV_{CI} and the intrachromosomal asymmetry M_{CA} are nothing but expected. Following the explanation of this index by Zuo and Yuan (2011), the higher price of CV_{CI} found here, belongs to *Fritillaria montana*, because the karyotype comprises of mostly metacentric chromosomes. On the contrary, the higher price of M_{CA} belongs to *F. epirotica*, as it has a typical asymmetrical karyotype according to Stebbins (1971).

In total, the multivariate analysis PCoA confirms all above findings. More precisely, it presents all the accessions belonging to the same species close to each other. The two cytotypes of *Fritillaria montana* overlap, while the two species are clearly separated (Fig. 7).

Generally, karyological characteristics, as chromosome number, ploidy level, centromere position, and the number and location of satellites and secondary constrictions, can be used in elucidating taxonomical relationships of several plant taxa (Bareka et al. 2008, 2012 see for references). Although, karyomorphometrics is able

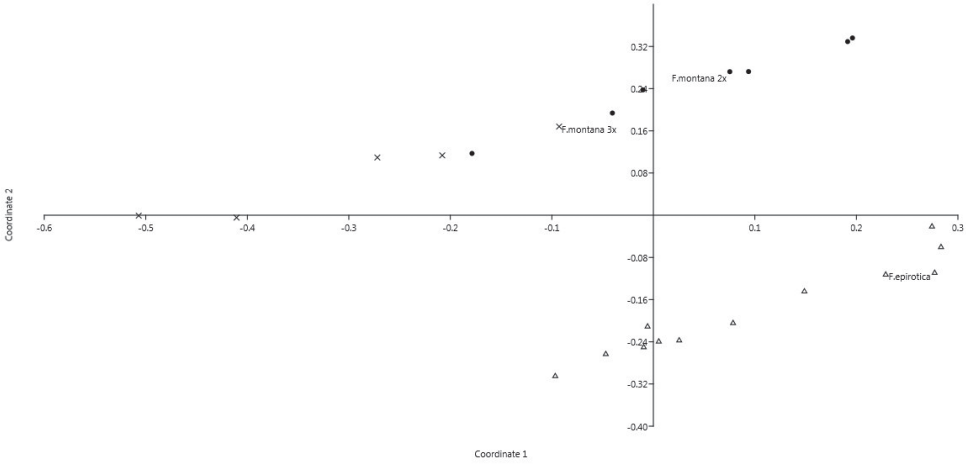

Figure 7. PCoA analysis based on six quantitative karyologial parameters. Triangle depicts *F. epirotica*, 2n = 2x = 24; dots *F. montana*, 2n = 2x = 18 and x *F. montana*, 2n = 3x = 27.

to provide more information about the studied taxa, the conclusions can be used only as additional evidences to the primary hypothesis. However, molecular chromosomal markers and fluorescence in situ hybridization (FISH) could provide additional information concerning genome organization in the genus and differentiation among its species and are recommended as a safer way to reveal whether our assumption for the origin of the reduced chromosome number 2n = 18 is correct. Moreover, this method is desirable to be carried out because it will unveil the type of polyploidy for 2n = 3x = 27, as an autopolyploidy or allopolyploidy (Bareka et al. 2012).

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Chromosomal and mitochondrial diversity in *Melitaea didyma* complex (Lepidoptera, Nymphalidae): eleven deeply diverged DNA barcode groups in one non-monophyletic species?

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Abstract

It is generally accepted that cases of species' polyphyly in *COI* trees arising as a result of deep intraspecific divergence are negligible, and the detected cases reflect misidentifications or/and methodological errors. Here we studied the problem of species' non-monophyly through chromosomal and molecular analysis of butterfly taxa close to *Melitaea didyma* (Esper, 1779) (Lepidoptera, Nymphalidae). We found absence or low interspecific chromosome number variation and presence of intraspecific variation, therefore we conclude that in this group, chromosome numbers have relatively low value as taxonomic markers. Despite low karyotype variability, the group was found to have unexpectedly high mitochondrial haplotype diversity. These haplotypes were clustered in 23 highly diverged haplogroups. Twelve of these haplogroups are associated with nine traditionally recognized and morphologically distinct species *M. chitralensis* Moore, 1901, *M. deserticola* Oberthür, 1909, *M. didymoides* Eversmann, 1847, *M. gina* Higgins, 1941, *M. interrupta* Colenati, 1846, *M. latonigena* Eversmann, 1847, *M. mixta* Evans, 1912, *M. saxatilis* Christoph, 1873 and *M. sutschana* Staudinger, 1892. The rest of the haplogroups (11 lineages) belong to a well-known west-palaearctic species *M. didyma*. The last species is particularly unusual in the haplotypes we obtained. First, it is clearly polyphyletic with respect to *COI* gene. Second, the differentiation in *COI* gene between these mostly allopatric (but in few cases sympatric) eleven lineages is extremely high (up to 7.4%), i.e. much deeper than the "standard" DNA barcode species threshold (2.7–3%). This level of

divergence normally could correspond not even to different species, but to different genera. Despite this divergence, the bearers of these haplogroups were found to be morphologically indistinguishable and, most importantly, to share absolutely the same ecological niches, i.e. demonstrating the pattern which is hardly compatible with hypothesis of multiple cryptic species. Most likely such a profound irregularity in barcodes is caused by reasons other than speciation and represents an extraordinary example of intra-species barcode variability. Given the deep level of genetic differentiation between the lineages, we assume that there was a long period (up to 5.0 My) of allopatric differentiation when the lineages were separated by geographic or/and ecological barriers and evolved in late Pliocene and Pleistocene refugia of north Africa, the Iberian and Balkan Peninsulas, the Middle East and Central Asia. We discuss the refugia-within-refugia concept as a mechanism explaining the presence of additional diverged minor haplogroups within the areas of the major haplogroups. We also provide the first record of *M. gina* in Azerbaijan and the record of *M. didyma turkestanica* as a new taxon for Russia and Europe.

Keywords

Biodiversity, butterflies, *COI*, chromosome, karyotype, mitochondrial DNA, monophyly, non-monopyletic species, Nymphalidae, phylogeography, Pleistocene refugium, taxonomy

Introduction

The *Melitaea didyma* (Esper, 1779) species complex, a group of taxa close to *M. didyma* (Bryk 1940, Higgins 1941, Kolesnichenko 1999, Kolesnichenko et al. 2011) is widely distributed in the Palaearctic region. This complex exhibits a high level of individual and seasonal variation, although distinction between described taxa and between different populations in wing pattern is often unclear (Higgins 1941, 1955, Lvovsky and Morgun 2007, Oorschot and Coutsis 2014). Simultaneously these butterflies are similar in male and female genitalia structure (Higgins 1941).

The significant reviews of this complex were published by Bryk (1940), Higgins (1941, 1955), Kolesnichenko (Kolesnichenko 1999, Kolesnichenko et al. 2011), Tuzov and Churkin (2000). More recently the whole genus *Melitaea* Fabricius, 1807 was revised by Oorschot and Coutsis (2014). However, available cytogenetic (Lukhtanov and Kuznetsova 1989), morphological (Lvovsky and Morgun 2007, Kolesnichenko et al. 2011, Oorschot and Coutsis 2014) and molecular (Wahlberg and Zimmermann 2000, Lukhtanov et al. 2009, Dincă et al. 2015) data show that the *M. didyma* species complex requires a more detailed study.

Combination of molecular and cytogenetic methods is a useful tool for detecting cryptic species (Lukhtanov et al. 2015) and can be a good addition to morphological analysis for ordering complex taxonomic structures (Lukhtanov et al. 2016). In our previous paper we applied analysis of DNA barcodes to demonstrate that *M. didyma* complex is a monophyletic group and is represented by multiple deeply diverged mitochondrial DNA haplogroups (Pazhenkova et al. 2015).

In the present study we use a combination of molecular and chromosomal markers to analyse additional material collected in Armenia, Bulgaria, Georgia, Greece, Iran, Israel, Kazakhstan, Kyrgyzstan, Russia, Slovenia, Syria and Turkey, in order to reveal

taxonomic and phylogeographic structure within the *M. didyma* species complex. In our opinion, this group includes the following species: *M. didyma* Esper, 1779, *M. chitralensis* Moore, 1901, *M. deserticola* Oberthür, 1909, *M. didymoides* Eversmann, 1847, *M. gina* Higgins, 1941, *M. interrupta* Colenati, 1846, *M. latonigena* Eversmann, 1847, *M. mixta* Evans, 1912, *M. saxatilis* Christoph, 1873 and *M. sutschana* Staudinger, 1892. This complex does not include the taxa of the *M. persea* complex (*M. persea* Kollar, 1849, *M. casta* Kollar, 1849, *M. eberti* Koçak, 1980 and *M. higginsi* Sakai, 1978) and the taxa of the *M. ala* complex (*M. ala* Staudinger, 1881, *M. bundeli* Kolesnichenko, 1999, *M. kotshubeji* Sheljuzhko, 1929, *M. acraeina* Staudinger, 1886, *M. enarea* Frühstorfer, 1917, *M. ninae* Sheljuzhko, 1935 and *M. didymina* Staudinger, 1895) which were shown to be strongly diverged with respect to genitalia structure (Higgins 1941, Kolesnichenko 1999, Oorschot and Coutsis 2014) and molecular markers (Leneveu et al. 2009).

Material and methods

We studied standard *COI* barcodes (658-bp 5' segment of mitochondrial *cytochrome oxidase subunit I*). We obtained *COI* sequences from 121 specimens collected in Armenia, Bulgaria, Georgia, Greece, Iran, Israel, Kazakhstan, Kyrgyzstan, Russia, Slovenia, Syria and Turkey. DNA was extracted from a single leg removed from each voucher specimen.

Legs from 21 specimens were processed at Department of Karyosystematics of Zoological Institute of the Russian Academy of Sciences. Primers and PCR protocol are given in our previous publications (Lukhtanov et al. 2014, Pazhenkova et al. 2015). Sequencing of double-stranded product was carried out at the Research Resource Center for Molecular and Cell Technologies of St. Petersburg State University. Legs from 100 specimens of *Melitaea* were processed at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using their standard high-throughput protocol described by deWaard et al. (2008). The set of voucher specimens of butterflies is kept in the Zoological Institute of the Russian Academy of Science (St. Petersburg).

The analysis involved 265 *COI* sequences (including outgroup) (Suppl. material 1). Among them there were 144 published sequences (Wahlberg and Zimmermann 2000, Vila and Bjorklund 2004, Leneveu et al. 2009, Lukhtanov et al. 2009, Dincă 2011, 2015, Hausmann et al. 2011, Ashfaq et al. 2013, Pazhenkova et al. 2015) collected from GenBank.

Within the studied samples, we are not completely sure of the identity of *M. chitralensis* specimens (their barcodes were obtained from GenBank) because we were not able to check these vouchers and used the identification of these samples accepted in Ashfaq et al. (2013). According to Kolesnichenko (1999), *M. chitralensis* is a member of the *M. ala* subgroup, but the analysed samples clearly clustered with *M. mixta*. Therefore, we can not exclude the possibility that these samples represent a north Pakistani population close to *M. mixta*, but not a true *M. chitralensis*.

Sequences were aligned using BioEdit software (Hall 1999). Mean uncorrected p-distances between haplogroups were calculated in MEGA7 (Kumar et al. 2015). Phylogenetic hypotheses were inferred using Bayesian inference (BI) as described previously (Vershinina and Lukhtanov 2010, Talavera et al. 2013a,b). Briefly, Bayesian analyses were performed using the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with default settings as suggested by Mesquite (Maddison and Maddison 2015): burn-in=0.25, nst=6 (GTR + I +G). Two runs of 10 000 000 generations with four chains (one cold and three heated) were performed. Chains were sampled every 10000 generations.

Karyotypes were obtained from fresh adult males and processed as previously described (Vershinina et al. 2015). Briefly, gonads were removed from abdomen and placed to freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing butterfly in the field. Testes were stored in the fixative for 1 month at +4°C. Then the gonads were stained in 2% acetic orcein for 7-10 days at +18-20°C. Haploid chromosome numbers (n) were counted in meiotic metaphase I (MI) and metaphase II (MII).

Results

Karyotype

The haploid chromosome number n=28 was found in prometaphase I, MI and MII cells of seven studied individuals (Table 1, Fig. 1). All chromosome elements formed a gradient size row. The karyotype contained no exceptionally large or small chromosomes.

Table 1. Chromosome number and localities of *Melitaea gina* samples collected in Iran (province West Azerbaijan) (Collectors: V. Lukhtanov, E. Pazhenkova and N. Shapoval).

Sample	Karyotype	Haplotype	Locality	Altitude	Date
Q153	n=28	M18	25 km E of Mahabad (vic. Darman): N36°45'00"; E45°51'37"	1900–2000 m	10 August 2016
Q155	n=28		25 km E of Mahabad (vic. Darman): N36°45'00"; E45°51'37"	1900–2000 m	10 August 2016
Q156	n=28	M14	25 km E of Mahabad (vic. Darman): N36°45'00"; E45°51'37"	1900–2000 m	10 August 2016
Q157	n=28	M15	25 km E of Mahabad (vic. Darman): N36°45'00,30"; E45°51'36,60"	1900–2000 m	10 August 2016
Q182	n=28		25 km E of Mahabad (vic. Darman): N36°45'00"; E45°51'37"	1900–2000 m	10 August 2016
Q183	n=28		25 km E of Mahabad (vic. Darman): N36°45'00"; E45°51'37"	1900–2000 m	10 August 2016
Q211	n=28		3 km W of Khalifen: N36°44'35"; E45°32'13"	2100–2200 m	11 August 2016

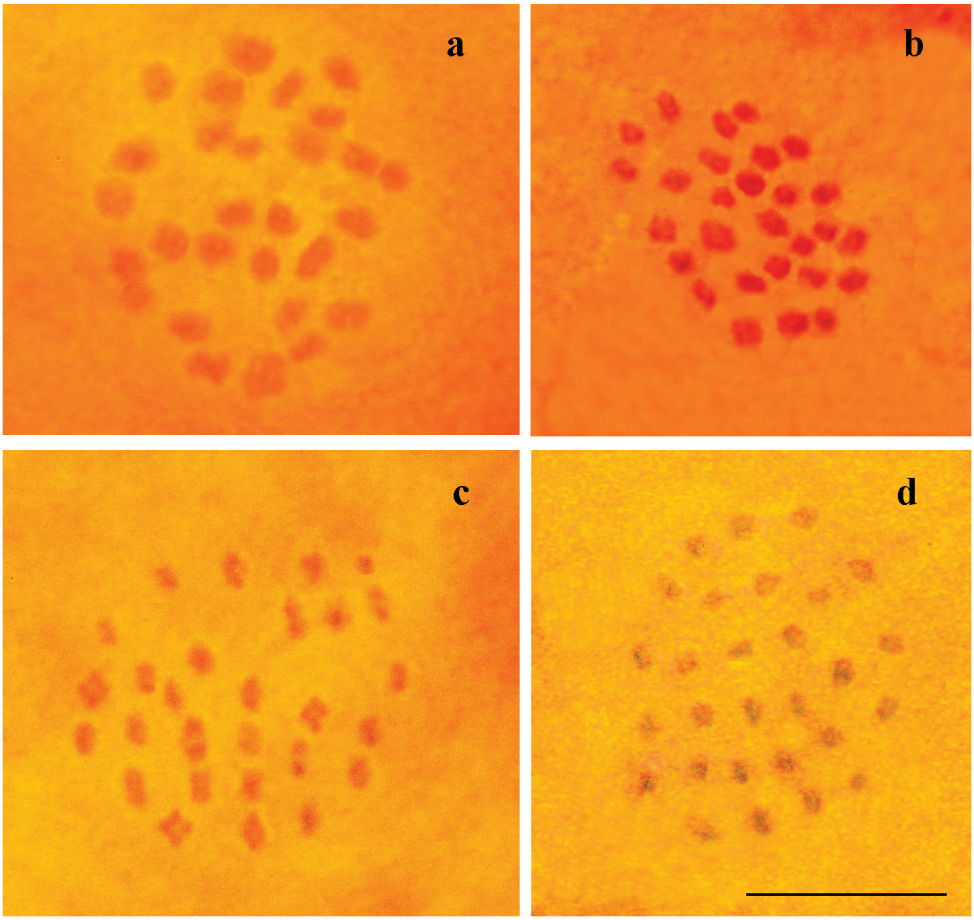

Figure 1. Karyotypes in male meiosis of *Melitaea gina* from Iran. **a** sample Q183, prometaphase I, n = 28 **b** sample Q153, late prometaphase I, n = 28 **c** sample Q183, MI, n = 28 **d** sample Q155, M I, n = 28. Scale bar corresponds to 10μ in all figures.

COI haplotypes and haplogroups

Bayesian analysis of the barcode region recovered the *M. didyma* complex as a monophyletic clade (Fig. 2), which agrees with Leneveu et al. (2009). Despite low karyotype variability, the clade was found to have unexpectedly high mitochondrial haplotype diversity. These haplotypes were clustered in 23 highly diverged haplogroups called *chitralensis*, *deserticola*, *didyma*, *didymoides*, *gina*, *gina2*, *interrupta*, *latonigena*, *liliputana*, *mauretanica*, *mixta*, *neera*, *neera2*, *occidentalis*, *protaeoccidentis*, *saxatilis*, *sutschana*, *sutschana2*, *sutschana3*, *turkestanica*, *turkestanica2*, *turkestanica3* and *turkestanica4* (Figs 2–6, Suppl. material 1). These haplogroups had high support (Bayesian posterior probability from 0.95 to 1) and were associated with particular geographical areas (Fig. 7).

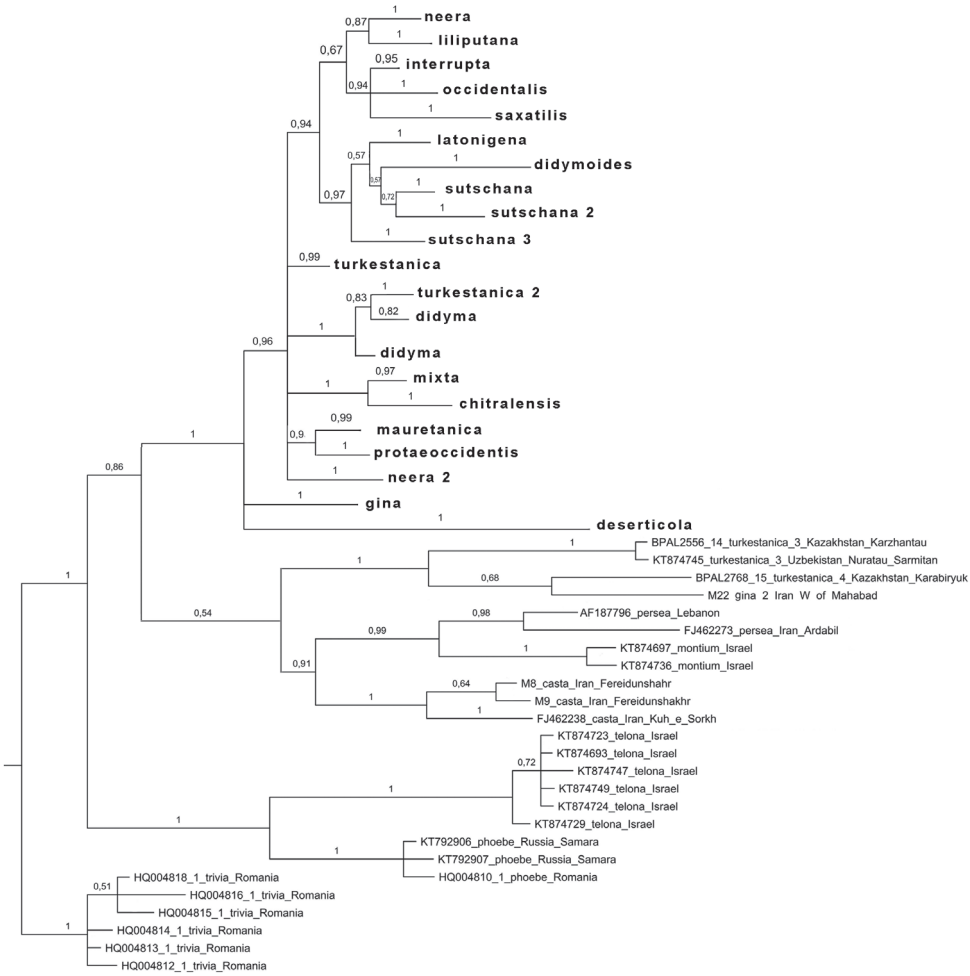

Figure 2. The Bayesian tree of *Melitaea* based on analysis of *the cytochrome oxidase subunit I* (*COI*) gene. Numbers at nodes indicate Bayesian posterior probability.

The uncorrected mean *p*-distances between the haplogroups were high (up to 9.1% between *turkestanica4* and *deserticola*) (Table 2). The majority of them were much higher than the 'standard' 2.7–3.0% DNA barcode threshold usually used for allopatric taxa as an indicator for their species distinctness (Lambert et al. 2005, Lukhtanov et al. 2015).

