CompCytogen 13(4): 325–338 (2019)
doi: 10.3897/CompCytogen.v13i4.37687
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Recurrent variation in the active NOR sites in the monkey frogs of the genus *Pithecopus* Cope, 1866 (Phyllomedusidae, Anura)

Joana Moura Gama[1], Camilla Borges Gazolla[1], Deborah Yasmin de Souza[1], Shirlei Maria Recco-Pimentel[2], Daniel Pacheco Bruschi[1]

1 *Programa de Pós-Graduação em Genética (PPG-GEN). Departamento de Genética, Universidade Federal do Paraná (UFPR), Setor de Biológicas, Av. Coronel Francisco H. Dos Santos, 100, 81531-980, Curitiba, PR, Brazil* **2** *Departamento de Biologia Estrutural and Funcional, Universidade Estadual de Campinas (UNICAMP), Cidade Universitária, 13083-863, Campinas, SP, Brazil*

Corresponding author: *Daniel Pacheco Bruschi* (danielpachecobruschi@gmail.com)

Academic editor: *L. Kupriyanova* | Received 25 June 2019 | Accepted 20 September 2019 | Published 21 October 2019

http://zoobank.org/4A76D35B-97A9-4824-B793-6A5B0553E77D

Citation: Gama JM, Gazolla CB, de Souza DY, Recco-Pimentel SM, Bruschi DP (2019) Recurrent variation in the active NOR sites in the monkey frogs of the genus *Pithecopus* Cope, 1866 (Phyllomedusidae, Anura). Comparative Cytogenetics 13(4): 325–338. https://doi.org/10.3897/CompCytogen.v13i4.37687

Abstract

Treefrogs of the genus *Pithecopus* Cope, 1866 exhibit expressive chromosomal homogeneity which contrasts with a high variation frequency of the nucleolus organizer region (NOR) related to the group. Currently, the genus contains eleven species and no chromosomal data are available on *P. palliatus* Peters, 1873, *P. ayeaye* Lutz, 1966 and *P. megacephalus* Miranda-Ribeiro, 1926. Here, we describe the karyotypes of these three species based on Giemsa staining, C-banding, silver impregnation and in situ hybridization (FISH). We were also analyze the evolutionary dynamic of the NOR-bearing chromosome in species of genus under a phylogenetic view. The results indicate that *P. palliatus*, *P. ayeaye*, and *P. megacephalus* have similar karyotypes, which are typical of the genus *Pithecopus*. In *P. palliatus* the NOR was detected in the pericentromeric region of pair 9p whereas in *P. ayeaye* and *P. megacephalus* we report cases of the multiple NOR sites in karyotypes. In *P. ayeaye* the NOR was detected in the pericentromeric region of pair 9p in both homologues and additional sites was detected in pairs 3q, 4p, and 8q, all confirmed by FISH experiments. Already in *P. megacephalus* the NOR sites were detected in pericentromeric region homologues of pair 8q and additionally in one chromosome of pair 13q. A comparative overview of all the *Pithecopus* karyotypes analyzed up to now indicates the recurrence of the NOR-bearing chromosome pairs and the position of the NORs sites on these chromosomes. We hypothesized that this feature is a result of a polymorphic condition present in the common ancestor of *Pithecopus*. In such case, the lineages derived from

polymorphic ancestor have reached fixation independently after divergence of lineages, resulting in a high level of homoplasy observed in this marker. Our findings help to fill the gaps in the understanding of the karyotype of the genus *Pithecopus* and reinforce the role of the evolutionary dynamics of the rDNA genes in karyotype diversification in this group.