Most of the haplogroups were found to be allopatric. However, in some cases barcodes' clusters did not correspond to the simple allopatric geographical distribution. The sample *Melitaea gina* M22 (haplogroup *gina2*) was found in sympatry with the haplogroup *gina* in north-west Iran. The distance between *gina* and *gina2* was 6.5%. Haplogroups *turkestanica4*, *turkestanica3* and *turkestanica2* were highly diverged (up to 7.4%) as compared with the haplogroup *turkestanica* and were found in sympatry

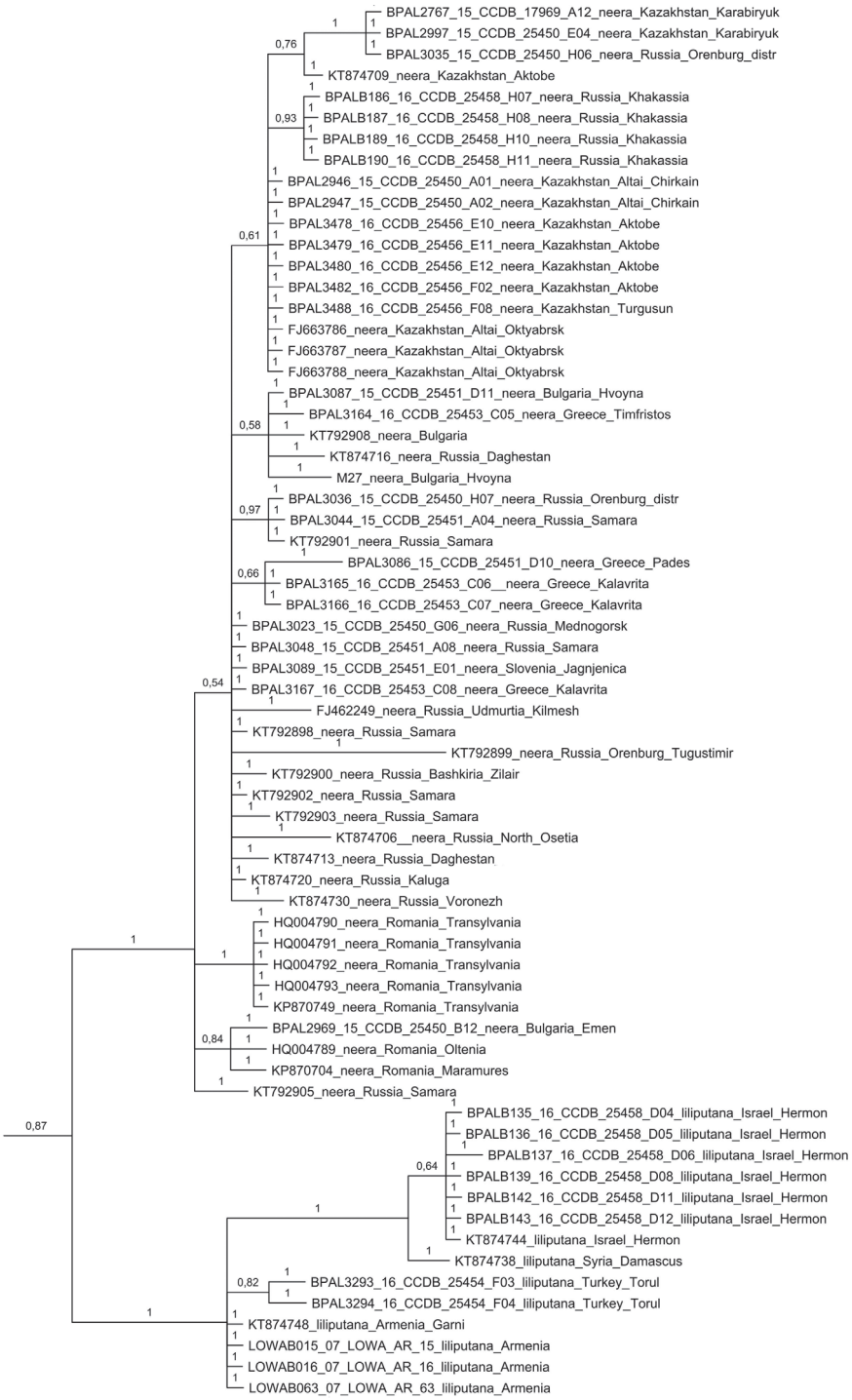

Figure 3. Fragment of the Bayesian tree of *Melitaea didyma* complex (haplogroups *neera* and *liliputana*) based on analysis of *COI* gene. Numbers at nodes indicate Bayesian posterior probability.

Table 2. Mean uncorrected *COI* p-distances between 23 haplogroups of the *M. didyma* species complex (%).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. chitralensis																						
2. deserticola	8.4																					
3. didyma	4.2	6.7																				
4. didymoides	6.3	6.9	4.7																			
5. gina	5.5	7.5	4.2	5.3																		
6. gina 2	6.4	9.5	6.5	7.2	6.5																	
7. interrupta	4.9	6.2	2.7	3.1	4.4	6.1																
8. latonigena	5.0	6.9	3.1	4.1	4.7	6.5	3.5															
9. liliputana	4.7	7.1	3.1	4.8	5.2	6.8	3.4	3.7														
10. mauretanica	4.1	6.3	2.1	4.2	4.8	7.1	2.2	3.1	3.5													
11. mixta	2.4	6.9	3.5	5.0	5.2	6.9	4.1	4.6	4.3	3.6												
12. neera	3.7	6.8	2.4	4.3	4.9	7.1	2.3	2.9	2.0	2.0	3.3											
13. neera 2	3.2	6.7	1.9	4.7	4.5	6.4	2.6	3.0	2.8	2.0	2.7	1.7										
14. occidentalis	4.9	6.9	2.9	3.9	4.6	6.4	1.8	3.8	3.9	2.4	4.0	2.8	2.4									
15. protaeoccidentis	3.3	5.6	2.1	4.1	4.2	6.7	2.7	2.7	3.0	2.1	2.9	2.1	2.0	3.0								
16. saxatilis	5.0	7.9	4.0	4.7	5.4	7.4	3.5	4.5	4.3	3.3	5.0	3.7	3.9	3.9	3.8							
17. sutschana	5.6	6.9	3.4	3.5	4.5	6.7	3.1	2.4	3.7	3.5	4.6	2.6	3.3	3.1	3.2	3.9						
18. sutschana 2	5.9	7.6	4.0	4.1	5.7	7.7	3.9	3.0	4.3	4.1	5.2	3.2	3.9	4.3	3.8	4.5	1.8					
19. sutschana 3	4.7	6.9	2.5	3.4	4.5	7.1	2.6	2.4	3.4	2.6	4.0	2.3	2.4	3.0	2.6	2.7	1.5	2.1				
20. turkestanica	3.4	7.0	2.3	4.4	4.3	7.0	3.0	3.4	3.1	2.4	2.7	2.1	1.6	3.1	2.3	3.7	3.6	4.3	2.8			
21. turkestanica 2	4.8	7.5	1.1	5.7	5.1	6.6	3.7	4.1	4.1	3.1	4.1	3.4	2.9	3.9	3.0	5.0	4.4	4.4	3.5	3.2		
22. turkestanica 3	7.0	8.9	5.8	8.9	6.4	6.4	4.8	6.1	7.1	7.0	6.3	7.9	6.9	5.8	6.0	6.2	7.0	6.9	7.0	6.6	6.4	
23. turkestanica 4	7.0	9.1	6.5	7.4	7.0	4.3	6.1	7.3	6.7	7.3	7.4	7.2	6.4	6.6	6.5	7.5	7.2	8.0	7.2	7.4	7.0	4.4

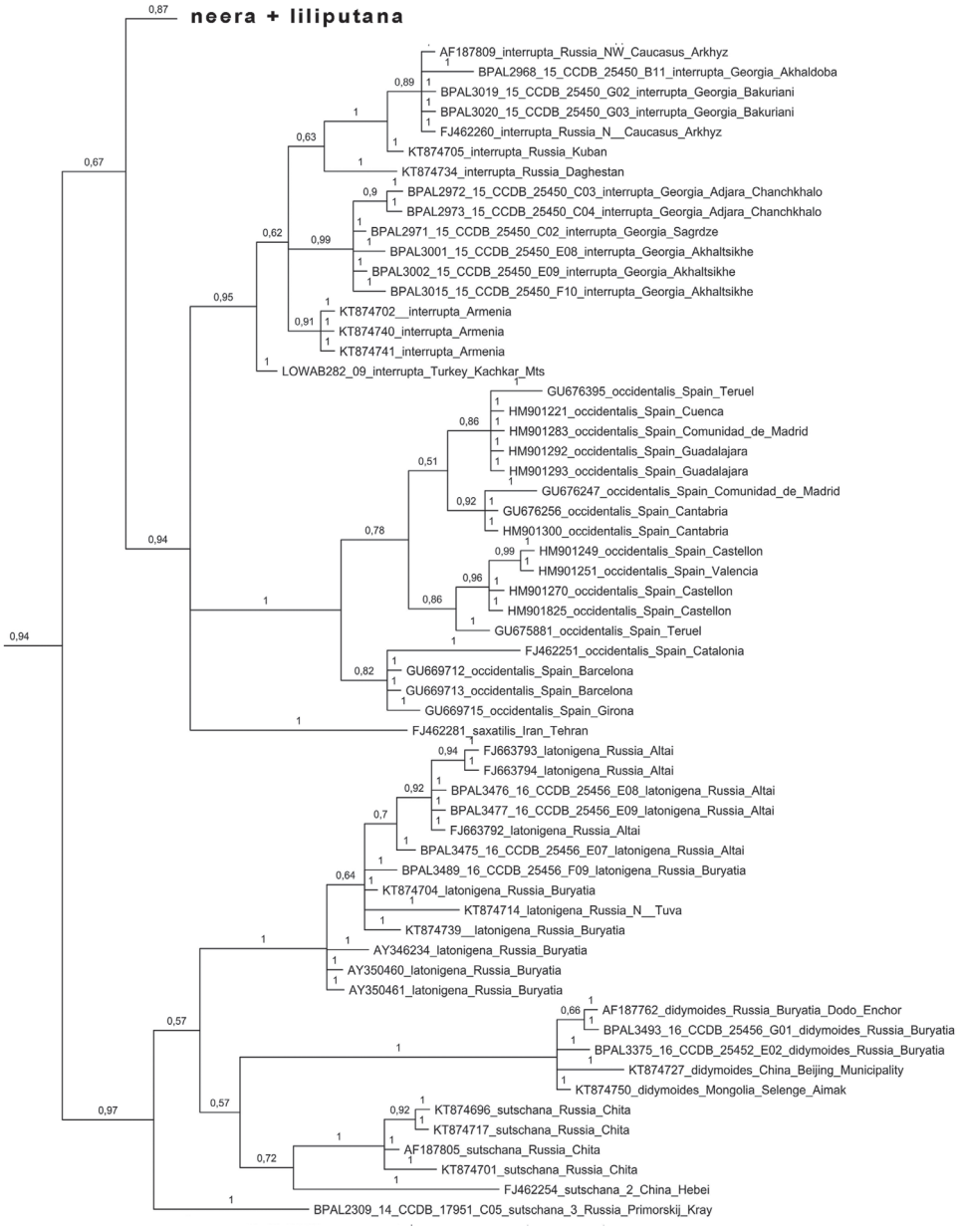

Figure 4. Fragment of the Bayesian tree of *Melitaea didyma* complex (haplogroups *interrupta*, *occidentalis*, *saxatilis*, *lathonigena*, *didymoides*, *sutschana*, *sutschana 2*, *sutschana 3*) based on analysis of *COI* gene. Numbers at nodes indicate Bayesian posterior probability.

with the haplogroup *turkestanica* (Fig. 8). In Slovenia, the specimen BPAL3090-15 (haplogroup *neera2*) was found together with the haplogroup *neera*. The distance between *neera* and *neera2* was 1.7%.

Figure 5. Fragment of the Bayesian tree of *Melitaea didyma* complex (haplogroups *turkestanica*, *turkestanica 2*, *didyma*) based on analysis of *COI* gene. Numbers at nodes indicate Bayesian posterior probability.

Two samples with the *turkestanica* haplotypes (haplogroup *turkestanica*), one from Aktobe (Kazakhstan) and one from Samara (Russia) were found in sympatry with *M. dimyma neera* haplotypes (haplogroup *neera*). In Karabiryuk (Kazakhstan), two samples with the *neera* haplotypes (haplogroup *neera*) were found in sympatry with *M. didyma turkestanica* haplotypes (haplogroup *turkestanica* and *turkestanica4*).

Discussion

Chromosome number variation

The genus *Melitaea* is known to be characterized by relatively low interspecific chromosome number variation. The representatives of basal clades (see phylogeny in Leneveu et al. 2009), the taxa of *M. cinxia* (Linnaeus, 1758), *M. diamina* (Lang, 1989), *M. athalia (*Rottemburg, 1775), *M. trivia* ([Denis & Schiffermüller], 1775) and *M. phoebe* ([Denis & Schiffermüller], 1775) species groups demonstrate n=30-31 (Federley 1938, de Lesse 1960, Robinson 1971, Larsen 1975, Hesselbarth et al. 1995). These haploid numbers are modal ones not only for *Melitaea*, but also for the family Nymphalidae and for the order Lepidoptera in whole (Robinson 1971, Lukhtanov 2000, 2014). Most likely, one of them (probably, n=31, see Lukhtanov 2014) represents an ancestral lepidopteran condition preserved in the basal lineages of *Melitaea*.

The younger lineages, the *M. fergana* Staudinger, 1882 and *M. didyma* species groups, were found to possess lower chromosome numbers varying from n=27 to n=29-30. Within the *M. fergana* species group, *M. athene* Staudinger, 1881, the only karyologically studied species, was found to have n=29 (with n=30 as a rare intra-individual variation) (Lukhtanov and Kuznetsova 1989). The species-rich *M. didyma* group consists of three complexes: a complex of taxa close to *M. ala*, a complex of taxa close to *M. persea* and a complex of taxa close to *M. didyma*. Within these complexes the following chromosome numbers were found: n=29 in *M. ala* (Lukhtanov and Kuznetsova 1989), n=27 in *M. persea* (de Lesse 1960) and different numbers from n=27 to n=29-30 in species of the *M. didyma* complex (Table 3).

Together with *M. deserticola* (n=29, Larsen 1975), *M. gina* occupies a basal position within the *M. didyma* complex (Fig. 6). Therefore analysis of *M. gina* was crucially important for understanding chromosome number evolution in this complex. Our study revealed *M. gina* to have n=28, a number previously observed in *M. didyma* from Italy (de Lesse 1960) and *M. didyma neera* from the Kazakh Altai (Lukhtanov and Kuznetsova 1989). Taking into account absence or relatively low level of interspecific chromosome number variation in the *M. didyma* complex and presence of intraspecific variation (Table 3), we conclude that in this group chromosome numbers have relatively low value as taxonomic markers (but see: Lukhtanov and Kuznetsova 1989).

Table 3. Chromosome numbers of taxa close to *M. didyma*.

Taxon	Chromosome number	Country	Locality	Reference
M. didyma ssp.	n=28	Italy	Abruzzi	de Lesse 1960
M. didyma neera	n=28	Kazakhstan	Altai	Lukhtanov and Kuznetsova 1989
M. didyma neera	n=27	Russia	N Caucasus, Pyatigorsk	Lukhtanov and Kuznetsova 1988
M. interrupta	n=29	Turkey		de Lesse 1960
M. interrupta	n=29	Azerbaijan, Nakhichevan	Zangezur Mts	Lukhtanov and Kuznetsova 1989
M. latonigena	n=29–30	Kazakhstan	Altai	Lukhtanov and Kuznetsova 1989
M. deserticola	n=29	Lebanon		Larsen 1975
M. gina	n=28	Iran	W Azerbaijan	This study

Note. We did not include in the Table 3 the following data: *M.* "*didyma*" (N Iran, Elburz, Demavend) n=28 (de Lesse 1960) because true *M. didyma* is not known from Iran (van Oorschot and Coutsis 2014), and the studied samples could represent *M. interrupta kendevana* or *M. gina*. *M.* "*didyma libanotica*" (Lebanon, Ain Zhalta Cedars) with n=27 (Larsen 1975) was also not included in the Table 2 since its identity remains unclear. The voucher samples for this count were larvae, and their identification was not certain. They actually could represent *M. persea* (n=27 is typical number for *M. persea*, including the population from Lebanon (de Lesse 1960).

DNA barcode haplogroups and problem of non-monophyletic species

Despite low level of chromosome number variability, the *M. didyma* complex was found to have unexpectedly high level of mitochondrial haplotype diversity. These haplotypes were clustered in 23 highly diverged haplogroups (Fig. 2). 12 of these haplogroups are associated with nine traditionally recognized and morphologically distinct species *M. deserticola*, *M. gina*, *M. didymoides*, *M. saxatilis*, *M. sutschana* (this species was devided recently in *M. sutschana* and *M. yagakuana* Matsumura, 1927, see Oorschot and Coutsis 2014), *M. latonigena* (this species was devided recently in *M. latonigena* and *M. latonigenides* Oorschot and Coutsis, 2014, see Oorschot and Coutsis 2014), *M. interrupta*, *M. mixta* and *M. chitralensis*.

The rest of the haplogroups belong to the well-known west-palearctic species *M. didyma*. Despite intrapopulation and seasonal variability, this species is very homogenous with respect to morphology, including the structure of genitalia, a character which is most useful for species separation in *Melitaea* (Suschkin 1913, Higgins 1941, Oorschot and Coutsis 2014). In accordance with this homogeinity, in the recent revision (Oorschot and Coutsis 2014) all populations of this species, except for Central Asian populations, were considered as members of the same subspecies *M. didyma didyma*. The populations from Central Asia were treated by Oorschot and Coutsis (2014) as a separate subspecies *M. didyma turkestanica*.

If we follow the opinion of experts in *Melitaea* taxonomy (Kolesnichenko et al. 2011, Oorschot and Coutsis 2014) and accept the traditional taxonomic treatment of the species *M. didyma*, we should acknowledge that this species is particularly unusual in the haplotypes we obtained. First, it is clearly polyphyletic with respect to *COI* gene, and

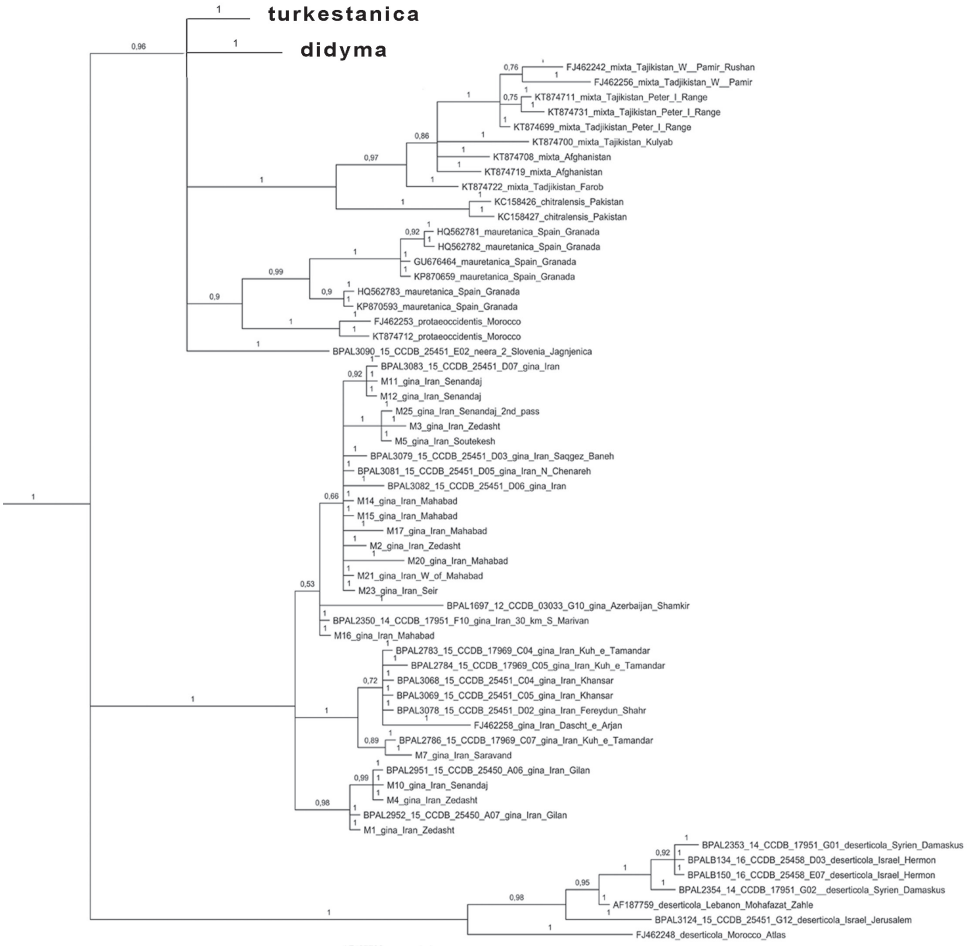

Figure 6. Fragment of the Bayesian tree of *Melitaea didyma* complex (haplogroups *mixta*, *chitralensis*, *mauretanica*, *protaeoccidentis*, *neera2*, *gina* and *deserticola*) based on analysis of the *COI* gene. Numbers at nodes indicate Bayesian posterior probability.

the lineages of *M. didyma* are intermixed with other well recognized species on the tree (Figs 2–6). Second, the number of distinct *COI* lineages within *M. didyma* is unusually high (11 lineages) and their genetic differentiation is extreme. The majority of these haplogroups are allopatric, but some of them have sympatric (*neera/neera1*, *turkestanica/turkestanica2*, *turkestanica/turkestanica3*, *turkestanica/turkestanica4*) or partially sympatric (*neera/turkestanica*, *occidentalis/didyma*) distribution. The mean uncorrected pairwise distances between the lineages is up to 7.4% if the lineages *turkestanica3* and *turkestanica4* are considered (Table 2). The lineages *turkestanica3* and *turkestanica4* are the most diverged lineages of *M. didyma*. Together with *gina2*, on the tree (Fig. 2) they have an intermediate position between the lineage (*M. didyma* + *M. deserticola* + *M. gina*) and the lineage (*M. persea* + *M. casta*). It even appears as a sister group to (*M. persea* + *M. casta*),

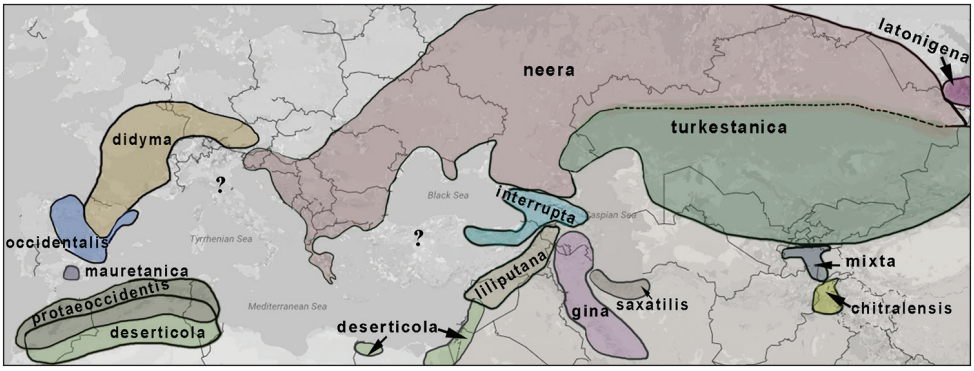

Figure 7. Distribution ranges of western *COI* haplogroups of *Melitaea didyma* complex.

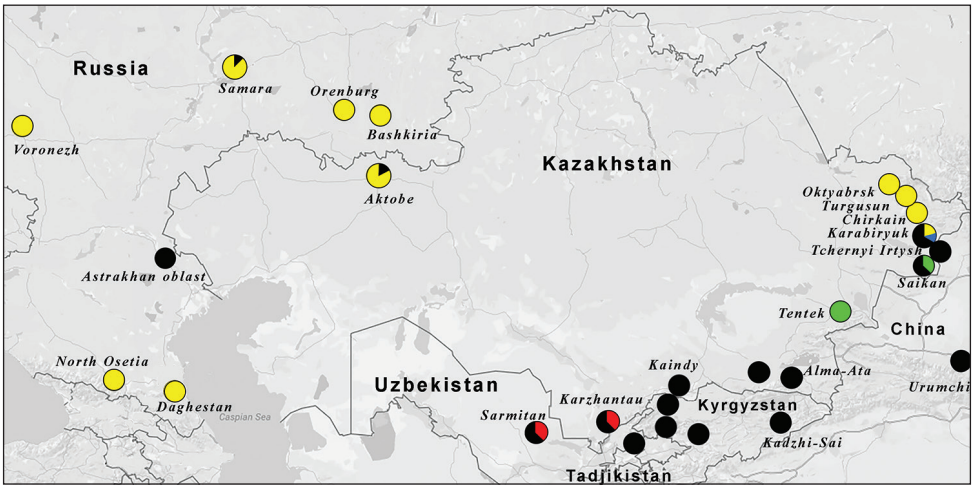

Figure 8. Localization of *neera* and *turkestanica* haplogroups (yellow circles – *neera*, black – *turkestanica*, green – *turkestanica2*, red – *turkestanica3*, blue – *turkestanica4*)

but with a very low support (0.54). However, even if the lineages *gina2*, *turkestanica3* and *turkestanica4* are not considered, the distances between *M. didyma* groupings remains high, up to 4.1% between *turkestanica2* and *liliputana*, i.e. much deeper than the "standard" DNA barcode species threshold (2.7-3%) (Hebert et al. 2003, Lukhtanov et al. 2016).

There are two theoretically possible explanations for this pattern. First, *M. didyma* sensu auctorum can be a mix of multiple species that mostly have allopatric distribution ranges, but some of them are sympatric. Second, the recovered haplogroups (at least the allopatric ones) can represent highly diverged intraspecific lineages. Of course, a combination of the first and the second hypotheses is possible, and a part of the haplogroups could represent different species, and another part of the haplogroups could represent intraspecific variations.

In our opinion, the second hypothesis seems to be more plausible. There are the following arguments for the second scenario. First, no morphological differences between the bearers of these haplogroups are known (except for lighter, more yellowish wing colour in the three *M. didyma turkestanica* haplogroups as compared with other haplogroups). The second (and the most convincing) argument is based on our field obseravtion of butterfly habitats and ecological preferences. In ecology the competitive exclusion principle, also known as Gause's law is one of the most important rule (Gause 1934, Hardin 1960). In complete accordance with this rule, in case of sympatry the most closely related species pairs, such as *M. didyma*/*M. interrupta*, *M. didyma*/*M. latonigena* and *M. gina*/*M. saxatilis* demonstrate clear niche differentiation (*M. didyma* and *M. gina* are more xerophilous, whereas *M. interrupta*, *M. latonigena* and *M. saxatilis* are more mesophilous taxa). This was not a case for sympatric haplogroups *neera*/*neera2*, *turkestanica*/*turkestanica*, *turkestanica*/*turkestanica3* and *turkestanica*/*turkestanica4* (Fig. 8). The bearers of these haplogroups were not only morphologically identical, but also were found to fly exactly syntopically and synchronously. This pattern is hardly compatible with non-conspecifity of these haplogroups.

M. didyma neera and *M. didyma turkestanica* are differentiated ecologically (Pazhenkova et al. 2015), however, there was no ecological separation between bearers of the *neera* and *turkestanica* haplogroups in cases of their sympatry. In Samara and Aktobe, where the haplogroup *neera* was predominant, both haplogroups were found in *M. didyma neera* biotope (steppe), and in Karabiryuk where the haplogroup *turkestanica* was predominant, both haplogroups were found in *M. didyma turkestanica* biotope (desert) (Fig. 8). This pattern corresponds more to a result of haplotype introgression than to co-habitation of two ecologically differentiated species.

Interestingly, the haplogroup *turkestanica2* is not related to the haplogroup *turkestanica* and is a derivative from West-European haplogroup *didyma*. This pattern can be treated as a result of ancient introgression. Generally, footprints of ancient and more recent introgression are both an evidence for transparency of boundaries between *M. didyma* populations.

The mega-analysis of species-level para- and polyphyly in DNA barcode gene trees was recently conducted by using a huge data set (4977 species and 41,583 specimens of European Lepidoptera) (Mutanen et al. 2016), however without in-depth-analyses of particular cases. This study resulted in conclusion that cases of species' polyphyly in *COI* trees arising as a result of deep intraspecific divergence were negligible, and the detected cases reflected misidentifications or/and methodological errors. Despite this, our analysis demonstrates that species-level polyphyly in DNA barcode based on deep intraspecific divergence may be a real phenomenon.

Distribution ranges and phylogeography

The *M. didyma* complex consists of at least 23 *COI* haplogroups, the majority of which demonstrated a strict attachment to particular geographic ranges: *chitralensis* (north Pa-

kistan); *deserticola* (north Africa, Israel, Jordan, Lebanon, Syria); *didyma* (west Europe); *didymoides* (Asian Russia, Mongolia, North China); *gina* (W Iran, Azerbaijan); *interrupta* (Caucasus, NE Turkey); *latonigena* (Asian Russia, north-east Kazakhstan, Mongolia, north-west China); *liliputana* (Armenia, Turkey, Syria, Lebanon, Israel); *mauretanica* (south Spain); *mixta* (Tajikistan, Kyrgyzstan, Uzbekistan, Pakistan, Afghanistan); *neera* (east Europe, north Caucasus, west Siberia, north Kazakhstan); *occidentalis* (Spain); *protaeoccidentis* (north Africa); *saxatilis* (north Iran); *sutschana* (Russian Far East, Korea, north-east China) and *turkestanica* (Kazakhstan, Kyrgyzstan, Uzbekistan, Tajikistan, west China). With few exceptions (e.g. *deserticola*/*protaeoccidentis*, *deserticola*/*liliputana*), the ranges of these haplogroups do not overlap substantially (Fig. 7), and we hypothesize that mitochondrial diversity was formed in allopatry. Given the deep level of genetic differentiation between the lineages, we assume that there was a long period of allopatric differentiation when the lineages were separated by geographic or/and ecological barriers. Under generally accepted maximum 2.3% (Brower 1994) and minimum 1.5% uncorrected pairwise distance per million years (Quek et al. 2004) for *COI* sequence of various arthropod taxa, this period can be estimated to be as long as 0.5–5.0 My. In our opinion, this is an evidence that each of these haplogroups evolved in one of the main west-palaearctic late Pliocene and Pleistocene refugia in north Africa (*protaeoccidentis*, *deserticola*), the Iberian Peninsula (*occidentalis*, *mauretanica*), the Balkan Peninsula (*neera*), the Middle East (*liliputana*, *saxatilis*, *gina*) and Central Asia (*turkestanica*, *mixta*, *chitralensis*). The presence of additional diverged minor haplogroups *neera2*, *turkestanica2*, *turkestanica3*, *turkestanica4*, *gina2*, which could originate allopatrically in small isolated spots, but currently exist in secondary sympatry with major haplogroups *neera*, *turkestanica* and *gina*, agrees well with the refugia-within-refugia concept (Gòmez and Lunt 2007, Karaiskou et al. 2014). Interestingly, the area of the most diverged haplogroup *turkestanica3* is close to the area of the recently described subspecies *M. didyma carminea* (Kolesnichenko et al. 2011).

Taxonomic interpretation

We tentatively suggest interpreting the main clusters discovered within *M. didyma* sensu stricto (*M. didyma didyma*, *M. didyma mauretanica*, *M. didyma occidentalis*, *M. didyma protaeoccidentis*, *M. didyma liliputana*, *M. didyma neera* and *M. didyma turkestanica*) as subspecies because each of them has its own distribution range and is distinct with respect to mtDNA (i.e. represents by a monophyletic lineage or a combination of two or three monophyletic lineages). As a result we propose the following classification:

M. didyma **(Esper, [1779])**
- ***M. didyma didyma*** **(Esper, [1779])**
- ***M. didyma mauretanica*** **Oberthür, 1909**
- ***M. didyma occidentalis*** **Staudinger, 1961**
- ***M. didyma protaeoccidentis*** **Verity, 1929**

***M. didyma liliputana* Oberthür, 1909**
***M. didyma neera* Fischer de Waldheim, 1840**
***M. didyma turkestanica* Sheljuzhko, 1929**
***M. didymoides* Eversmann, 1847**
***M. sutschana* Staudinger, 1892**
***M. latonigena* Eversmann, 1847**
***M. interrupta* Colenati, 1846**
***M. mixta* Evans, 1912**
***M. chitralensis* Moore, 1901**
***M. deserticola* Oberthür, 1909**
***M. saxatilis* Christoph, 1873**
***M. gina* Higgins, 1941**

New records

We provide the first record of *M. gina* in Azerbaijan (sample BPAL1697-12, Azerbaijan, Shamkir, 27 June 2011, collector V. Tikhonov).

We also record *M. didyma turkestanica* as a new taxon for Russia and Europe (samples BPAL3168-16, BPAL3169-16, BPAL3170-16, BPAL3173-16 Russia, Astrakhanskaya oblast, Bogdinsko-Baskunchaksky zapovednik, 24 May 2008, collector S. Nedoshivina).

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Supplementary material 1

Table S1

Authors: Elena A. Pazhenkova, Vladimir A. Lukhtanov

Data type: Microsoft Office Excel file

Explanation note: List of *Melitaea* samples used in this study.

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Analysis of the karyotype structure in *Ricolla quadrispinosa* (Linneus, 1767): inferences about the chromosomal evolution of the tribes of Harpactorinae (Heteroptera, Reduviidae)

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Abstract

The subfamily Harpactorinae is composed of six tribes. Phylogenetic studies bring together some of Harpactorinae tribes, but by and large the data on evolutionary relationships of the subfamily are scarce. Chromosome studies are of great importance for understanding the systematics of different groups of insects. For Harpactorinae, these studies are restricted to some subfamilies and involved only conventional chromosome analysis. This work analyzed cytogenetically *Ricolla quadrispinosa* (Linneus, 1767). The chromosome number was determined as $2n = 24 + X_1X_2Y$ in males. In metaphase II the autosomal chromosomes were organized in a ring with the pseudo-trivalent of sex chromosomes in its center. After C-banding followed by staining with DAPI, AT-rich blocks in autosomes were observed and the negatively heteropycnotic sex chromosomes. The data obtained, together with existing data for other species of the group, indicated that different chromosomal rearrangements are involved in the evolution of the species. In addition, a proposal of karyotype evolution for the subfamily, based on existing phylogenetic studies for the group is presented.

Keywords
holokinetic chromosomes, speciation, DAPI, heterochromatin, reproductive isolation, chromosomal rearrangements

Introduction

Reduviidae are the largest family of predatory insects of the suborder Heteroptera, consisting of approximately 7000 species (Kaur et al. 2009, Weirauch et al. 2014). Harpactorinae is the largest subfamily of Reduviidae, and is composed of six tribes: Apiomerini, Diaspidiini, Ectinoderini, Harpactorini, Tegeini and Rhaphidosomatini (Schuh and Slater 1995, Zhang et al. 2015). However, some authors consider Dicrotelini as a tribe (Miller 1954, Tomokuni and Cai 2002, Weirauch et al. 2014). Phylogenetic studies suggest that the first three tribes form a separate clade from the last three tribes (Davis 1969, Coscaron and Melo 2003, Zhang and Weirauch 2014, Zhang et al. 2015).

In Harpactorinae cytogenetic studies are restricted to only three of the six tribes: Apiomerini, Dicrotelini, and Harpactorini (Table 1), showing diploid numbers ranging from 12 to 30, a predominance of 24 autosomes and several sex systems (XY, XnY) (Table 1) (Kuznetsova et al. 2011, Kaur and Kaur 2013). Probable, the cytogenetical variations result from chromosomal rearrangements in autosomes and sex chromosomes. This type of alteration is an important factor in the speciation process, since causing dramatic effects on fertility (Spirito 1998, Rieseberg 2001, Livingstone and Rieseberg 2003, Nosil et al. 2009, Macaya-Sanz et al. 2011).

Evolutionary relationships related to karyotype changes are poorly known for Harpactorinae, and the majority of karyological reports in Harpactorinae are restricted to conventional analysis without the application of banding techniques (Cai and Tomokuni 2003). The present study analyzed cytogenetically, for the first time, *Ricolla quadrispinosa* (Linneus, 1767) (Harpactorini) in order to elucidate its karyotype structure and relate this to existing data on Harpactorinae. In addition, we presented different proposals for the phylogenetic relationships of this group based on the chromosomal data available so far.

Material and methods

Samples and collection sites

Fifteen male specimens of *R. quadrispinosa* were collected from Iguaçu National Park - Foz do Iguaçu - Brazil - 25°37'40.67"S; 54°27'45.29"W (DDM). Each individual was identified and deposited at the Federal University of Pará (UFPA).

Chromosome preparations and conventional staining

The gonads of the adult specimens were dissected in physiological solution for insects (7.5g NaCl, 2.38g Na_2HPO_4, 2.72g KH_2PO_4 in 1L of distilled water). The testes were treated with tap water for 3 min and fixed in methanol:acetic acid (3:1) for 30 min. Chromosome preparations were performed through cellular suspension by maceration in a drop of 60% acetic acid, with each gonad previously treated with 45% acetic acid. These preparations were submitted to conventional staining with Giemsa 3% and also to chromosome banding techniques. Chromosome measurements were carried out using the computer application MicroMeasure version 3.2 (Reeves and Tear 2000).

Chromosome banding

The distribution of heterochromatin was analyzed by Giemsa C-banding according to Sumner (1972), after treatment with 0.2M HCl for 10 min at room temperature, Ba $(OH)_2$ for 1 min and 40 s at 60 °C, and 2× SSC for 1 hour at 60 °C. The AT-rich bands were detected with 4'-6-diamino-2-phenylindole (DAPI), respectively, according to Schweizer et al. (1983). The slides were stained with 2µg/mL DAPI for 30 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer (pH 7.0) 1:1, plus 2.5mM $MgCl_2$. All images were acquired with a Leica DM 4500 B microscope, equipped with a DFC 300FX camera and Leica IM50 4.0 software, and optimized for best contrast and brightness with iGrafx Image software.

Results

The males of *R. quadrispinosa* presented 2n = 24 + X_1X_2Y. In metaphase II, the autosomes are arranged in ring while the three sex chromosomes form a pseudo-trivalent in the center (Fig. 1a, b, d, e). After C-banding, sex chromosomes were shown to be negatively heteropycnotic at all stages (Fig. 1a, b). The C-banding followed by conventional staining highlighted positive heteropycnotic blocks in the interphase nuclei (Fig. 1c). It was also possible to observe positive heteropycnotic blocks in terminal regions of the majority of autosomes and in interstitial region of one pair of chromosomes (Fig. 1d).

The fluorochrome staining with DAPI performed after the C-banding revealed several AT-rich blocks in the autosomes, which were located in both the terminal and interstitial regions of the autosomes while the sex chromosomes were shown to be negatively heteropycnotic (Fig. 1e).

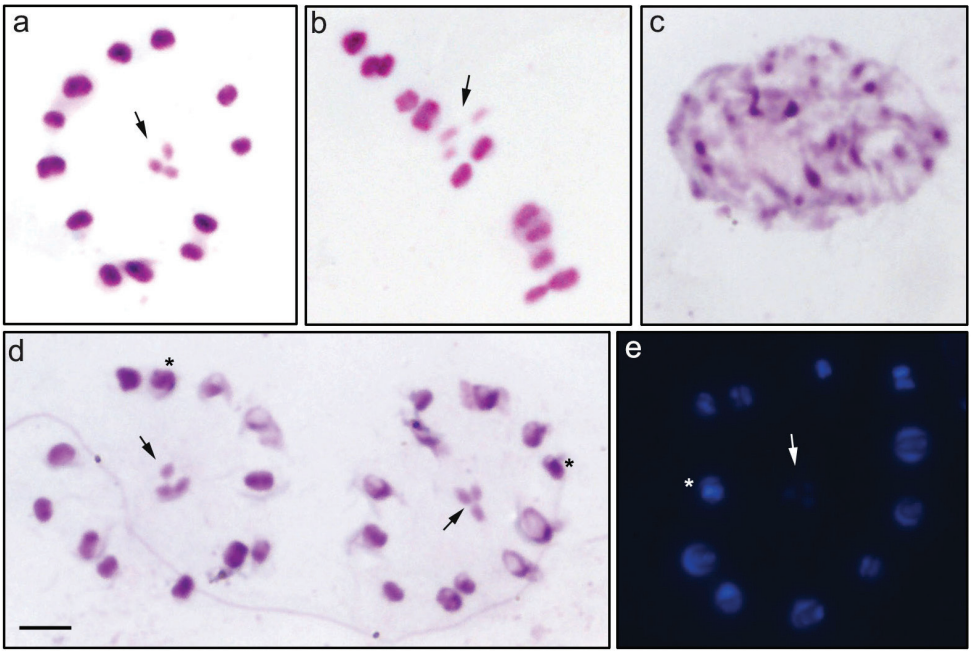

Figure 1. Meiocytes of *Ricolla quadrispinosa.* **A, B** conventional staining: metaphase II **C** conventional staining: interphase nucleus **D** Giemsa C-banding: metaphase II **E** DAPI staining: metaphase II. Sex chromosomes indicated by arrows. Interstitial heterochromatic block indicated by asterisk. Scale bar: 5μm.

Discussion

The number of autosomes observed in *R. quadrispinosa* (24) was similar to that revealed in the most species of the tribe Harpactorini (Table 1), and represents the karyotype conservation regarding the number of autosomes in this group. On the contrary, the multiple sex system observed in *R. quadrispinosa* (X_1X_2Y) has only been reported in another eight species (Table 1) (Kaur and Kaur 2013, Jande 1959, Dey and Wangdi 1988, Payne 1909, Satapathy and Patnaik 1989). Cytogenetic data exist only for three tribes: Apiomerini, Dicrotelini, and Harpactorini (Table 1). These studies are scarce considering the great diversity of the subfamily, with approximately 2800 species (Weirauch et al. 2014).

Within Harpactorinae, there is a very striking karyotype conservation in the Apiomerini tribe, where all species studied so far have presented 2n = 22 + XY (Table 1). As of now, these data support the proposed phylogeny for the group (Tomokuni and Cai 2002, Weirauch 2008, Hwang and Weirauch 2012, Zhang et al. 2015), where Apiomerini form a clade separate from Harpactorini. Analyzing the existing cytogenetic data and those obtained by us, a large karyotype variation within Harpactorini can be seen with 2n = 12 to 2n = 30 and different sex systems (Table 1), which reinforces its phylogenetic distance from Apiomerini.