Keywords
chromosomal evolution, *Pithecopus*

Introduction

Duellman et al. (2016) recognized the genus *Pithecopus* Cope, 1986 (the monkey frogs) as a distinct taxon from the genus *Phyllomedusa* Wagler, 1930, with which it had previously been synonymized, and Frost (2019) concluded that the genus contains 11 valid species. The genus is distributed throughout Central America from east of the Andes and northern Argentina (Frost 2019). Molecular inferences (Faivovich et al. 2010; Bruschi et al. 2014; Duellman et al. 2016; Haga et al. 2017) have recovered two well-supported clades in *Pithecopus* with a strong biogeographic component. One clade includes primarily lowland species (*Pithecopus azureus* Cope, 1862, *Pithecopus araguaius* Haga, Andrade, Bruschi, Recco-Pimentel & Giaretta, 2017, *Pithecopus hypochondrialis* Daudin, 1800, *Pithecopus palliatus* Peters, 1873 and *Pithecopus nordestinus* Caramaschi, 2006), while the second clade encompasses species that inhabit highland regions and plateaus (*Pithecopus ayeaye* Lutz, 1966, *Pithecopus centralis* Bokermann, 1965, *Pithecopus megacephalus* Miranda-Ribeiro, 1926, *Pithecopus oreades* Brandão, 2002, and *Pithecopus rusticus* Bruschi, Lucas, Garcia & Recco-Pimentel, 2014), with the exception of *Pithecopus rohdei* Mertens, 1926, which is distributed throughout the altitudinal gradient of the Brazilian Atlantic Forest. Interestingly, high levels of endemism (Magalhães et al. 2018) and cryptic diversity (Faivovich et al. 2010, Ramos et al. 2019) have been reported in the "highland" clade. Cytogenetic data have already indicated interpopulational variability in *P. rohdei* (Barth et al. 2009, Paiva et al. 2009, Bruschi et al. 2012), which could be the first step to speciation. Population genetic divergence was recently confirmed by a molecular analysis using nuclear and mitochondrial markers (Ramos et al. 2019), which emphasizes the potential contribution of karyotype data as complementary evidence for the identification of cryptic diversity.

No published chromosomal data are available on *P. palliatus*, *P. ayeaye*, and *P. megacephalus*. *Pithecopus palliatus* is a member of the lowland clade (Faivovich et al. 2010, Duellman et al. 2016), and inhabits temporary pools in the tropical rainforests of the upper Amazon basin in Ecuador, Peru, northern Bolivia and western Brazil (Frost 2019). By contrast, *P. ayeaye* and *P. megacephalus* have more restricted geographic ranges in southeastern Brazil, where they form small, highly structured and isolated populations with a discontinuous distribution in mountaintop isolates ("sky islands") in highland Rockfield ("*campo rupestre*") ecosystems (Magalhães et al. 2018, Ramos et al. 2018).

Pithecopus ayeaye is endemic to high altitudes in southeastern Brazil. This species is listed as critically endangered (CR) by the International Union for Conservation of

Nature, IUCN (Caramaschi et al. 2016), although reports of new occurrence localities (Araújo et al. 2007, Baêta et al. 2009) led to the removal of the species from the Brazilian List of Endangered Species (ICMBio 2014). Magalhães et al. (2018) recently identified three different evolutionary significant units (ESUs) of *P. ayeaye* in distinct *campo rupestre* ecosystems using multilocus DNA sequences and emphasized the need for the inclusion of the genetic profile of this species in the definition of regional conservation policies.

Pithecopus megacephalus occurs at high elevations (above 800 m a.s.l.) in the *campo rupestre* systems of the Southern Espinhaço Mountain Range (Oliveira et al. 2012). Using multilocus analyses, Ramos et al. (2018) found considerable genetic structuring among three *P. megacephalus* populations from different "sky islands" in the Espinhaço Range, and evidence of low gene flow among these populations.

Here, we advance our understanding of the cytogenetics of the genus *Pithecopus* and compile the karyotype data available on the genus to discuss its chromosomal features from a phylogenetic perspective.

Material and methods

Biological samples

We analyzed populations of *P. ayeaye*, *P. megacephalus* and *P. palliatus* sampled in Brazilian localities (Table 1). Specimen collection was authorized by the Biodiversity Information System (SISBIO) of the Chico Mendes Institute for Biodiversity Conservation (ICMBio), through license 45183-3. Voucher specimens were deposited in the "Prof. Dr. Adão José Cardoso" Museum of Zoology (**ZUEC**) at University of Campinas (**UNICAMP**), in São Paulo state, Brazil.