According to Poggio et al. (2007), the ancestral chromosome number in Reduviidae is 2n = 28, with XY system, while the karyotypes with 22 autosomes are more

Table 1. Cytogenetic studies in Harpactorinae.

Tribe	Species	Diploid number (♂)*	Reference
Apiomerini	*Apiomerus lanipes*	22A+XY	Poggio et al. 2007
	Apiomerus crassipes	22A+XY	Payne 1912
	Apiomerus flaviventris	22A+XY	Ueshima 1979
	Apiomerus spissipes	22A+XY	Ueshima 1979
	Apiomerus sp.	22A+XY	Ueshima 1979
	Heniartes huacapistana	22A+XY	Ueshima 1979
Dicrotelini	*Henricohahnia typica*	$24A+X_1X_2X_3Y$	Kaur and Kaur 2013
Harpactorini	*Acholla ampliata*	$24A+X_1X_2X_3Y$	Payne 1909
	Acholla multispinosus	$20A+X_1X_2X_3X_4X_5Y$	Troedsson 1944
	Arilus cristatus	$22A+X_1X_2X_3Y$	Payne 1909
	Coranus fuscipennis	$24A+X_1X_2Y$	Jande 1959
	Coranus sp.	$24A+X_1X_2Y$	Kaur and Kaur 2013
	Cosmoclopius nigroannulatus	$24A+X_1X_2X_3Y$	Poggio et al. 2007
	Cosmoclopius poecilus	$24A+X_1X_2X_3Y$	Poggio et al. 2007
	Cydnocoris crocatus	$24A+X_1X_2Y$	Dey and Wangdi 1988
	Euagoras erythrocephala	$24A+X_1X_2Y$	Kaur and Kaur 2013
	Euagoras plagiatos	$24A+X_1X_2Y$	Kaur and Kaur 2013
	Fitchia spinulosa	$24A+ X_1X_2Y$	Payne 1909
	Harpactor fuscipes	$24A+X_1X_2X_3Y$	Ueshima 1979
	Irantha armipes	$24A+X_1X_2X_3Y$	Kaur and Kaur 2013
	Lophocephala guerini	$24A+X_1X_2Y$	Satapathy and Patnaik 1989
	Montina confusa	12+XY	Bardella et al. 2014
	Polididus armatissimus	10A+XY	Banerjee 1958
	Polididus sp.	10A+XY	Manna and Deb-Mallick 1981
	Pselliopus cinctus	$24A+ X_1X_2X_3Y$	Payne 1912
	Repipta flavicans	18A+XY	Bardella et al. 2014
	Repipta taurus	$24A+ X_1X_2X_3Y$	Kaur et al. 2012
	Ricolla quadrispinosa	$24+X_1X_2Y$	Present study
	Rhynocoris costalis	$24A+X_1X_2X_3Y$	Kaur and Kaur 2013
	Rhynocoris fusicipes	$24A+ X_1X_2X_3Y$	Dey and Wangdi 1988
	Rhynocoris kumarii	$24A+X_1X_2X_3Y$	Kaur and Kaur 2013
	Rhynocoris marginatus	$24A+ X_1X_2X_3Y$	Satapathy and Patnaik 1989
	Rhynocoris sp.	$24A+X_1X_2X_3Y$	Kaur and Kaur 2013
	Rocconota annulicornis	$24A+X_1X_2Y$	Payne 1909
	Sinea complexa	$24A+X_1X_2X_3Y$	Payne 1909
	Sinea confusa	$24A+X_1X_2X_3Y$	Payne 1909
	Sinea rileyi	$24A+X_1X_2X_3 X_4X_5Y$	Payne 1912
	Sinea spinipes	$24A+X_1X_2X_3Y$	Payne 1909
	Sphedanolestes himalayensis	$24A+X_1X_2X_3Y$	Kaur and Kaur 2013
	Sycanus collaris	$24A+X_1X_2X_3Y$	Jande 1959
	Sycanus croceovittatus	$24A+X_1X_2X_3Y$	Kaur and Kaur 2013
	Sycanus sp.	$24A+X_1X_2X_3Y$	Manna 1951
	Velinus nodipes	$24A+X_1X_2X_3Y$	Toshioka1936
	Velinus annulatus	$24A+X_1X_2X_3Y$	Kaur and Kaur 2013
	Vesbius purpureus	24A+XY	Manna and Deb-Mallick 1981
	Zelus exsanguis	24A+XY	Payne 1909
	Zelus sp. close to *Z. leucogrammus*	24A+XY	Poggio et al. 2007
	Zelus laticornis	24A+XY	Bardella et al. 2014

* ♂ – males; A – autosomes; XY – sex system XY

common in Reduviidae (Ueshima 1979). Considering this, two evolutionary trends may be proposed for Harpactorinae: (i) reduction in the number of autosomes through episodes of chromosomic fusion, and (ii) increase in the number of sex chromosomes due to chromosomic fission events. Thus the occurrence of fissions and fusions probably gave rise to the karyotype *R. quadrispinosa*, and put the Apiomerini species in a condition closer to an ancestral karyotype

In the Harpactorini, twenty-one species present 2n = 24 + $X_1X_2X_3Y$ and 9 species present 2n = 24 + X_1X_2Y (Table 1). Karyotypes with multiple systems with a larger number of X chromosomes are observed only in two species, *Acholla multispinosus* (De Geer, 1773) (2n = 20 + $X_1X_2X_3X_4X_5Y$) and *Sinea rileyi* Montandon, 1893 (2n = 24 + $X_1X_2X_3X_4X_5Y$). Although the variation in the sex chromosome systems is large, the number of autosomes is the same in the majority of species. In Heteroptera, the most common sex mechanism is XX/XY (Papeschi and Bressa 2006). Two hypotheses regarding the evolution of sex systems in Heteroptera have been proposed. The first hypothesis suggests that advanced Heteroptera, derived from the extinct group Gerromorpha, still have the plesiomorphic condition X0; thus, the XX/XY system is derived (Ueshima 1979). In contrast, the second hypothesis suggested that the X0 system is derived from the ancestral system XY (Nokkala and Nokkala 1983, 1984, Grozeva and Nokkala 1996). This last hypothesis appears to be plausible, since studies by Grozeva et al. (2014) in *Xenophyes cacus* Bergroth, 1924 (Peloridiidae, the sister group of Heteroptera) show a tendency to lose the Y chromosome during evolution. Regarding the origin of multiple sex systems, Ueshima (1979) and Papeschi and Bressa (2006) state that they are probably the result of fragmentations of the original sex chromosomes. This would likely be the origin of the multiple sex systems of *R. quadrispinosa*, which have originated by breaks in the XY sex systems of ancestors.

For Dicrotelini, the only species have been cytogenetically studied, *Henricohahnia typical* Breddin, 1900 with 2n = 28 (Kaur and Kaur 2013). If consider only the diploid number, this species would be closer to the species of the Harpactorini. However, according to the phylogeny proposed by Zhang et al. (2015), the Dicrotelini form a separate clade, closer to Apiomerini than Harpactorini. In this way, due to lack of cytogenetic studies in the group, it is not possible to trace an evolutionary line within the tribe. The analysis of more species of Dicrotelini could help to elucidate this hypothesis.

Even taking into account the phylogenetic studies for the group proposed by Zhang et al. (2015), cytogenetic analyzes corroborate the differentiation of Apiomerini from Harpactorini, the former being the more conserved tribe. It is possible to group the species with similar karyotypes within Harpactorini, where those with low diploid numbers and simple sex system form separate branches from those with a higher diploid number and multiple sex systems. Considering the above, coupled with the chromosomal number found in the sister group and most of the species of the subfamilies of Reduviidae, we propose an ancestor with 2n = 24 (22 + XY) for Harpactorinae (Fig. 2). Apiomerini would have remained closer to the ancestral karyotype. Observing Figure 2, it is possible to note that an autosome fusion event (event A) would have given rise to the karyotypes of the species with 2n = 12 + XY, and then a second fusion event (event

B) would have originated the karyotypes of the species with 2n = 10 + XY. These karyotypes are observed in *Montina confusa* (Stål, 1859) (Bardella et al. 2014) and two species of the genus *Polididus* Stål, 1858 (Banerjee 1958, Manna and Deb-Mallick 1981), respectively. This result can be confirmed by molecular analysis, where these genera are grouped forming a separate clade within Harpactorinae (Zhang and Weirauch 2014).

Another chromosomal alteration, the fission of autosomal chromosomes (C event) would have led to a new branch within the Harpactorini, originating 2n = 24 + XY (*Zelus* Fabricius, 1803 and *Vesbius* Stål, 1866 species) (Table 1). Also in this branch, chromosomal fusion events (D event) would originate the karyotypes with 2n = 18 + XY (Fig. 2), observed in *Repipta flavicans* (Amyot and Serville, 1843) (Bardella et al. 2014). Phylogenetically *Zelus* and *Repipta* are close (Zhang and Weirauch 2014) and occupy a separate branch within the Harpactorini distant from other species with simple sex systems, which allows us to put them in this position with respect to the karyotypic evolution. Variations in this karyotype were observed in *R. taurus* (Fabricius, 1803) (Kaur et al. 2012).

The multiple sex systems would have arisen by the fission of the X chromosomes of the ancestral XY system (event E) to give the karyotype 2n = 24 + X_1X_2Y, observed in nine species of the tribe (Table 1). A second fission event of sex chromosomes (F event) would have led to the most common karyotype observed in Harpactorini, 2n = 24 + $X_1X_2X_3Y$, with maintenance of the number of autosomes revealed in twenty-one species of the tribe (Table 1). This explains the intermediate position of the species with these karyotypes in the phylogeny of Harpactorini (Zhang and Weirauch 2014). So far only the one species of Harpactorini, *Sinea rileyi* (Payne 1912), has a different karyotype with 2n = 24 + $X_1X_2X_3X_4X_5Y$, that probably represents an isolated event of karyotypic variation, since all other species of the genus have 28 chromosomes (Table 1).

In addition to differences in the number of autosomes and sex chromosomes, in *R. quadrispinosa* the sex chromosomes are presented as negatively heteropycnotic. Also in metaphase II it is possible to notice several AT rich blocks occupying the terminal and, rarely, interstitial regions of the autosomes. Different patterns of heterochromatin have been reported in other 5 Harpactorinae species (Bardella et al. 2014). Thus in *Apiomerus lanipes* (Fabricius, 1803) the presence of terminal C-DAPI$^+$/CMA$_3^+$ bands in the terminal region was shown, and the heterochromatic sex chromosomes of this species exhibit different florescent patterns. In *Montina confusa* (Stål, 1859) C-DAPI$^+$/CMA$_3^+$ bands were observed in both terminal regions of the two largest autosomes and sex chromosomes. *M. confusa* also showed the third autosomal pair with a C-DAPI$^+$/CMA$_3^+$ band in only one terminal region, whereas the three smaller pairs were totally C-DAPI$^+$/CMA$_3^+$. In *Cosmoclopius nigroannulatus* Stål, 1860 and *Zelus laticornis* (Herrich-Schaeffer, 1853) only one of the sex chromosomes in each species was totally DAPI$^+$ and CMA$_3^+$ in each species. *Repipta flavicans* (Amyot & Serville, 1843) has not demonstrated fluorescent bands in autosomes and sex chromosomes (Bardella et al. 2014). Thus, in Harpactorinae a wide variety of different patterns of C-heterochromatin distribution between the chromosomes was revealed, that implying a large divergence in the karyotypic evolution of species of this subfamily.

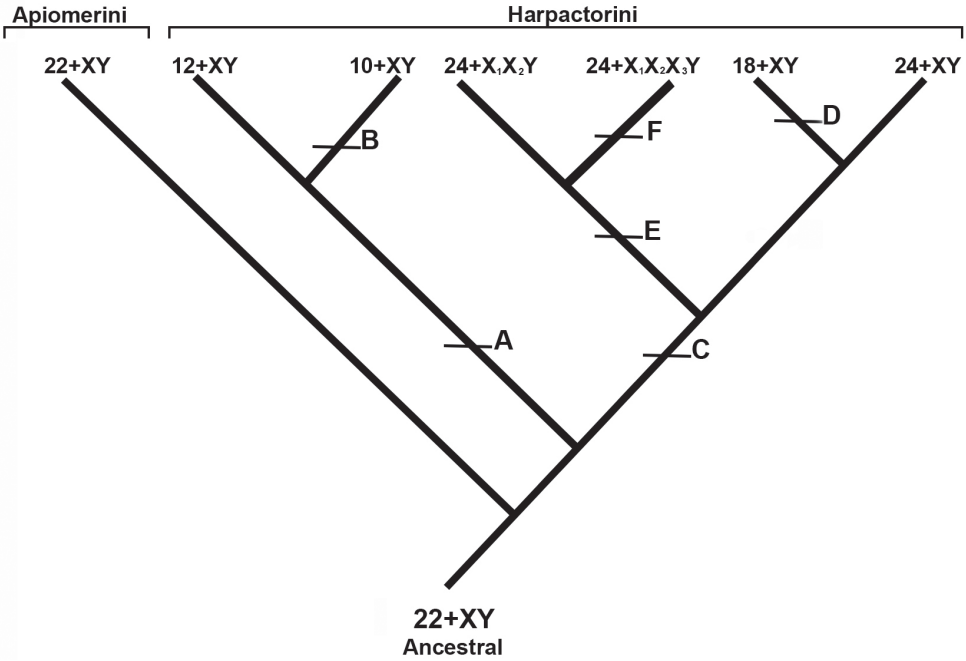

Figure 2. Chromosomal evolution in Apiomerini and Harpactorini. Evolutionary events marked by caps: **A** fusion of autosomes **B** fusion of autosomes **C** fission of autosomes **D** fusion of autosomes **E** fission of sex chromosomes **F** fission of sex chromosomes. Chromosomal formulae represent the diploid number. The tribe Dicrotelini was not included in scheme because only one species has been studied cytogenetically in this tribe.

Considering the influence of the chromosomal rearrangements in the speciation processes, particularly those involved in the differentiation of sex chromosomes, we can suggest that these alterations were fundamental as mechanisms of pre-zygotic reproductive isolation. It is probable that these chromosomal alterations caused the separation of groups, as different species are observed in the same geographical region, leading to a process of sympatric speciation.

Acknowledgments

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Comparison of different cytogenetic methods and tissue suitability for the study of chromosomes in *Cimex lectularius* (Heteroptera, Cimicidae)

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Abstract

In the article we summarize the most common recent cytogenetic methods used in analysis of karyotypes in Heteroptera. We seek to show the pros and cons of the spreading method compared with the traditional squashing method. We discuss the suitability of gonad, midgut and embryo tissue in *Cimex lectularius* Linnaeus, 1758 chromosome research and production of figures of whole mitosis and meiosis, using the spreading method.

The hotplate spreading technique has many advantages in comparison with the squashing technique. Chromosomal slides prepared from the testes tissue gave the best results, tissues of eggs and midgut epithelium are not suitable. Metaphase II is the only division phase in which sex chromosomes can be clearly distinguished. Chromosome number determination is easy during metaphase I and metaphase II. Spreading of gonad tissue is a suitable method for the cytogenetic analysis of holokinetic chromosomes of *C. lectularius*.

Keywords

holokinetic chromosomes, spreading method, squashing method, testes, midgut, karyogram

Introduction

Insect chromosome research is more than 130 years old (White 1973). Large polytene chromosomes of Diptera (*Chironomus* spp., *Drosophila* spp., *Sciara* spp.) were the first subjects studied (e.g. Korschelt 1884, Koller 1935). Gradually, cytogenetic studies became more and more common, so that now insect cytogenetics is a well-established field of science using various modern enhanced methods (e.g. Cabral-de-Mello et al. 2010, Novotná et al. 2011, van't Hof et al. 2011, Bueno et al. 2013).

Historically, classical histology was the first method used for preparing arthropod chromosomes, including insect ones, when the tissue in paraffin wax was cut into sections 7-20 microns in thickness (McClung 1899, Montgomery 1901, Darlington 1939, Slack 1939, Parshad 1957, Piza 1957, and others). This method is no longer used for study of insect chromosomes. The next method developed was a squashing technique (Sáez 1950), which began to be widely used in second half of the 20th century (e.g. Leston 1957, Piza 1957, Warren et al. 1960, Ueshima 1963) and it is still considered as a classical method by the majority of insect cytogeneticists including heteropterologists (e.g. Bressa et al. 2002a, 2003, Poggio et al. 2006, Grozeva et al. 2010, Yang et al. 2012).

The most recent method "hotplate spreading" (only spreading hereinafter) was originally used only for vertebrate chromosomes studies. The whole method was then modified by Crozier (1968) and used also for insect chromosome research (namely ants and dipterans). Traut (1976) developed other modifications of the spreading for lepidopteran chromosome analysis. Recently, this method has been used more frequently not only for study of chromosomes of various insect taxa (Bressa et al. 2009, 2015, van't Hof et al. 2011, Paladino et al. 2013, Sadílek et al. 2013, Chirino et al. 2015) but also for all other main arthropod evolutionary lineages such as arachnids (e.g. Šťáhlavský and Král 2004, Forman et al. 2013, Adilardi et al. 2014, Sadílek et al. 2015), myriapods (e.g. Green et al. 2016), and crustaceans (e.g. Kořínková and Goldyn 2011).

However, the use of the squashing method still strongly prevails over spreading in present Heteroptera cytogenetic studies. As the first, Angus (1982) optimised spreading after Crozier (1968) and applied it for chromosome analysis of Hydrophilidae (Coleoptera). Angus routinely used colchicine to block spindle formation. Later, this method was used in the study of the nepomorphan families Notonectidae (Angus et al. 2004) and Corixidae (Waller and Angus 2005). The spreading technique was also modified by Traut (1976) for male and female meiotic studies in lepidopteran species. Following his procedure, spreading was used successfully for chromosome studies of other heteropteran taxa, namely Corixidae: Micronectinae (Ituarte and Papeschi 2004), Reduviidae: Hammacerinae (Poggio et al. 2011), Triatominae (Morielle-Souza and Azeredo-Oliveira 2007, Poggio et al. 2013a), Reduviinae (Poggio et al. 2013b), Coreidae (Bressa et al. 2008), Pyrrhocoridae (Bressa et al. 2009), and Belostomatidae (Bardella et al. 2012, Chirino et al. 2013, 2014).

One of the very frequently studied Heteroptera is the obligatorily ectoparasitic genus *Cimex* Linnaeus, 1758 (Cimicidae), which includes parasitologically and medi-

cally important species. This genus is characterised by possession of the all-important heteropteran cytogenetic features: holokinetic chromosomes (e.g. Wolf et al. 1997, Mola and Papeschi 2006, Papeschi and Bressa 2006, Guerra et al. 2010, Poggio et al. 2014), achiasmatic male meiosis of collochore type (Nokkala and Nokkala 1983, Nokkala and Grozeva 2000, Grozeva and Nokkala 2002, Ituarte and Papeschi 2004, Grozeva et al. 2008, 2010, Poggio et al. 2009, 2014, Kuznetsova et al. 2011), postreductional inverted male sex chromosome meiosis (Viera et al. 2009, Kuznetsova et al. 2011), and the diffuse stage (Kuznetsova and Maryańska-Nadahowska 2000, Bressa et al. 2002b, Rebagliati et al. 2005, Lanzone and Souza 2006). However the cytogenetic research on *Cimex* species is difficult because of some other chromosome characteristics, such as the small size, high morphological similarity and superspiralization during almost the whole period of chromosomal division (e.g. Ueshima 1966, Manna 1984). Holokinetic chromosomes lack a primary constriction and thus a localized centromere, which facilitates structural rearrangements of the karyotype by nonlethal chromosomal fusions and fragmentations. Fusions in this type of chromosomes do not result in dicentric chromosomes. Chromosome fragments are able to attach to spindle fibres and migrate normally during mitosis or meiosis, which enables them to go through further cell division (e.g. Motzko and Ruthmann 1984, Howe et al. 2001, Mandrioli and Manicardi 2003, Schvarzstein et al. 2010).

In addition to the above mentioned features, the important human ectoparasite model species *Cimex lectularius* Linnaeus, 1758 shows intraspecific variability in number of sex chromosomes from three (X_1X_2Y) to 21 (X_1X_2Y+18 extra Xs) (e.g. Darlington 1939, Slack 1939, Ueshima 1966, Sadílek et al. 2013). In the family Cimicidae, the sex is determined by the presence of an XX/XY (female/male) simple sex chromosome system in 53 cytogenetically analysed species. Most cimicid species, including the majority of *Cimex* species, also possess a multiple sex chromosome system $X_1X_1X_2X_2$/X_1X_2Y (except *C. antennatus* Usinger & Ueshima, 1965, *C. latipennis* Usinger & Ueshima, 1965 and *C. incrassatus* Usinger & Ueshima, 1965 with the basic XX/XY system) (Poggio et al. 2009, Grozeva et al. 2010, Kuznetsova et al. 2011, Sadílek et al. 2013). Four cimicid species possess constantly three X chromosomes ($X_1X_2X_3Y$, male) (*Paracimex capitatus* Usinger, 1966, *P. inflatus* Ueshima, 1968, *P. philippinensis* Usinger, 1959 and *Hesperocimex coloradensis* List, 1925) and two species four X chromosomes ($X_1X_2X_3X_4Y$, male) (*Cimex adjunctus* Barber, 1939 and *C. brevis* Usinger & Ueshima, 1965) (Ueshima 1979, Kuznetsova et al. 2011).