Cytogenetic analyses

Metaphase cells were obtained from the intestines and testes of animals previously treated with 2% colchicine (*Sigma – Aldrich*; 0.02 ml per 1 g of body weight), following procedures modified from King and Rofe (1976) and Schmid (1978). Prior to the removal of the organs, the animals were anesthetized profoundly with 5% Lidocaine,

Table 1. Details of the *Pithecopus* species and specimens sampled for the cytogenetic analyses presented in this study.

Species	Number of specimens	Locality/State[1]	Geographic coordinates	ZUEC[2] number
P. ayeaye	03 ♂	Brumadinho/MG	20°29'S, 44°19'W	16403–16405
P. megacephalus	03 ♂	Santana do Riacho/MG	19°10'S, 43°42'W	In the accept
P. palliatus	12 ♂ + 3 ♀	Boca do Acre/AM	8°44'S, 67°23'W	17037–17051

[1]AM = Amazonas; MG = Minas Gerais; [2]ZUEC = "Prof. Dr. Adão Cardoso" Museum of Zoology at University of Campinas (UNICAMP).

applied to the skin, to minimize suffering, as recommended by the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists (available at http//www.asih.org/publications). The chromosome preparations were stained with 10% Giemsa and C-banded (Sumner 1972). The C-banded chromosomes of *P. ayeaye* were stained with fluorochrome AT-specific DAPI and GC-specific Mytramycin (MM).

The nucleolus organizer regions (NOR) were revealed by the silver nitrate impregnation technique (Ag-NOR) following Howell and Black (1980). Fluorescent *in situ* hybridization (FISH) was used to confirm the presence of multiple NORs in the *P. ayeaye* karyotype. The FISH assays followed the protocol of Viegas-Péquignot (1992). The 28S rDNA probe were isolated from *Pithecopus hypochondriasis*, cloned and sequenced by Bruschi et al. (2012) and sequence is available in GenBank database under accession number HM639985. The probe was labeled with digoxigenin 11-dUTP (Roche Applied Science). The hybridized signals were detected using an anti-digoxigenin antibody conjugated with rhodamine (600 ng/mL) and counterstained with 0.5 mg/ml of DAPI.

We analyzed twenty metaphase plates per individual for each of the applied methods. The metaphases were photographed under an Olympus microscope and analyzed using the Image Pro-Plus software, version 4 (Media Cybernetics, Bethesda, MD, USA). The chromosomes were ranked and classified according to the scheme of Green and Sessions (1991).

Results

All three species analyzed here had a diploid number of 26 chromosomes. The chromosomal complement of all three species (Figs 1A, 2A, and 3A) consisted of the four metacentric pairs (1, 4, 8 and 11), six submetacentric pairs (2, 3, 5, 6, 12 and 13), and three subtelocentric pairs (7, 9 and 10). A secondary constriction was detected in the pericentromeric region of the short arm of the homologs of pair 9 in *P. ayeaye* and *P. palliatus*, although in the *P. megacephalus* karyotype, the secondary constriction was observed in the pericentromeric region of the long arm of the homologs of pair 8. Additional secondary constrictions were observed heterozygously in the interstitial region of the long arms of chromosomes 3 and 8 in all the individuals analyzed, as well as in the pericentromeric region of the short arm of chromosome 4 (Fig. 1A).

The heterochromatin revealed by the C-banding was arranged in centromeric blocks in the karyotypes of all three species studied here (Figs 1B, 2B and 3B). In *P. ayeaye*, we detected C-positive bands in the pericentromeric region of the long arm of pairs 6 and 8, and in the short arm of pair 11 (Fig. 1B). In *P. ayeaye* karyotype C-banded chromosomes were sequentially stained with DAPI and MM fluorochromes to reveal the A:T and C:G richness and resulted in brilliant signals in regions coincident with heterochromatic blocks detected by C-banding (Fig. 1C). We also detected MM-positive fluorescence signals that coincided with the secondary constrictions observed by conventional staining (Fig. 1E).