Intraspecific variability in the number of X chromosomes has been described in three cimicid species from the subfamily Cimicinae, *Paracimex borneensis* Usinger, 1959 (2X; 5-9X), *P. capitatus* (2-6X) and *C. lectularius* (2-20X) (summary in Ueshima 1966, 1968, 1979). The numbers of *C. lectularius* X chromosomes can differ among different populations (localities), or among specimens within one population. Even a single specimen can contain cells with different numbers of X chromosomes (Ueshima 1966, 1979, Sadílek et al. 2013). Preliminary study has also indirectly indicated the possibility of a variable number of X chromosomes in an obligatory bat parasite *Cimex pipistrelli* Jenys, 1839 (Sadílek et al. 2013). Therefore, it seems possible that intraspe-

cific variability of X chromosomes could be a general feature of the genera *Cimex* and *Paracimex* Kiritshenko, 1913, or even possibly a wider spectrum of Cimicidae species.

Cimex lectularius became an intensively studied species by a wide spectrum of scientific approaches due to its recent massive global expansion (e.g. Hwang et al. 2005, Romero et al. 2007, Reinhardt et al. 2008, Weeks et al. 2010, Balvín et al. 2012, Booth et al. 2015), including cytogenetic studies using modern methods by Grozeva et al. (2010, 2011) and the detailed analysis of variable karyotype by Sadílek et al. (2013). As it is generally very important to improve methodological approach to research, we used the spreading method for preparing *C. lectularius* chromosome slides.

The main aim of the present study is to compare results of the spreading method, used for the first time in the Cimicidae, with the traditional squashing method. We aimed to find out if the spreading method resulted in different or more conclusive data and could be therefore more suitable for analysis of cimicid holokinetic chromosomes. The use of spreading is currently quite rare even within researches of other Heteroptera species but it is also recommended for cytogenetic studies of the other insect orders. The present paper also makes comparisons of the suitability of different tissues for cytogenetic study, and of distinct cell division phases, chromosome size measurement and assembly of *C. lectularius* karyograms.

Material studied and equipment used

Material studied

220 specimens of *C. lectularius* collected from 65 localities in 10 European countries in the period 2010–2012 were studied, for geographical origins see Sadílek et al. (2013). Live specimens were mostly collected by pest exterminators from human dwellings. They were either studied immediately or were kept alive in the refrigerator at 4 °C without any blood meal. They could survive in good health in such conditions even more than a half of year. Gonad tissue from 115 adult males, 81 adult females and 24 larvae was studied cytogenetically. From those specimens 116 slides of mesenteron (1 slide per specimen) and 13 slides of eggs/embryos (1 slide from a few eggs per female) were also analysed.

Equipment used

The chromosome slides were examined using the Olympus Provis AX 70 light microscope and selected cells and stages of division were documented by the digital imaging system Olympus DP 72 and software QuickPHOTO CAMERA 2.3. Karyograms were made in graphic editor Corel DRAW X5. For assembly of karyograms, chromosomes were cut out from photographs, measured and sorted by size in software ImageJ 1.47 with Levan plugin (http://imagej.nih.gov/ij/).

Results and discussion

Hotplate spreading

The basic principle of the hotplate spreading technique is to turn extracted tissue into a suspension and let cells to adhere to the surface of a microscope slide (optimal is SuperFrost quality slide) as the drop was moved on the slide by pushing it with fine tungsten needles and evaporated. The resulting semipermanent slide (without cover slip) is characterized by its long durability (for years), stored at 4 °C for basic Giemsa staining or -20 °C to -80 °C for further molecular analysis (e.g. FISH). *Cimex lectularius* specimens were dissected in hypotonic solution 0.075 M KCl immediately after killing, to keep the gonad tissue hydrated and remove debris of other tissues. During hypotonisation, the cells receive additional water due to osmosis, making them larger, the contents of the cell are loosened and chromosomes become more individualized. Chromosomes can be damaged or washed away during final dissociation in case of excessive hypotonic treatment. However, chromosomes are still too compact and are not analysable in insufficiently hypotonised cells. Several time periods of tissue hypotonisation were tried: 10, 15, 20, 25 and 30 minutes. The best results were obtained from samples after 25 minutes of fresh hypotonic solution treatment.

Tissue fixation in methanol: glacial acetic acid 3:1 was the next step, methanol can be replaced by 99.9% ethanol. Alcohol causes immediate death of cells and acetic acid penetrates the membrane for quick ideal preservation of inner structures especially chromosomes. Two types of fixation were tested, one step fixation for 5, 10, 15 or 20 minutes, and two step fixation for 5+10, or 10+20 minutes. However, the duration of fixation had a minor effect on the final quality of chromosomes on slides. Two step fixation for 5+10 minutes was found to be optimal, the tissue dehydration effectiveness increased because in the second fixation step dilution by water from hypotonic solution was reduced to minimum.

Fixed tissue was mechanically suspended on the slide with tungsten needles and cells were chemically released by adding of 1–2 drops of 60% acetic acid. Undissociated clusters of tissue were removed. The slides with suspension were put on a warm (45 °C) histological plate and the drop was moved all around the slide with the needle. Adhering cells can create hardly diagnosable clusters without that movement. The chromosome sets are very often overlapping in those clusters. Suspension movement also contributes to evenly distributed chromosomal material on the slide surface. The slides were stained on the second day, allowing them to dry properly and to avoid loss of chromosomes. The staining was carried out using a 5% Giemsa solution in Sörensen phosphate buffer (pH = 6.8) for 10, 15, 20, 30 or 40 minutes (optimum in 30 minutes). The stained slides were stored in a refrigerator at 4 °C. The mechanism of cell adherence is described in detail by Imai et al. (1988).

The squashing technique is the more widely used method in Heteroptera cytogenetics. Usually, living specimens are directly fixed in ethanol: glacial acetic acid or methanol: glacial acetic acid (3:1) and can be stored at 4 °C for later use. Dissected gonad tissue is squashed under a cover slip in a drop of 45% acetic acid, which is then frozen using dry ice (solid CO_2) (e.g. Kuznetsova and Nadachowska 2000, Grozeva

et al. 2010, Kuznetsova et al. 2015), or freezing in liquid nitrogen (e.g. Pérez et al. 2004, Bardella et al. 2010) to allow removal of the coverslip. There are also frequent modifications of squashing method for example with use of acetic haematoxylin (e.g. Bressa et al. 2005) or iron propionic haematoxylin (e.g. Rebagliati et al. 2001). After removing cover slips with a razor blade, the slides are dehydrated in fresh fixative for 15 min and air-dried (e.g. Grozeva and Nokkala 2002, Grozeva et al. 2010). The slides are stained with Feulgen Giemsa (e.g. Grozeva and Nokkala 1996).

An undoubted advantage of the squashing method is a possibility to fix material right in the field and then keep it in 70% ethanol at 4 °C for a long time (months, years), but the gonads kept longer period in cold become harder and the squashing of tissue would be more difficult. Material cannot be preserved before use of spreading method, because chromosomes from fixed cells cannot be spread. The major advantage of spreading is easier methodology. In particular, independence of dry ice or liquid nitrogen (hard to supply in the field) makes it possible to use this method outside the laboratory, with the only demand being for electricity or even without hotplate at the room temperature - higher temperature fasten the evaporation and the efficiency of the plate spreading technique.

The spreading needs manual skill in suspension droplet movement on slide after dissociation. Unsuitable manipulation could lead to loss, damage or overlap of chromosomes. On the other hand, a squashed tissue could be easily insufficiently spread and then the chromosomes on slides could be poorly, or not at all analyzable, or even the tissue can be lost during coverslip removing. The use of squashing can be very problematic in organisms with high chromosome number.

The spreading is generally an easier technique, which provides slightly better results than the squashing technique and often provides abundant slides with well-dispersed cells suitable for further analysis. Therefore, gonad tissue spreading is a suitable method for the cytogenetic analysis of Heteroptera, particularly with focus on the small, variable and numerous holokinetic chromosomes of *C. lectularius*. The main advantages and disadvantages of the two methods are summarised in Table 1.

Table 1. Summary of general advantages and disadvantages of the hotplate spreading and squashing methods of chromosome preparation.

	Spreading	**Squashing**
Material	- must be killed freshly	+ can be fixed in field
	- keep it alive, store it for short time (month)	+ store it for months or longer
Equipment	+ possible to perform it in the field (need of electricity)	- not possible to perform it in the field (need of solid CO_2 or liquid N)
Overall difficulty	+ lower	- higher
	+ just handle to move with droplet on slide properly with fine tungsten needles	- cells must be in chromosomes on slide is hardly analyzable single layer, if not
Results	+ even on material rich slides is only single layer of cells	- on material rich slides is high probability of overlap
	+ i.e. more analyzable nuclei	- i.e. fewer analyzable nuclei

Tissue suitability and results obtained

Cimex lectularius reproduction is acyclic, which is why it is almost impossible to find out the exact age or physiological condition of wild specimens. Negative results from specimens with inactive gonads (absence of cell division) could be caused just by starving. Exact age and condition could be known only in laboratory reared specimens and it is also possible to use eggs or larvae of specific age.

Chromosome slides were made from tissues with the highest mitotic index, which express amount of dividing cells. Meiotic chromosomes could be isolated only from gonad tissue, but mitotic chromosomes should be obtained from all types of proliferating tissues as in insect e.g. hemolymph, epithelium of digestive tract and in holometabolous insect imaginal disc.

Gonads. Generally, tissue of gonads is used for cytogenetical studies, mainly testes (Fig. 1A, D) (e.g. Kuznetsova et al. 2004, Bressa et al. 2009, Grozeva et al. 2010, Poggio et al. 2011, 2014), sometimes ovaries (Fig. 1B, C, E) (e.g. Angus et al. 2004, Waller and Angus 2005). We obtained chromosomes in all various stages of spermatogenesis (mitosis and meiosis) from *C. lectularius* testes, and only mitotic chromosomes in its ovaries. However, also frequent meiotic pachytene cells (Fig. 2E) were recorded in ovaries. This could mean that the female pachytene is a prolonged resting phase when immature oocytes stop meiosis until feeding or mating. In the contrast to females, the pachytene stage in *C. lectularius* males is very short and its finding is extremely rare.

Gonads from 4th and 5th instar larvae were analyzed as well as those of adults (Fig. 1A–C). Gonads of the 4th larval instar are always very small, any manipulation of them is quite difficult as well as a correct determination of sex. Size of the 5th larval instar gonads (Fig. 1D, E) can be different in wide spectrum, from miniature as in the 4th larval instar to large and well developed in sub adult specimens, in which also sex can be distinguished easily. In the older 5th instar larvae, nuclei from mitosis to meiotic metaphase II can be seen (Fig. 2A, B, L).

In *C. lectularius* feeding directly initiates mating behaviour and cell division in gonads, thus this is a required condition for gonad growth and gamete production (Usinger 1966). In our study, small gonads and therefore negative slides were recorded even from recently (approximately 7 days) fully engorged specimens, which probably could not digest their meal and start gamete production.

Testes tissues were shown to be very suitable for the *C. lectularius* cytogenetic research. They contain large quantities of cells in all stages of meiotic and mitotic division and provide enough information for complete karyotype analysis. Ovarian tissue is suitable in cases of lack of males or as a reference in samples with a higher chromosomal variability, and to confirm the sex chromosome system in comparison with chromosomes of males. In samples of *C. lectularius* with variable karyotype, it is interesting to observe complementarity of chromosome number between males and females, and it is also possible to study females with varying X chromosome numbers in oocytes, originating from breeding of specimens with different karyotypes (Sadílek et al. 2013).

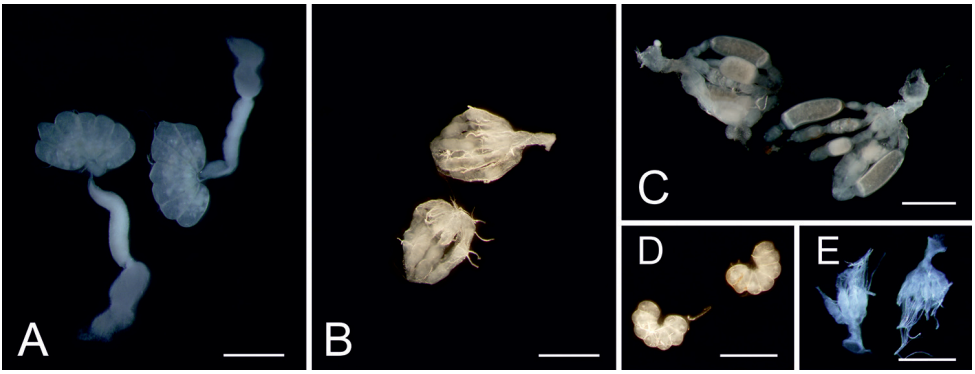

Figure 1. Adult and 5th instar larva *Cimex lectularius* gonads. **A** Adult testes **B** Adult ovaries, without eggs **C** Adult ovaries, with well-developed eggs **D** 5th instar larva testes, well-developed, probably sub adult specimen **E** 5th instar larva ovaries. Scale bar = 1 mm.

The absence of meiotic metaphases in adult females suggests meiotic division in an earlier instar. However, because of quite frequently recorded pachytene nuclei (Fig. 2E) the whole meiotic division has to take place even in adult females. In the contrast, in testes pachytene chromosomes were recorded very rarely, thus it is possible to propose a different length of the pachytene stage between sexes. It is very possible that the whole phenomenon is connected to male achiasmatic meiosis. Male pachytene checkpoint is missing because of male recombination absence that means the male pachytene is very fast and hard to record (e.g. Tung et al. 2000).

Heteroptera cytogenetics is studied usually on male gonads. Detailed study of female karyotype is often problematic, because there is much lower abundance of dividing cells in ovaries than in testes and because all female meiotic stages are almost impossible to record. These are the main reasons for the absence of information about female cytogenetics especially meiosis (Kuznetsova et al. 2011). Nevertheless, in a case of complicated variable karyotypes of *C. lectularius*, the analysis of female cytogenetics results is important and highly recommended.

Midgut epithelium. This tissue should be suitable for cytogenetic study due to continual wasting of digestive cells, followed by intensive mitotic division and differentiation of the regenerative (= stem) cells (e.g. Azevedo et al. 2009, Rost-Roszkowska et al. 2010a, 2010b). Nevertheless, midgut epithelium slides of *C. lectularius* contained no countable mitotic chromosomes. Even specimens with rich chromosome slides from gonads provided no records of any chromosomal division in midgut epithelium. We found only one poor nucleus with mitotic chromosomes from 116 slides analysed. It is very interesting that absence of mitosis in midgut is not connected either with presence or absence of food in midgut lumen. Negative slides without distinct particular chromosomes for karyotype study were from specimens with completely full, through all situations, to empty midgut. It could be similar to the case recently described in Ceratopogonidae (Diptera) (Urbanek and Rost-Roszkowska 2015). In

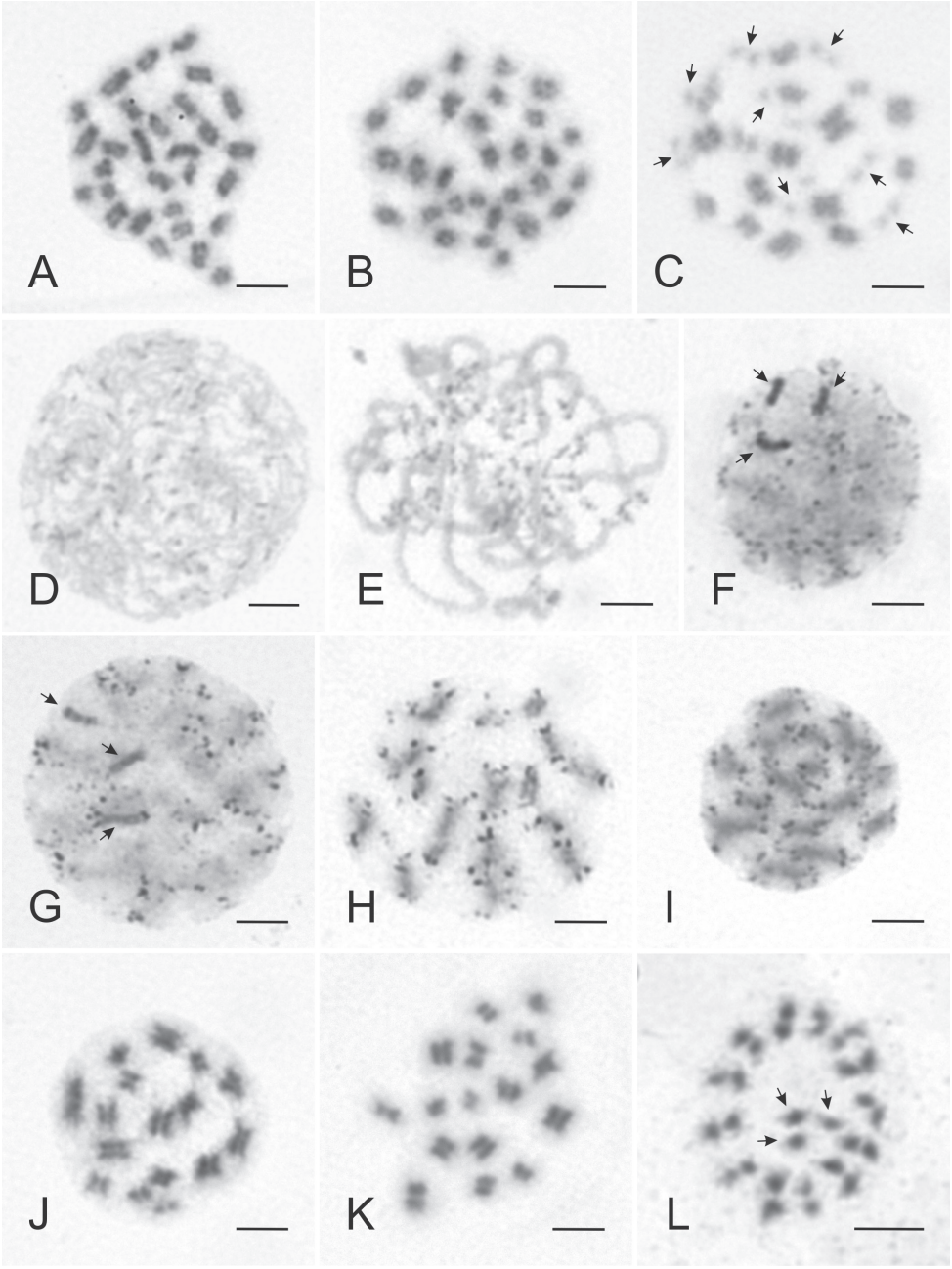

Figure 2. Various stages of mitotic and meiotic *Cimex lectularius* chromosomes with basic karyotype 2n = 26+X_1X_2Y (**A, B, D–L**) and karyotype 2n = 26+X_{1-10}Y (**C**), stained with Giemsa. **A** Mitotic prometaphase ♂ **B** Mitotic metaphase ♂ **C** Metaphase I ♂ **D** Leptotene ♀ **E** Pachytene ♀ **F** Diffuse stage ♂ **G** Diffuse stage - postpachytene transition ♂ **H** Postpachytene ♂ **I** Late postpachytene ♂ **J** Prometaphase I ♂ **K** Metaphase I ♂ **L** Metaphase II ♂. Arrow = sex chromosome (**F, G, L**) or fragments of supposedly sex chromosomes (**C**). Scale bar = 5 μm.

studied dipterans, gonad maturing induces degeneration of digestive cells of midgut epithelium, which are used as accumulated nutrients and not apparently replaced, because in adult females regenerative cells are very rare. The mitotic division of regenerative cells has not been observed even in larvae in this case, in which the cells are only differentiated. Recently, no mitotic divisions and differentiations of the regenerative cells were observed in midgut epithelium of two *Cimex* species, *C. lectularius* and *C. pipistrelli* (Rost-Roszkowska et al. 2016).

It is more complicated to obtain mitotic chromosomes from midgut epithelium than from gonad tissue in general. The use of colchicine or other mitosis-inhibiting agents, which abolish spindle formation and leave the chromosomes free in the cell, as in the studies of Angus et al. (2004) and Waller and Angus (2005) is necessary for clear chromosome preparations. Colchicine is not a mitostatic when applied to whole insects or embryos, but allows the chromosomes to continue their mitotic cycle (contraction, separation of chromatids, re-elongation) while lying free in the cell. However, our completely negative results suggest that the *C. lectularius* midgut tissue is not suitable for chromosome research even with colchicine treatment.

Eggs. This stage of insect generally contains many tissues with a large amount of mitotic cells of the growing embryo. However, we were not successful in recognizing of these cells on spreaded slides. Three low quality mitoses were recorded on only a single slide from 13 slides analyzed. A serious complication is the unpredictable presence of eggs in wild *C. lectularius* females, and also the impossibility of distinguishing in advance sex of the embryos. We are sure the sex of embryos only in case of the male basic karyotype $2n = 26+X_1X_2Y$, otherwise we are not able to distinguish between male with one more supernumerary chromosome ($X_1X_2X_3Y$) and basic karyotype of female $2n = 26+X_1X_1X_2X_2$.

The use of eggs is not common in Heteroptera cytogenetics, but for example in study of holokinetic chromosomes in parthenogenetic Psocoptera (Nokkala and Golub 2006), parthenogenetic psyllids and of monocentric chromosomes of Hydrophilidae (Coleoptera) was use of embryonic tissues successful (Angus 1982, Shaarawi and Angus 1991). However, the authors in Hydrophilidae studies used a different modified spreading technique after Crozier (1968).

The karyotype was successfully determined in 128 out of 220 specimens of *C. lectularius* (58%), 80 males and 48 females, from 140 positive chromosomal slides (34%) (with cells in division) out of 412 examined. Slides prepared from testes tissue gave the best results, 90 positive slides out of 170 (53%). Ovarian tissue contains only mitosis with a lower number of 50 positive slides out of 111 (45%). However, the tissues of midgut and eggs were surprisingly unsuccessful, with only 2 positive slides out of 125 (1.6%). All slides were treated identically, therefore a ratio between positive and negative slides could show percentage of specimens in ideal physiological state for getting mitotic and meiotic chromosomes.

Chromosome division phases studied

The following stages of cell division were observed with various frequencies in *C. lectularius* males. Mitotic cells were recorded especially in metaphase and prometaphase stages (Fig. 2A, B) in 80% of specimens. Leptotene and pachytene stages were detected only in two specimens. In late prophase I, the most frequent meiotic cells were diffuse stage in 90% of specimens and postpachytene in 30% of specimens (Fig. 2F–I). Less frequently, cells in metaphase I (Fig. 2K) were observed in 25% of specimens, and cells in metaphase II (Fig. 2L) were the most rare, only in 20% of specimens. Metaphases I were frequently very abundant in the specimens, in a contrast short lasting stages of prometaphase I (Fig. 2J) and II were observed always in small amounts and only in a four specimens.

On slides from ovary cells in mitotic metaphase stage (100% of specimens) only early prophase I (leptotene and pachytene) (Figs 2D, E) from meiotic division were detected. Leptotene nuclei were recorded only in 10% of specimens, pachytene nuclei were observed in 50% of specimens in small densities only. In females no cells were observed in late meiosis, which was the main stage of male cells.

Leptotene (Fig. 2D) and pachytene (Fig. 2E) nuclei are isopycnotic and did not show any distinct features. At diffuse stage (Fig. 2F), autosomes are decondensed and the sex chromosomes are distinctly positive heteropycnotic. During transition from diffuse stage to postpachytene (Fig. 2G), the sex chromosomes become isopycnotic and cannot be distinguished from autosomes. Postpachytene may be considered as meiotic prophase stage that substitutes diplotene and diakinesis in organisms with achiasmatic meiosis where no recombination occurs. During postpachytene, autosomes condensate again and dark terminal spots on telomeric regions of each chromatid appear (Fig, 2G–I). The dark spots disappear at the end of postpachytene, and from prometaphase onwards the chromosomes are isopycnotic (Fig. 2J) and continue in condensation until metaphase I.

In metaphase I (Fig. 2K), nucleus with basic karyotype $2n = 26+X_1X_2Y$ shows 13 autosomal bivalents and three sex chromosomes, which do not pair with each other. Male metaphase II is radial, the 13 autosomes dispose in a ring configuration and the X_1, X_2 and Y chromatids form a pseudotrivalent, which lies at the centre of it (Fig. 2L), in concordance with observation of Ueshima (1967), Grozeva et al. (2010) and Sadílek et al. (2013). Metaphase II is the only stage in which it is possible to definitely distinguish autosomes and sex chromosomes. The chromosome arrangement of metaphase II precisely matches the inverted meiosis of sex chromosomes, in which reductional division of autosomal bivalents occurs in anaphase I whereas the sex chromosomes segregate chromatids (equational division). In anaphase II, autosomes segregate sister chromatids, and the X_1 and X_2 chromatids segregate from the Y (Ueshima 1966, 1979, Grozeva et al. 2010), even with 20 X supernumerary sex chromosomes (Sadílek et al. 2013) (Fig. 2C). Only the metaphase II reflects clearly number of sex chromosomes in *C. lectularius* with supernumerary sex chromosome fragments, because this is the only phase where autosomes and sex chromosomes can be distinguished.

Chromosome number determination is notably easier in meiotic metaphase I and II than in mitosis, because chromosomes are paired and superspiralized. These results show that, using the spreading method, it is possible to get mitotic and meiotic chromosome slides in high quality for further analysis.

Karyogram assembly

Well-spread mitotic chromosomes can be used to assemble karyograms. A particular requirement here is that chromosomes are not physically stretched in the course of preparations, as can happen with squashes. A karyogram represents standard format of species karyotype image that helps us to distinguish chromosomes, generally specific pairs of autosomes and sex chromosomes (e.g. Angus et al. 2015). The chromosomes are usually ordered by size, position of centromere and some specific marker on chromosomes (e.g. C-bands, G-bands, DAPI/CMA_3 fluorescent bands, Ag-NOR bands or position of specific genes visualized by FISH) (Marco et al. 2009, Maryańska-Nadachowska et al. 2012, Chirino et al. 2015). However, in Heteroptera the holokinetic organization and the chromosome composition do not allow to use many of these cytogenetic features. Besides, some characters may be so variable that the comparison is very complicated, especially between different stages of mitotic or meiotic divisions. For example, the size of chromosomes can vary distinctively according spiralization in various phases. That is a reason the relative size of chromosomes (percentage of single chromosome length from whole karyotype) is used more frequently. In this case, it is necessary to measure all the chromosomes in a great number of cells at the same division stage, i.e. at metaphase I, or at metaphase II, or at spermatogonial metaphase (e.g. Sakamoto and Zacaro 2009, Chirino et al. 2013, 2014, Sadílek et al. 2015).

We assembled three examples of karyograms from *C. lectularius* mitotic chromosomes from different chromosome number of 2n = 29, 33 and 37 (Fig. 3A–C) and two male meiotic karyograms from prometaphase II and metaphase II, 2n = $26+X_1X_2Y$ (Fig. 3D) and 2n = $26+X_{1\text{-}7}Y$ (Fig. 3E), respectively. In these cases the size of chromosomes was measured trying to find out the fragmentation events - decreasing size of the X chromosomes during increasing of their number. Nuclei in mitotic prometaphase provide the most relaxed and still quite compact chromosomes, thus the best stage for getting karyograms (Fig. 3A–C). In the contrast, chromosomes in both meiotic metaphases I and II are globular and very similar to each other (Fig. 3D, E). Moreover, *C. lectularius* chromosomes do not show any strong morphological pattern, and distinguishing pairs of autosomes and sex chromosomes is not easy. In the mitotic prometaphase, we are able to put together some chromosomal pairs according to heteropycnotic regions on the ends of chromosomes visible just after regular Giemsa stain (Fig. 3A–C). The pattern of chromosomes change a little among different karyograms, so it is not possible to use it as a clear diagnostic feature. The sex chromosomes can be quickly recognised only in metaphase II (Figs 2L, 3D, E).

Figure 3. Male mitotic and meiotic karyograms of *Cimex lectularius* chromosome variants. **A-C** Mitotic prometaphase. **A** 2n = 26+X_1X_2Y **B** 2n = 26+X_{1-6}Y **C** 2n = 26+X_{1-10}Y **D** Prometaphase II, 2n = 26+X_1X_2Y **E** Metaphase II, 2n = 26+X_{1-7}Y. Scale bar = 5 µm.

In each of *C. lectularius* karyotypes the size of chromosomes gradually decreases. That is a reason why the size expressed only as a percentage is not very suitable for karyotype comparison among congeneric species with different diploid chromosome numbers because they have different distribution of length. However, in case of *C. lectularius* chromosome fragments we can predict their very small size as on example of metaphase I with 2n = 26+X_{1-10}Y (Fig. 2C). In the contrast two karyograms show additional sex chromosomes (Fig. 3B, C) of almost the same size as the sex chromosomes in karyotype with basic chromosome number (Fig. 3A). It suggests an occurrence of non-disjunction or even possibility of chromosome fragments different spiralization. Another explanation could be the fragments resulted from fragmentation in different parts of the original sex chromosomes. If fragments origin is independent in various populations, they simply cannot be identical and must differ by size and content. All these hypotheses need further study. The karyogram assembly brought us at least rough chromosome size comparison of *C. lectularius* various karyotypes.

Conclusion

The hotplate spreading technique has many advantages in comparison with the squashing technique. It is suitable for use by cytogenetic beginners as they need only to

get the manual skill in suspension droplet movement on slide. One disadvantage of spreading exists – material has to be prepared freshly after killing, either in the field or after keeping alive in a lab. However, *C. lectularius* is capable to survive in good health several months without any meal. The spreading technique seems to be ideal for study of specimens with numerous holokinetic chromosomes.

Tissue of testes, the usual material for insect cytogenetic studies, appeared to be the most suitable also in chromosome study of *C. lectularius*. Ovaries sometimes also show some interesting results. But the tissue of midgut and eggs – supposedly suitable, did not show any satisfactory results.

Results based on ovarian tissue could be easily misinterpreted. During dissociation, cells from ovaries and developing male and female embryos resulted from mating with unknown karyotype male could be mixed. Thus it is possible to observe artificial heterogenic sample of three karyotypes, which can be misleadingly considered as a variability in one female karyotype. This is made possible thanks to cimicid specific traumatic insemination and egg fertilization directly in ovarioles, whole effect could be also magnified by low abundance of mitotic nuclei in ovarian tissue in general. To avoid this problem would be necessary to separate only germarium, part of ovaries where mitosis give rise to primary oocytes.

Meiotic metaphase II is the best division phase for study of chromosomes in *C. lectularius*, but mitotic prometaphase and metaphase I are also usable. Our suggestion that the abundant nuclei in diffuse stage could serve for quick diagnosis of sex chromosome number was not proved. Nuclei of specimens with higher number of sex chromosomes did not show clear number of heteropycnotic sex chromosome elements. The explanation could be either that spiralization of sex chromosome fragments has changed so they are no more positively heteropycnotic during diffuse stage, or too small size of fragments.

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RESEARCH ARTICLE

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Comparative cytogenetics of tree frogs of the *Dendropsophus marmoratus* (Laurenti, 1768) group: conserved karyotypes and interstitial telomeric sequences

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Abstract

The diploid number 2n = 30 is a presumed synapomorphy of *Dendropsophus* Fitzinger, 1843, although a noticeable variation in the number of biarmed/telocentric chromosomes is observed in this genus. Such a variation suggests that several chromosomal rearrangements took place after the evolutionary origin of the hypothetical ancestral 30-chromosome karyotype; however, the inferred rearrangements remain unknown. Distinct numbers of telocentric chromosomes are found in the two most cytogenetically studied species groups of *Dendropsophus*. In contrast, all three species of the *Dendropsophus marmoratus* (Laurenti, 1768) group that are already karyotyped presented five pairs of telocentric chromosomes. In this study, we analyzed cytogenetically three additional species of this group to investigate if the number of telocentric chromosomes in this group is not as variable as in other *Dendropsophus* groups. We described the karyotypes of *Dendropsophus seniculus* (Cope, 1868), *D. soaresi* (Caramaschi & Jim, 1983) and *D. novaisi* (Bokermann, 1968) based on Giemsa staining, C-banding, silver impregnation and *in situ* hybridization with telomeric probes. *Dendropsophus seniculus*, *D. soaresi* and *D. novaisi* presented five pairs of telocentric chromosomes, as did the remaining species of the group previously karyotyped. Though the species of this group show a high degree of karyotypic similarity, *D. soaresi* was unique in presenting large blocks

of het-ITSs (heterochromatic internal telomeric sequences) in the majority of the centromeres. Although the ITSs have been interpreted as evidence of ancestral chromosomal fusions and inversions, the het-ITSs detected in the karyotype of *D. soaresi* could not be explained as direct remnants of ancestral chromosomal rearrangements because no evidence of chromosomal changes emerged from the comparison of the karyotypes of all of the species of the *D. marmoratus* group.

Keywords
Chromosomes, Anura, telomeric sequence

Introduction

Faivovich et al. (2005) resurrected the genus *Dendropsophus* Fitzinger, 1843 to accommodate all Neotropical hylid species known or suspected to have a diploid chromosome number 2n = 30. This cytogenetic character state was later confirmed as a synapomorphy for this genus by Suárez et al. (2013) after the description of a 2n = 24 karyotype for *Xenohyla* Izecksohn, 1998, the sister genus of *Dendropsophus* (see Faivovich et al. 2005, Pyron and Wiens 2011, Duellman et al. 2016). Based on preliminary data, Bogart (1973) hypothesized that centric fission events may have been involved in the origin of an ancestral 30-chromosome karyotype, which is a hypothesis that was also considered by Suárez et al. (2013). However, the chromosomes that are putatively involved in these events have not yet been recognized, and this hypothesis remains to be validated.

Although all of the *Dendropsophus* species karyotyped to date show 2n = 30 (see review in Catroli and Kasahara 2009, Medeiros et al. 2013, Suárez et al. 2013, Oliveira et al. 2016), a noticeable variation in the number of biarmed/telocentric chromosomes is observed among them, suggesting that several chromosomal rearrangements took place after the evolutionary origin of the hypothetical ancestral 30-chromosome karyotype. Karyotypes with only biarmed chromosomes [as in *D. minutus* (Peters, 1872) (Gruber et al. 2005) and *D. leali* (Bokermann, 1964) (Bogart 1973)] and karyotypes with up to five pairs of telocentric/subtelocentric chromosomes [as in *D. labialis* (Peters, 1863) (Bogart 1973), *D. sanborni* (Schmidt, 1944) and *D. jimi* (Napoli & Caramaschi, 1999) (Medeiros et al. 2013)] may be observed. However, the chromosomes and events involved in these rearrangements also remain undiscovered because most *Dendropsophus* species karyotypes are not yet described, and few chromosomal markers are available for the known karyotypes, preventing reliable hypotheses of chromosome homeology.

Of the nine species groups recognized in *Dendropsophus* (for a review of the *Dendropsophus* groups, see Faivovich et al. 2005), the *D. microcephalus* (Cope, 1886) group is the most species-rich (currently with 40 species—Frost 2016) and the most studied cytogenetically (17 species karyotyped—review by Catroli and Kasahara 2009, Medeiros et al. 2013, Oliveira et al. 2016). It is noteworthy that karyotypes without any telocentric chromosome (in *D. leali*—Bogart 1973) and with one [as in *D. bipunctatus* (Spix, 1824)—Bogart 1973], two [as in *D. phlebodes* (Steineger, 1906)—Kaiser et al. 1996], three [as in *D. cruzi* (Pombal & Bastos, 1998)—Gruber et al. 2005], four [as in

D. nanus (Boulenger, 1889)—Medeiros et al. 2003] or five (as in *D. jimi*—Medeiros et al. 2013) telocentric chromosome pairs are observed in this group. Karyotypes with distinct numbers of telocentric chromosomes were also found in the *D. leucophyllatus* (Beireis, 1783) group (Bogart 1973, Kaiser et al. 1996, Gruber et al. 2005), which currently has 11 species (see Frost 2016) and is the second most cytogenetically studied species group of *Dendropsophus* (four of the named species are karyotyped). In contrast, all of the three species of the *D. marmoratus* group that are already karyotyped (i.e. *D. marmoratus*, *D. melanargyreus* and *D. nahdereri*) present five pairs of telocentric chromosomes (Bogart 1973, Gruber et al. 2005, Suárez et al. 2013).

Chromosomal sites composed of telomeric repeats localized apart from the telomeres, also known as interstitial or intrachromosomal telomeric sequences (ITSs) or repeats (ITRs), have been detected in several animals (Meyne et al. 1990, Nanda et al. 2002, Rovatsos et al. 2015, Schmid and Steinlein 2016) and plants (Tek and Jiang 2004, He et al. 2013). Based on the genomic location and sequence organization, especially in the number of telomeric repeats, Ruiz-Herrera et al. (2008) classified the ITSs in short ITSs (s-ITSs) and heterochromatic ITS (het-ITS). The s-ITSs [called short interstitial telomeres, short ITs, by Azzalin at al. (2001)] are short stretches of telomeric hexamers distributed at internal chromosomal positions, presumably present in all vertebrate species, whereas het-ITSs are large blocks of telomeric-like repeats localized mainly in centromeric and pericentromeric regions (Ruiz-Herrera et al. 2008). The s-ITSs probably originated from the insertion of telomeric repeats during the repair of DNA double-strand breaks, as was originally proposed by Nergadze et al. (2004, 2007). On the other hand, the het-ITSs have been widely considered to be remnants of ancestral chromosomal rearrangements as fusions (e.g., Lee et al. 1993, Slijepcevic 1998, Ropiquet et al. 2010, Paço et al. 2013, Young et al. 2013) and inversions (e.g., Farré et al. 2009, Ocalewicz et al. 2013, Paço et al. 2013). Recently, Schmid and Steinlein (2016) proposed an additional category of ITS, named euchromatic-ITSs (eu-ITSs), to accommodate the large ITSs that are not revealed as heterochromatic sites by C-banding or staining with base-specific fluorochromes.

The *Dendropsophus marmoratus* group currently includes eight species, i.e., *D. marmoratus*, *D. acreanus* (Bokermann, 1964), *D. dutrai* (Gomes & Peixoto, 1996), *D. melanargyreus*, *D. nahdereri*, *D. novaisi*, *D. seniculus* and *D. soaresi* (Faivovich et al. 2005). Some adult and larval morphological synapomorphies of this species group may be recognized (Faivovich et al. 2005); however, its cladistic proximity with other *Dendropsophus* species groups as well as the internal phylogenetic relationships of this group remain unclear (Faivovich et al. 2005; Pyron and Wiens 2011, Fouquet et al. 2011, Medeiros et al. 2013). To date, up to three of the eight species of the *D. marmoratus* group have been included in phylogenetic analysis (Fouquet et al. 2011).

In this study, we analyzed cytogenetically three additional species of the *Dendropsophus marmoratus* group to investigate if the number of telocentric chromosomes in this group is not as variable as in other *Dendropsophus* groups. Because karyotypic variation in number of telocentric chromosomes may result from rearrangements involving telomeric sequences (review in Ruiz-Herrera et al. 2008), we included here

the mapping of telomeric sequences in the karyotypes of two of the analyzed species. Additionally, we provided the nucleotide sequence of a fragment of the 16S ribosomal RNA gene of one exemplar for each of the species that were analyzed cytogenetically to yield a reliable association of the chromosomal data set with a DNA data set that has been remarkably useful for taxonomic and phylogenetic studies of anurans.

Material and methods

Specimens

Four male exemplars of *Dendropsophus seniculus* from Ribeirão Grande, state of São Paulo, Brazil, nine *D. soaresi* males from Barreiras, state of Bahia, Brazil and one female of *D. novaisi* from Jequié, state of Bahia, Brazil were analyzed cytogenetically. The specimens were collected under a permit issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (#32483), and deposited at the amphibian collection of the Museu de Zoologia "Prof. Adão José Cardoso" at the Institute of Biology – University of Campinas, Campinas, Brazil, under the accession numbers ZUEC 17225–17228 (*D. seniculus*), ZUEC 16867–16875 (*D. soaresi*) and ZUEC 17858 (*D. novaisi*).

Cytogenetic analyses

Animals were injected intraperitoneally with 2% colchicine (Sigma – Aldrich; 0.02 mL per 1 g body weight) for an "in vivo" treatment that lasted at least 4 hours. The animals were deeply anesthetized with lidocaine gel 2% and their intestines were removed and used for obtaining chromosomal preparations according to the method of King and Rofe (1976). Chromosomes were conventionally stained with 10% Giemsa and sequentially submitted to C-banding (Sumner 1972) and silver staining by the Ag-NOR method (Howell and Black 1980).

To localize telomeric sequences, the karyotypes were *in situ* hybridized with the probe $(CCCTAA)_3$ (PNA – Peptid Nucleic Acid TelC-Cy3; PNA Bio Inc.), following the manufacturer's instructions.

Mitochondrial DNA sequences

Samples of genomic DNA were obtained from *Dendropsophus seniculus* (ZUEC 17225), *D. soaresi* (ZUEC 16867) and *D. novaisi* (ZUEC 17858) following the procedure reported by Medeiros et al. (2013). A fragment of approximately 1300 bp of the 16S ribosomal RNA gene was PCR-amplified using the primers 12L13(L) (Feller

and Hedges 1998) and 16Sbr(H) (Palumbi et al. 1991). The amplified products were purified with the GFX PCR and Gel Band DNA purification Kit (GE Healthcare) and directly sequenced in an automatic DNA ABI/Prism sequencer (Applied Biosystems) using BigDye Terminator kits (Applied Biosystems) and the primers 12L13 (Feller and Hedges 1998), TitusI(H) (Titus 1992), Hedges16L2a (Hedges 1994), Hedges16H10 (Hedges 1994), 16Sar(L) (Palumbi et al. 1991) and 16Sbr(H) (Palumbi et al. 1991). DNA sequences were aligned using ClustalW option implemented in BioEdit v. 7.2.5 (Hall 1999) and compared to each other and to the 16S rDNA sequence of *D. seniculus* available at GenBank (AY843666).