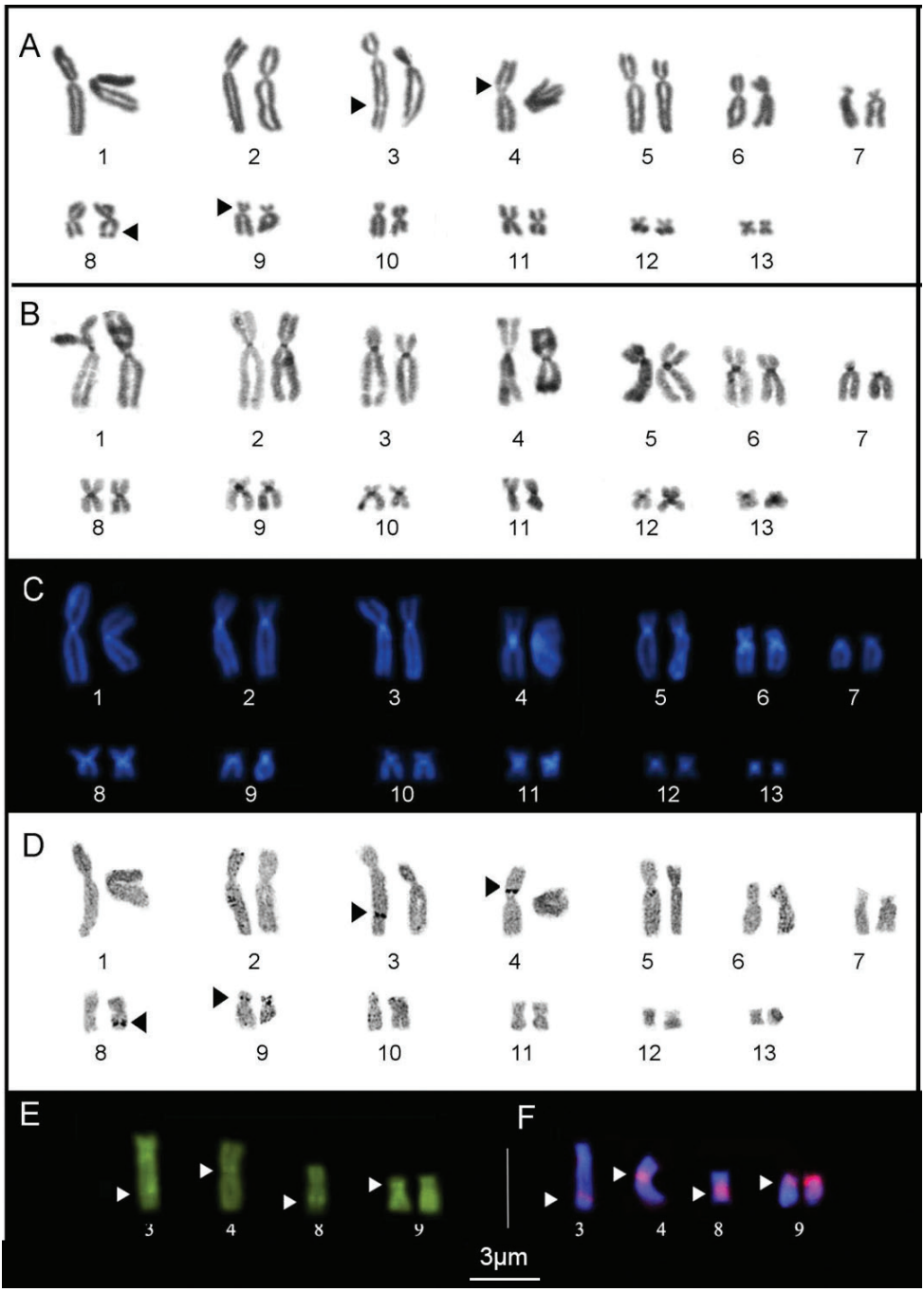


Figure 1. Karyotype of *P. ayeaye* prepared by conventional Giemsa staining (**A**) C- banding (**B**) Ag-NOR (**D**) DAPI staining after C-banding (**C**). Chromosomes submitted to Mytramicim (MM) (**E**) and FISH experiments with a nucleolar 28S rDNA probe (**F**). The arrow indicates indicates secondary constrictions; the arrowheads indicate multiple NOR site.