Results

Cytogenetic analyses

The karyotypes of *Dendropsophus seniculus*, *D. soaresi* and *D. novaisi* were very similar and presented three pairs (pairs 1, 2 and 4) of submetacentric chromosomes, seven pairs (pairs 3, 8–12 and 14) of metacentric chromosomes and five pairs (pairs 5–7, 13 and 15) of telocentric chromosomes (Figures 1–3). The nucleolus organizer region (NOR) was detected by silver staining in the long arm of chromosome 9 of the three species (insets in Figures 1–3). C-banding only detected the centromeric region of all the chromosomes of *D. seniculus* (Figure 1b), *D. soaresi* (Figure 2b) and *D. novaisi* (Figure 3b).

In situ hybridization detected telomeric sequences in all of the telomeres of *Dendropsophus seniculus* and *D. soaresi* (Figure 4). Additionally, interstitial telomeric sequences (ITSs) were detected in the centromeres of the chromosomes of *D. soaresi*, except in two of the five pairs of telocentric chromosomes (pairs 5 and 6) (Figure 4b).

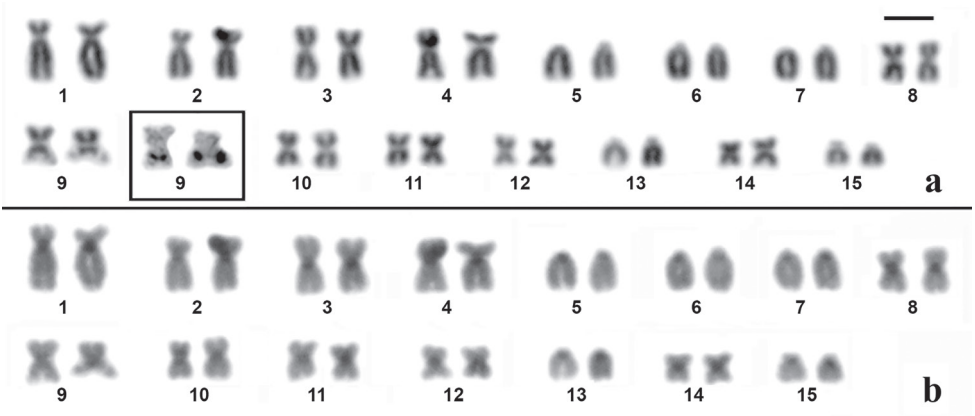

Figure 1. Karyotype of *Dendropsophus seniculus* stained with Giemsa (**a**) and C-banded (**b**). In the inset in (a), the NOR-bearing chromosome pair 9 after silver staining. Bar = 5 μm.

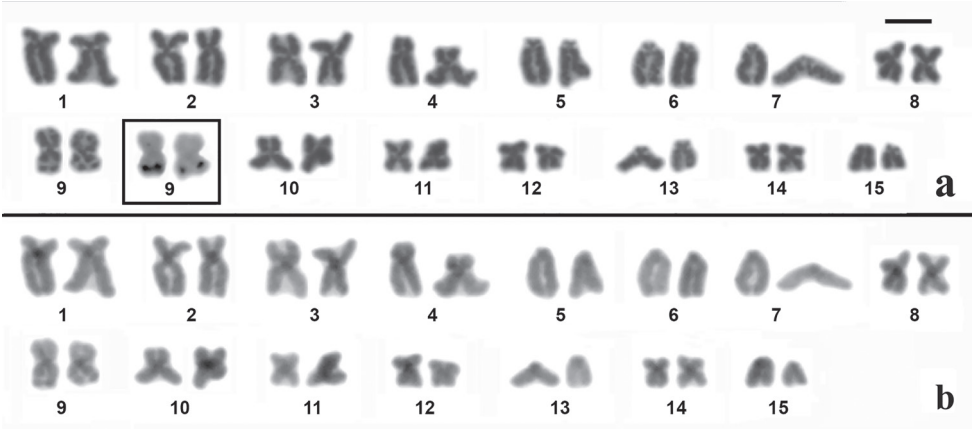

Figure 2. Karyotype of *Dendropsophus soaresi* stained with Giemsa (**a**) and C-banded (**b**). In the inset in (**a**), the NOR-bearing chromosome pair 9 after silver staining. Bar = 5 µm.

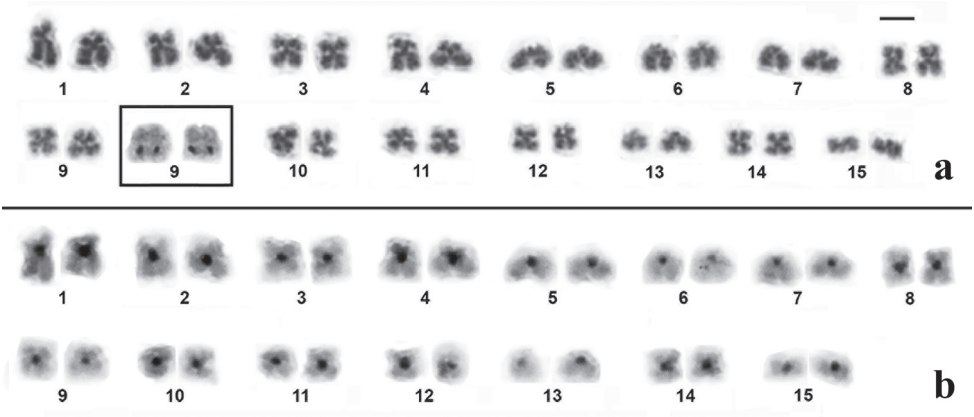

Figure 3. Karyotype of *Dendropsophus novaisi* stained with Giemsa (**a**) and C-banded (**b**). In the inset in (**a**), the NOR-bearing chromosome pair 9 after silver staining. Bar = 5 µm.

Mitochondrial DNA sequences

The nucleotide sequence (1312 bp) (see Suppl. material 1) of the 16S rDNA of the specimen of *Dendropsophus seniculus* that we analyzed was highly similar (99.6%) to the corresponding sequence available at GenBank (AY843666; Faivovich et al. 2005) from a specimen of *D. seniculus* from Angra dos Reis, Rio de Janeiro State, Brazil. The sequences obtained from *D. soaresi* (1314 bp) and *D. novaisi* (1310 bp) (see Suppl. material 1) were 86.4% similar to each other, and 88.4% and 90.7% (average value) similar to the sequences of *D. seniculus*, respectively.

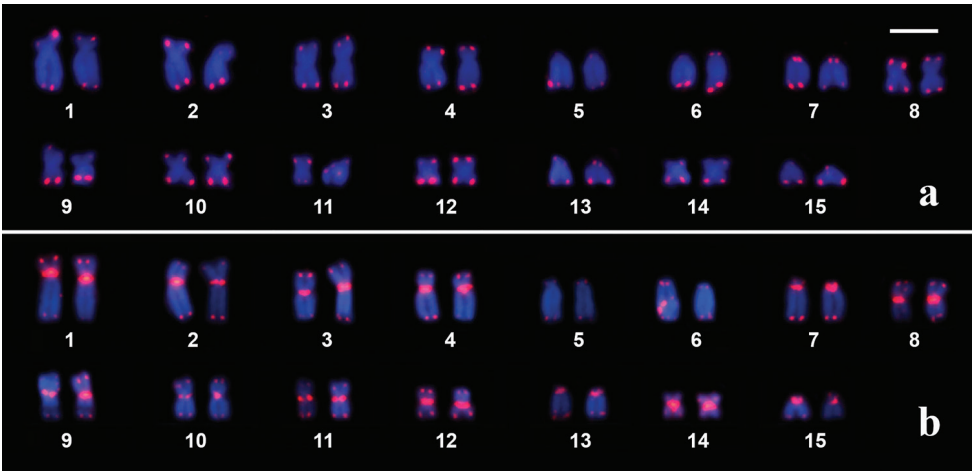

Figure 4. Karyotypes of *Dendropsophus seniculus* (**a**) and *Dendropsophus soaresi* (**b**) hybridized with telomeric probe.

Discussion

Karyotypic comparisons

The three species analyzed showed karyotypes composed of five pairs of telocentric chromosomes, similarly to the other three species of the *D. marmoratus* group previously studied cytogenetically [i.e. *D. marmoratus* (Bogart 1973, Suárez et al. 2013), *D. melanargyreus* (Suárez et al. 2013) and *D. nahdereri* (Gruber et al. 2005)]. The interspecific morphological conservation of the karyotypes of the species of the *D. marmoratus* group contrasts with the variation found in other *Dendropsophus* groups. The *D. microcephalus* group, for instance, includes species with zero to five pairs of telocentric chromosomes (see Bogart 1973, Kaiser et al. 1996, Gruber et al. 2005, Medeiros et al. 2003). Variation in the number of telocentric chromosomes could also be found in the *D. leucophyllatus* group, although only four named species of this group have been karyotyped (see Bogart 1973, Kaiser et al. 1996, Gruber et al. 2005).

According to the estimated dates of divergence provided by Duellman et al. (2016), the *D. marmoratus*, *D. microcephalus* and *D. leucophyllatus* groups arose at similar times in the mid-Miocene (17.0, 17.2 and 18.7 Mya, respectively). Therefore, differential time for divergence does not justify the different levels of karyotypic variation observed among the three aforementioned species groups. Further analyses of the chromosomal rearrangements involved in the karyotypic variations in *Dendropsophus* combined with phylogeographic studies are still necessary to elucidate about the high conservation in the number of telocentric chromosomes in the *D. marmoratus* group.

With respect to the number and relative size of the telocentric chromosomes, the karyotypes of the species of the *D. marmoratus* group are similar to that of *D. labialis* (Bogart 1973), a species included in the *D. labialis* group. This morphological similarity suggests that the telocentric chromosomes of these karyotypes could be homeologous,

although a better characterization of these chromosomes is fundamental to test this hypothesis. According to the most comprehensive phylogenetic analysis of *Dendropsophus* (Duellman et al. 2016) and assuming the telocentric chromosomes of the species of the *D. marmoratus* group and *D. labialis* are homeologous, it is possible to hypothesize that this karyotype configuration is plesiomorphic with respect to those constituted by other numbers and/or relative sizes of the telocentric chromosomes. However, internal relationships within *Dendropsophus* are consistently poorly supported and small taxonomic additions cause huge impacts (e.g., Fouquet et al. 2015, Duellman et al. 2016).

Five pairs of telocentric chromosomes were also observed in the karyotypes of *D. jimi* and *D. sanborni* (Medeiros et al. 2013), which are species that belong to the *D. microcephalus* group. In these karyotypes, however, the telocentric pairs were classified as pairs 5, 6, 12, 13 and 15, whereas the telocentric chromosomes of the karyotypes of *D. labialis* and the species of the *D. marmoratus* group are numbered as pairs 5, 6, 7, 13 and 15. Because only a few chromosomal markers are available for a comparison of these karyotypes, it is still not possible to determine if the telocentric chromosomes of all of these species are homeologous. Therefore, we cannot discard the possibility that chromosomes 12 of *D. jimi* and *D. sanborni* are homeologous to chromosomes 7 of *D. labilais* and the species of the *D. marmoratus* group, although these chromosomes differ by the presence of NOR in chromosomes 12 of *D. jimi* and *D. sanborni* (Medeiros et al. 2013).

The similarities among the karyotypes of the species of the *Dendropsophus marmoratus* group are not restricted to the number of telocentric chromosomes. *Dendropsophus seniculus*, *D. soaresi* and *D. novaisi* also share with *D. marmoratus* and *D. melanargyreus* the location of the NOR at a distal site of the long arm of chromosome 9, which differs from *D. nahdereri*, whose NOR is located on the short arm of the submetacentric chromosome 1 (Gruber et al. 2005).

C-banding did not reveal any differential band that could be considered exclusive to the karyotypes of *D. seniculus*, *D. soaresi* or *D. novaisi*, since only the centromeric regions were detected by this technique (present work). Conspicuous non-centromeric C-bands were also absent in the karyotypes of *D. marmoratus* and *D. melanargyreus*, the other two species of the *D. marmoratus* group whose karyotypes were already C-banded, although Suárez et al. (2013) reported the presence of some distal and interstitial C-bands in those karyotypes.

Despite the high similarity of the karyotypes of the species of the *Dendropsophus marmoratus* group with respect to the number and morphology of the chromosomes, C-banding pattern and location of NOR (except for *D. nahdereri*), the karyotype of *D. soaresi* stands out because of the presence of internal telomeric sequences in addition to the terminal telomeric sequences.

Interstitial telomeric sequences

Large and short ITSs are likely to play a role in karyotypic evolution. Several studies support the hypothesis that, in addition to possibly representing relics of chromosomal

changes, the het-ITSs may themselves induce chromosome breakage and subsequent chromosomal rearrangements (reviewed in Ruiz-Herrera et al. 2008 and Bolzán 2012). Similarly, experimental and associative studies have also suggested the involvement of s-ITSs with genomic instability or chromosomal hot spots of recombination (Aksenova et al. 2013, Wood et al. 2015).

The het-ITSs detected in the present study in the karyotype of *Dendropsophus soaresi* cannot be explained as direct remnants of ancestral chromosomal rearrangements because no evidence of chromosomal changes has emerged from the comparison of the karyotypes of all species of the *D. marmoratus* group already known (Bogart 1973, Gruber et al. 2005, Suárez et al. 2013, present work). Although it is very similar to the karyotypes of the other species of the group, the karyotype of *D. soaresi* is unique in presenting large blocks of centromeric ITSs because the karyotypes of *D. seniculus* (present study), *D. melanargyreus* and *D. marmoratus* (Suárez et al. 2013) showed only telomeric sites hybridized with telomeric probes.

The occurrence of het-ITSs at the majority of the centromeres of the karyotype of *Dendropsophus soaresi* is also remarkable and suggests the expansion and homogenization of telomeric sequences throughout the repetitive elements that compose these centromeric regions. Repetitive DNA, such as centromeric satellite DNA, is expected to expand in the genome and evolve in concert by a series of mechanisms, including unequal crossing-over, gene conversion, rolling circle replication and reinsertion, and transposon-mediated exchange (see Dover 1982, 1986 and the review by Plohl et al. 2008). The telomeric repeats present in heterochromatic sites should be subject to the same evolutionary forces (see Ruiz-Herrera et al. 2008). In contrast, the absence of het-ITS in the centromere of two chromosome pairs (telocentric chromosome pairs 5 and 6) of *Dendropsophus soaresi* suggests that these centromeres do not evolve in concert with the remaining centromeric regions of the genome. The reason for such differential behavior is intriguing and remains unknown.

Similar to observations of *Dendropsophus soaresi*, large blocks of centromeric/pericentromeric ITSs that were widely distributed throughout the genome were previously found in four other hylid species [i.e. *Aplastodiscus albofrenatus* (Lutz, 1924), *A. arildae* (Cruz & Peixoto, 1987) and *A. eugenioi* (Carvalho-e-Silva & Carvalho-e-Silva, 2005)—Carvalho et al. 2009, Gruber et al. 2012a; *Hypsiboas faber* (Wied-Neuwied, 1821)—Schmid and Steinlein 2016]. In the hylid *Itapotihyla langsdorffii* (Duméril & Bibron, 1841), ITSs were also observed in several centromeres (Gruber et al. 2012b), but in this case the het-ITSs are not as large as those previously mentioned. In addition to the aforementioned hylids, other fifteen hylid species showed ITSs in their karyotypes (Meyne et al. 1990, Wiley et al. 1992, Suárez et al. 2013, Mattos et al. 2014, Bruschi et al. 2014, Schmid and Steinlein 2016), which suggests that the appearance of this type of sequence is recurrent in the Hylidae family. Only the centromeric ITS found in chromosome 3 of *Scarthyla goinorum* (Bokermann, 1962) was clearly interpreted as a remnant of a chromosomal fusion that in that case could respond to the reduced chromosome number observed in this species (Suárez et al. 2013). The insertion of telomeric repeats during the repair of double-strand

breaks in DNA as a phenomenon putatively involved in the origin of ITS in Hylidae remains unexplored.

It is worth noticing that in the sample of metaphases analyzed in this paper, large signals of the telomeric probe were detected at a subterminal non-heterochromatic site of some chromosomes of *Dendropsophus seniculus* (Figure 4a, at the long arm of the right homologous of chromosome 9). This hybridization pattern resembles that pattern interpreted by Wood et al. (2014, 2015) as cytological evidence of the occurrence of t-loops formed between telomere and s-ITS. However, studies designed to search for s-ITSs in hylid karyotypes have not yet been performed, and the prevalence of ITSs in Hylidae remains an intriguing question to be assessed in further studies.

Association between cytogenetic data and 16S rDNA sequences

The high similarity between the 16S rDNA sequence of *Dendropsophus seniculus* we provided and that previously obtained by Faivovich et al. (2005) enables a reliable association between the cytogenetic data shown here and the analyses hitherto conducted with the previously available sequence, including the studies of Fouquet et al. (2015) and Duellman et al. (2016). On the other hand, the nucleotide sequences obtained here from *Dendropsophus soaresi* and *D. novaisi* were the first report of 16S rDNA sequences for these species.

Dendropsophus systematics are in flux and even comprehensive datasets are unable to provide a stable historical hypothesis (Fouquet et al. 2011; Peloso et al. 2016). The association between the cytogenetic dataset and 16S rDNA sequences may be very helpful in future analyses, especially because the species-level taxonomy of *Dendropsophus* has been subject to several changes. A number of *Dendropsophus* species has been described in the last few years (Rivera-Correia and Orrico 2013, Ortega-Andrade and Ron 2013, Orrico et al. 2014, Fouquet et al. 2015, Peloso et al. 2016) as well as species synonymizaton has been proposed (Guarnizo et al. 2012, Orrico et al. 2013). Therefore, a reliable association between different sets of data is fundamental for further integrative studies.

Conclusion

All of the karyotypes found in the *Dendropsophus marmoratus* group to date showed five pairs of telocentric chromosomes and were also similar in the location of NORs (except for the *D. nahdereri* karyotype, described by Gruber et al. 2005) and C-banding pattern. Because of this karyotypic conservatism, the het-ITSs present in the majority of the centromeres of the karyotype of *D. soaresi* may not be interpreted as direct remnants of ancestral chromosomal rearrangements.

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Supplementary material 1

16S rDNA sequences

Authors: Lívia S. R. Teixeira, Karin Regina Seger, Cíntia Pelegrineti Targueta, Victor G. Dill Orrico, Luciana Bolsoni Lourenço

Data type: Tif file

Explanation note: Alignment of 16S rDNA fragments obtained from specimens of *Dendropsophus seniculus*, *D. novaisi* and *D. soaresi* that were used in cytogenetic analyses. GenBank accession numbers: KY053469 (*D. seniculus*), KY053470 (*D. novaisi*), and KY053471 (*D. soaresi*).

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Chromosome numbers and DNA content in some species of *Mecardonia* (Gratiolae, Plantaginaceae)

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Abstract

Cytogenetic characterization and determination of DNA content by flow cytometry of five species of *Mecardonia* Ruiz et Pavon, 1798 (Gratiolae, Plantaginaceae) was performed. This is the first study of nuclear DNA content carried out in the genus. Mitotic analysis revealed a base chromosome number x = 11 for all entities and different ploidy levels, ranging from diploid (2n = 2x = 22) to hexaploid (2n = 6x = 66). The results include the first report of the chromosome numbers for *M. flagellaris* (Chamisso & Schlechtendal, 1827) (2n = 22), *M. grandiflora* (Bentham) Pennell, 1946 (2n = 22), *M. kamogawae* Greppi & Hagiwara, 2011 (2n = 66), and *Mecardonia* sp. (2n = 44). The three ploidy levels here reported suggest that polyploidy is common in *Mecardonia* and appear to be an important factor in the evolution of this genus. The 2C- and 1Cx-values were also estimated in all the species. The 2C-values ranged from 1.91 to 5.29 pg. The 1Cx-values ranged from 0.88 to 1.03 pg. The general tendency indicated a decrease in the 1Cx-value with increasing ploidy level. The significance of the results is discussed in relation to taxonomy of the genus.

Keywords

Gratiolae, chromosome number, DNA content, flow cytometry, polyploidy

Introduction

Mecardonia Ruiz & Pavon, 1798 belongs to the tribe Gratiolae (Plantaginaceae) and is distributed across the America, reaching its southernmost distribution in Argentina. The species are erect or creeping herbs, annual or perennial, much branched, mostly glabrous, sometimes blackening on drying, gland dotted, and yellow and white flowers (D'Arcy 1979). Since the description of the genus (Ruiz and Pavon 1794), there have been a few problems in establishing its generic and infrageneric circumscription. Rossow (1987) considered only 10 species, which had been previoulsy placed in *Bacopa* Aublet, 1775 under subgenus *Mecardonia* (Descole and Borsini 1954). More recently, Souza (1997) and Souza and Giuletti (2009) carried out some taxonomic modifications to Rossow's clasiffication. The demarcation of the genus is variable depending on the author consulted. Following Rossow's classification, the genus includes five species growing in Argentina: *M. flagellaris* (Chamisso & Schlechtendal, 1827), *M. grandiflora* (Bentham) Pennell, 1946, *M. procumbens* Small, 1903, *M. serpylloides* (Chamisso & Schlechtendal, 1891) and *M. tenella* (Chamisso & Schlechtendal, 1891). Recently, *M. kamogawae* Greppi & Hagiwara, 2011 was described by as an endemic species of Corrientes Province (Argentina).

Mecardonia has ornamental value because some cultivars developed from native species from Northern Argentina were recently introduced in the trade of ornamental plants (Greppi 2012). Therefore, researches on genetic improvement are carried out in this genus.

Cytological and cytogenetic studies have proved useful data for taxonomic and evolutionary analyses, which are widely used in processes of conventional or biotechnological genetic improvement (Poggio et al. 2004). Characters such as chromosome number, morphology, and meiotic behavior, as well as nuclear DNA content, have been used as taxonomic markers helping to circumscribe taxa and infer their relationships (Kron et al. 2007, Guerra 2008, Loureiro et al. 2010, Castro et al. 2012). At present, only two species of *Mecardonia* have been evaluated cytologically. Lewis et al. (1962) reported 2n = 42±2 for a Northamerican species *M. acuminata* (Walter, 1891) Small, 1903. Kaul (1969) determined 2n = 2x = 22 for *M. procumbens* Small, 1903 (as *M. dianthera* (Swartz 1900, Pennell 1946). Therefore, to increase the knowledge of *Mecardonia*, other species were cytologically analyzed in this study.

Nuclear DNA content, understood as genome size, is very variable across angiosperm, and has been revealed as an important character in biodiversity. In *Mecardonia* species there are no reported measurements of DNA content, but genome size variation has been explored in some genera of Plantaginaceae. DNA C-values are currently available for 204 species belonging to 18 genera of this family, including *Callitriche* Linnaeus, 1753, *Penstemon* Schmidel, 1762, *Plantago* Linnaeus, 1753 and *Veronica* Linnaeus, 1753 with a range of variation of 0.32–4,.63 pg (Albach and Greilhuber 2004, Broderick et al. 2011, Bennett and Leitch 2012, Wong and Murray 2012, Prančl et al. 2014, Meudt et al. 2015). Herein we used flow cytometry to estimate the nuclear DNA content in five species of *Mecardonia* for the first time. Additionally, we report original chromosome numbers of some of them. The results are discussed in relation to the taxonomy and evolution of the genus.

Materials and methods

We examined six populations from five species of *Mecardonia* collected in Argentina. Information about the studied material and the voucher specimens is provided in Table 1. Vouchers are deposited at the herbarium BAB of the Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina.

Mitosis analysis

Mitotic chromosome preparations were made from root meristems obtained from rooted stems. The roots were pretreated for about 4 h in 0.002 M 8-hydroxyquinoline solution at room temperature, fixed in 5:1 absolute alcohol/lactic acid, and then stained using Feulgen's technique. Permanent microscope slides were prepared by mounting in Euparal. In all samples at least 20 counts of 7–10 individuals were made to verify the observations.

Permanent microscope slides were examined and photographed using Zeiss Axioplan microscope Carl Zeiss with digital camera Canon Power Shot A 640.

Nuclear DNA measurements

DNA content (in picograms) was estimated by flow cytometry using fresh young leaves. The measurements were calculated from three replicates per individuals. In total we analyzed three individuals per species. The leaves of *Zea mays* Linnaeus, 1753 cv. 'CE-777' (2C = 5.43 pg., Doležel et al. 1998) were used as internal standard for almost all entities. While, *Hordeum vulgare* Linnaeus, 1753 cv. 'New Golden' (2C = 10.4 pg., Bennett

Table 1. *Mecardonia* species analyzed in this study, with their respective chromosome numbers (2n), locations, and voucher specimens.

	Species	2n	Location, voucher specimen
*	*M. flagellaris* (Cham. & Schldlt.) Rossow	2n = 2x = 22	Argentina. Entre Ríos, Dept. Federación, in front of complejo turístico Irupé. Greppi et al. 1411 (BAB).
		2n = 2x = 22	Argentina. Entre Ríos, Dept. Federación, complejo turístico Irupé Greppi et al. 1190 (BAB).
*	*M. grandiflora* (Benth.) Pennell	2n = 2x = 22	Argentina. Misiones, Dept. Guaraní, Ayo. Pepirí Miní o Yabotí. Greppi et al. 1189 (BAB).
*	*M. kamogawae* Greppi & Hagiwara	2n = 6x = 66	Argentina. Corrientes, Dept. Paso de los Libres, Paso de los Libres to national route 14, Greppi et al. 1081 (BAB).
	M. procumbens (Mill.) Small	2n = 2x = 22	Argentina. Córdoba. Dept. Unión, Monte Leña, national route 9, km 491, Greppi 681(BAB).
*	*Mecardonia* sp. n.	2n = 4x = 44	Argentina. Corrientes. Dept. Empedrado. Greppi and Hagiwara 1410 (BAB)

* First chromosome count.

and Leitch 2005) was used as the standard of hexaploid species. The selection of these internal standards was made since they are the common standards used in the laboratory where the flow cytometer is situated (Instituto de Floricultura, INTA Castelar, Buenos Aires, Argentina). To release nuclei from the cells, 0.5 cm^2 of leaf tissue of *Mecardonia* was chopped together with 0.5 cm^2 of leaf tissue of the internal standard in 0.5 ml buffer (High resolution DNA kit, Partec GmbH, Münster, Germany). Subsequently, 5 U ml^{-1} of RNAse were added and incubated for 2–5 min at room temperature. The suspension was filtered through a 30 µm nylon mesh. After this period, 1.5 ml of staining solution containing 1µg $µl^{-1}$ propidium iodide was added. Within 1 h of staining, measurements were performed with a CyFlow Ploidy Analyzer, Partec cytometer (green laser 532 nm, 30 mW). About 10,000 nuclei were measured for each sample.

The absolute value of DNA content (2C) of each sample was calculated by the formula: ($\bar{X}$ peak of sample × G1 DNA content (2C) of the standard)/$\bar{X}$ G1 peak of the standard (Doležel and Bartos 2005).

The monoploid genome size (1Cx) was calculated dividing the 2C-value by the ploidy level (Greilhuber et al. 2005).

Data analysis

The mean, standard deviation and the coefficient of variation of 2C-value were calculated for each species from three different individuals. Differences in 1Cx-value between species were tested by one-way analysis of variance (ANOVA) at a significance level of 5% (a = 0.05). The Tukey 5% post hoc test was used to test differences between each pair of species.

Pearson correlation coefficient was calculated to test whether the 2C–and 1Cx-values were related to chromosome number. Scatter plot was performed to evaluate the relationship between the 1Cx-values and the chromosome numbers (2n) of species. All statistical analyses were performed using the InfoStat software version 2013 (Di Rienzo et al. 2013).

Results

The chromosome numbers of six populations belonging to five species of *Mecardonia* were determined. The analyzed species and their chromosome numbers are given in Table 1. Four species were analyzed for the first time. The chromosome number observed in the remaining taxon is in agreement with previous studies. All species analysed shared the same base chromosome number (x = 11), and chromosome numbers ranged between 2n = 22 to 2n = 66. Of these, only three species were diploids: *M. flagellaris* (Fig. 1 A), *M. grandiflora* (Fig. 1 B) and *M. procumbens*. The remaining species were polyploids, *Mecardonia* sp. (Fig. 1 C) was tetraploid with 2n = 44 and *M. kamogawae* was hexaploid with 2n = 66 (Fig. 1 D).

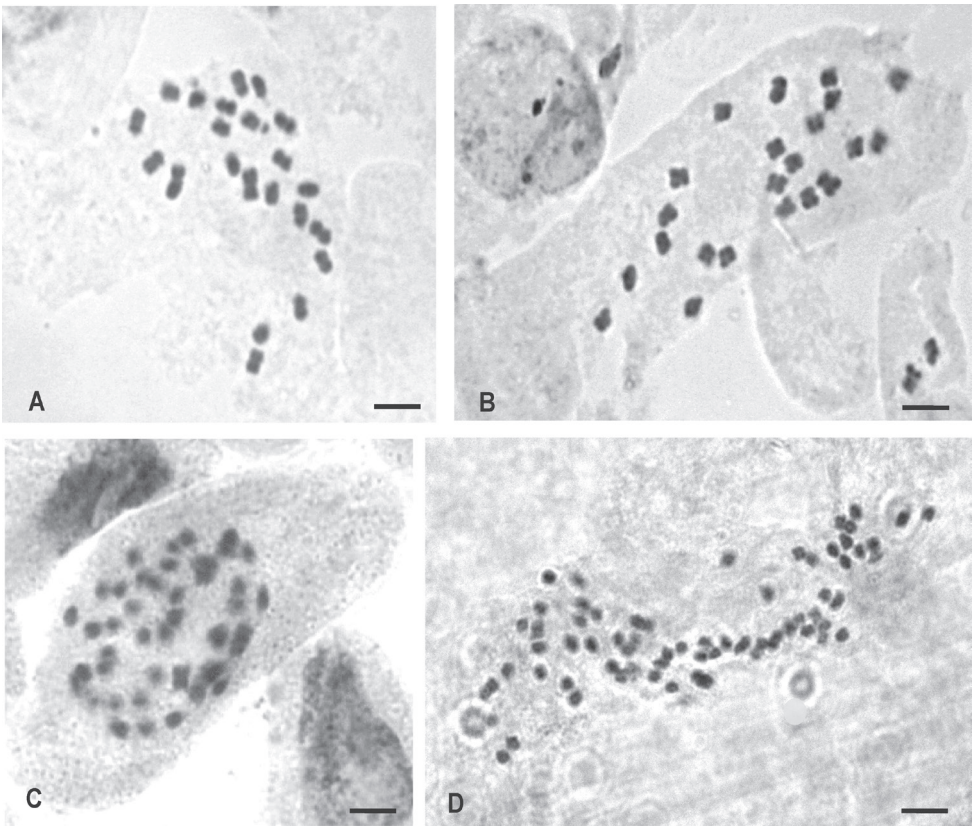

Figure 1. Somatic chromosomes of *Mecardonia* species. **A** *M. flagellaris:* 2n = 2x = 22 **B** *M. grandiflora:* 2n = 2x = 22 **C** *Mecardonia* sp. n.: 2n = 4x = 44 **D** *M. kamogawae*: 2n = 6x = 66. Bar = 5 μm.

Genomic DNA content

The DNA amounts determined for five species of *Mecardonia* are shown in Table 2. The flow cytometric measurements of all species and the standards resulted in well defined and sharp peaks. In all cases, the coefficients of variation were lower than 5% (Table 2), supporting the reliability of the flow cytometric assessments.

The 2C-values of the species here analyzed varied from 1.91 pg in *M. procumbens* (2x) to 5.29 pg in *M. kamogawae* (6x). The 2C-values were strongly and significantly correlated with chromosome number (r= 0.99; P= < 0.0001).

The 1Cx-values, which indicated the DNA content per genome, ranging from 1Cx= 0.88 pg in *M. kamogawae* to 1Cx= 1.03 pg in *M. flagellaris* (Table 2). The ANOVA showed significant differences for 1Cx-values (F= 357.52; P= <0.0001) between the species. The correlation between values of 1Cx and chromosome number was negative and significant (r= -0.86; P= <0.0001, Fig. 2).

Table 2. Chromosome number (2n), ploidy level, 2C-value (pg), CV (coefficient of variation), 1Cx-value (pg) of the *Mecardonia* species analyzed.

Species	Chromosome number (2n)	Ploidy level	2C (pg)	CV	1Cx (pg)
M. flagellaris	22	2x	2.06 ± 0.16	0.029	1.03[d]
M. grandiflora	22	2x	2.05 ± 0.08	0.010	1.02[d]
M. procumbens	22	2x	1.91 ± 0.06	0.012	0.95[c]
Mecardonia sp. n.	44	4x	3.71 ± 0.05	0.053	0.92[b]
M. kamogawae	66	6x	5.29 ± 0.10	0.061	0.88[a]
ANOVA					(F=357.52; P= <0.0001)

For ANOVA results, different lower-case letters indicate significant differences among species for mean values of each parameter at 5% level using Tukey's test.

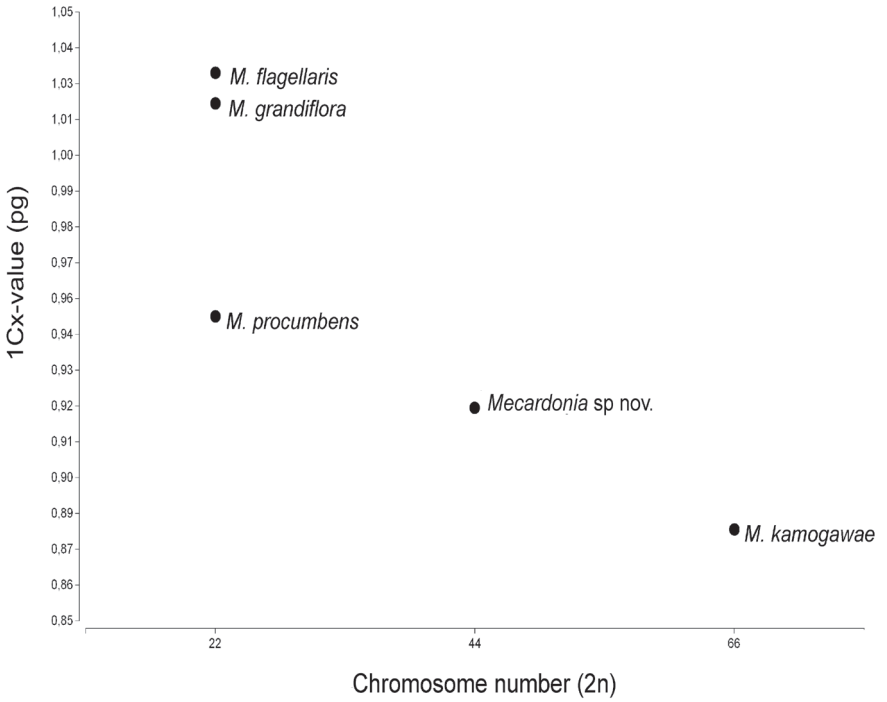

Figure 2. Scatter plot between 1Cx-value and chromosome number (2n).

Discussion

The chromosome number 2n = 22 found in *M. procumbens,* is consistent with the chromosome counts recorded in a previous cytological study (Kaul 1969). Sinha (1987) reported B chromosomes for this taxon; however, the populations here analyzed did not show these accessory chromosomes. Chromosome counts for *M. flagellaris* (2n = 22), *M. grandiflora* (2n = 22), *M. kamogawae* (2n = 66) and *Mecardonia* sp. (2n = 44) are

described here for the first time. Our results showed diploid and polyploid species for the genus. Polyploidization has long been recognized as an important process in plant evolution (Otto and Whitton 2000, Soltis et al. 2004). In Plantaginaceae, polyploidy is a common phenomenon occuring in many genera, such as *Antirrhinum* Linnaeus, 1753, *Chaenorhinum* (DC.) Reichenbach, 1828, *Cymbalaria* Hill, 1756, *Chelone* Linnaeus, 1753, *Digitalis* Linnaeus, 1753, *Linaria* Miller, 1754, *Plantago*, *Nuttallanthus* D.A. Sutton, 1988, *Stemodia* Linnaeus, 1759, *Veronica* (Hair 1966, Subramanian and Pondmudi 1987, Sosa and Seijo 2002, Sosa et al. 2009, 2011, Wolfe et al. 2002, Vargas et al. 2004, Murray et al. 2010, Castro et al. 2012, Wong and Murray 2012, Ranjbar and Nouri 2015). Our results evidenced the presence of multiple cytotypes in *Mecardonia*, hence suggesting polyploidy as a key driver of the evolution of the genus. The present analysis, in addition to previous chromosome number reports, revealed that the genus have exclusively the basic chromosome number of x = 11.

The interest on the study of genome size increased in the last decade. These studies focused on the use of genome size as a taxonomic marker (Castro et al. 2012, Angulo and Dematteis 2013, Galdeano et al. 2016) and on finding correlations between ecological and environmental variables and this character (Chalup et al. 2014, Vega and Dematteis 2015). However, there are still many families and genera being neglected, including *Mecardonia*, for which the present study is the first analysis of genome size for the genus. The estimates 2C- and 1C-values calculated for the species in this study are within ranges of variation found in Angiosperms and Plantaginaceae (Leitch and Bennett 2004, Meudt et al. 2015). Based on the available genome size data, *Mecardonia* falls into the categories "very small" genomes (2C = <2.8 pg) to "small" genomes (2C = <7.0 pg) according to values reported by Leitch et al. (1998) and Soltis et al. (2004).

The 2C-values of *Mecardonia* species revealed a positive and significant correlation with chromosome number (r = 0.99, P = < 0.0001). Therefore, in the genus there is a trend for increasing 2C–value with increasing ploidy level. On the other hand, the variation of 1Cx-values is negative and significantly (r = -0.86; P = <0.0001) correlated with chromosome number. Consequently, the values of 1Cx of the species decrease in inverse proportional to the ploidy level. Our data reflect that both polyploids (tetraploid 1Cx = 0.92 pg and hexaploid 1Cx = 0.88 pg) have lesser values of monoploid genome size than diploid species (mean of Cx = 1.00 pg). Many polyploid angiosperms undergo *genome downsizing* and so have smaller average genome sizes than their diploid relatives (Leitch and Bennett 2004, Leitch et al. 2008) and *Mecardonia* seems not to be an exception. Several studies have indicated that during polyploidization different balancing processes at genomic level occur which may promote variation in nuclear DNA content. These changes point towards a possible need for harmonization of genome and removal of some unnecessary genomic redundancies (Petrov 2001; Bennetzen et al. 2005, Pellicer et al. 2010). Mechanisms leading to a decrease in genome size in polyploids may include non-random elimination of chromosome- and genome-specific sequences (Ozkan et al. 2003, Shaked et al. 2001), illegitimate crossing over (Devos et al. 2002) or unbalanced deletion–insertion rates (Petrov 2001, 2002). Counterbalancing mechanisms are probably also involved to reduce the genetic and structural instabilities that accompany DNA loss (Pellicer et al. 2010).

Recently, Meudt et al. (2015) established a relationship between the genome downsizing with diversification in polyploid lineages of *Veronica* (Plantaginaceae), but they do not know how general this pattern might be or what causes it. Several hypotheses have been proposed to explain this relationship. Kraaijeveld (2010) suggested that organisms with small genomes have more stably inherited mutations, or a nucleotypic effect, in which organisms with small genomes and shorter genes have a general advantage as a result of faster replication and transcription. The genome size changes in *Mecardonia* are probably related with the diversification of the species. Further studies comparing this genus with the closest extant relative to determine what aspect of genome downsizing facilitate diversification are needed.

Taxonomic implications

The genus *Mecardonia* is currently under revision and some closely related species with intermediate morphological characteristics were found. It has been well documented in many plants that chromosome numbers and genome size can be used as extra taxonomic characters for discriminating between closely related taxa, helping to clarify the taxonomy of some species in problematic genus (Guerra 2008, Castro et al. 2012, Sosa and Dematteis 2014). For example, *Mercardonia* sp. is closely related to *M. flagellaris*. A detailed morphological analysis along with chromosome number here reported showed that it should be considered as different species. *Mecardonia* sp. is tetraploid with 2n = 44, while *M. flagellaris* is diploid with 2n = 22. Thus, both species differ in chromosome number and morphological features, such as aspect of plant, leaf shape, and trichome types of corolla. In addition, the new species has more restricted distribution to North of Argentina. However, *M. flagellaris* is expanding from Mato Grosso do Sul (Brazil) to Chubut (Argentina), arrived to Chile, Paraguay and Uruguay.

Another case is *M. kamogawae* that is morphologically related to *M. procumbens* from which it differs in the life-form, root types, leaf texture, and size of bracteoles and pedicels. Regarding chromosome number, *M. kamogawae* is hexaploid with 2n = 66, while *M. procumbens* is diploid with 2n = 22. Therefore, both species can be distinguished by morphological features, as well as by the chromosome number.

Mecardonia procumbens and *M. flagellaris* were diploids with 2n = 22. Although the chromosome number does not distinguish both species, differences in 2C-values were observed. *Mecardonia flagellaris* had higher value (2C = 2.06 pg.) than *Mecardonia procumbens* (2C = 1.91 pg.). D'Arcy (1979) and Souza (1997) placed *M. flagellaris* under the synonymy of *M. procumbens* by having similar morphological characteristics. We considered, however, that both species are morphologically distinct. *Mecardonia procumbens* differs by aspect of the plant, shape and length of leaf, and calyx pieces. Also, *M. procumbens* has a wider distribution as it extends from the South of the United States of America to the Argentine Northwest. However, *M. flagellaris* grow in Paraguay, South of Brasil, Uruguay and Northeast of Argentina.

Conclusion

The results of this study suggest that chromosome number is useful in distinguishing species of *Mecardonia*. The different ploidy levels of the taxa showed the importance of polyploidy in the evolution of the genus. The results here obtained combined with those reported previously confirm that the *Mecardonia* genus has basic number x = 11.

Regarding to the variation of genome size, decreases in DNA content have occurred during the evolution of genome size in the *Mecardonia* species.

Our results showed that differences in morphological features along with chromosome numbers and DNA content values support Rossow's criterion (1987).

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