In all the karyotypes, the secondary constrictions revealed by conventional Giemsa staining coincided with the NOR sites detected by the Ag-NOR method. In *P. ayeaye* the NORs were detected in the pericentromeric region of the short arm of the both homologs of pair 9 (Fig. 1D), besides of the additional sites in the interstitial region of the long arm of chromosomes 3 and 8 and in pericentromeric region of the short arm of chromosome 4 (Fig. 1D). The additional sites (pairs 3, 4 and 8) were found in all the individuals analyzed, invariably in the heterozygous condition. The FISH assays realized in *P. ayeaye* confirmed additional NOR sites in the pair 9 (Fig. 1F), which are MM-positive, as is typical of the anuran chromosome. In the *P. palliatus* the NOR sites also were detected in the pericentromeric region of the short arm of the homologs of pair 9 (Fig. 2C). Already in *P. megacephalus* the NORs were located in the pericentromeric region of the long arm of the homologs of pair 8 (Fig. 3C) and additionally in one homologue of pair 13 (Fig. 3C).

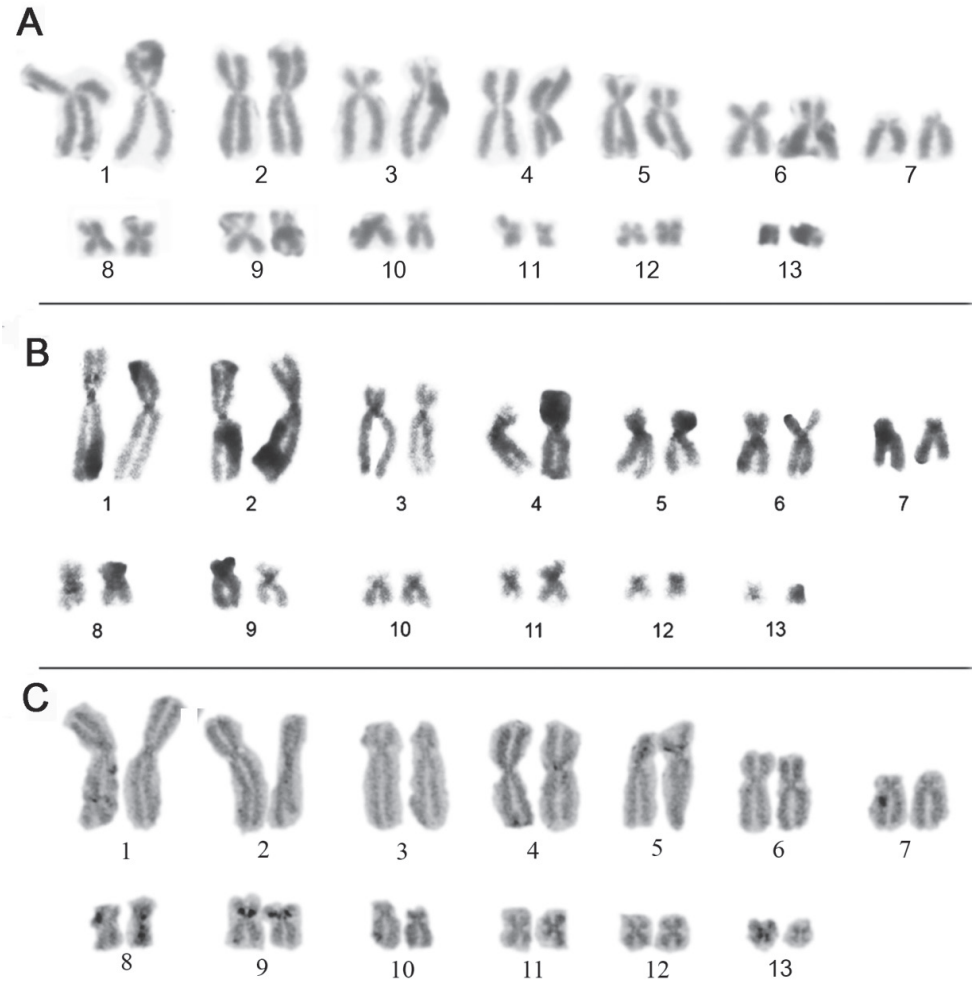


Figure 2. Karyotype of *P. palliatus* prepared by conventional Giemsa staining (**A**) C-banding (**B**) and Ag-NOR method (**C**). Secondary constrictions observed coincided with the Ag-NOR sites (**C**).

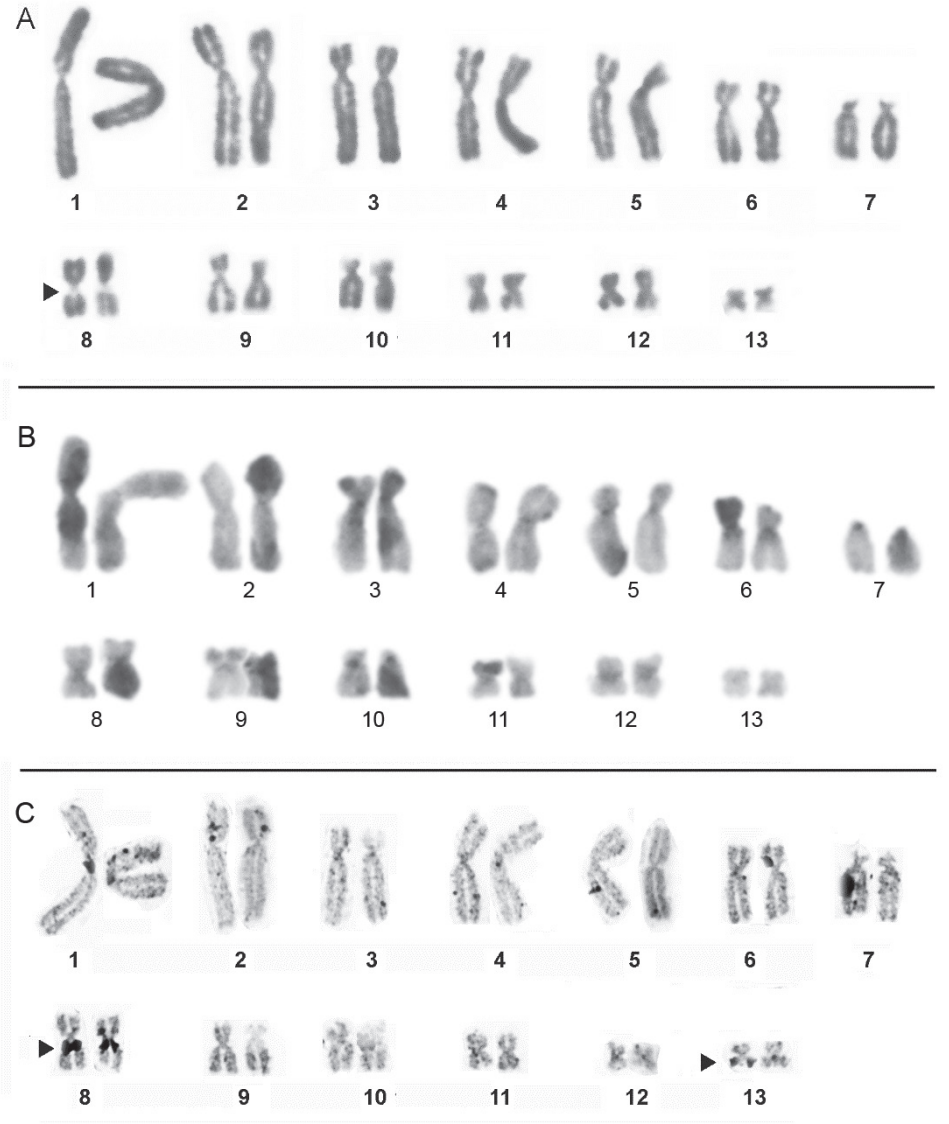


Figure 3. Karyotype of *P. megacephalus* prepared by conventional Giemsa staining (**A**) C-banding (**B**) and Ag-NOR method (**C**). The arrow indicates secondary constrictions in the pair 8 correspond to NOR sites. Note the additional NOR in one homologue of pair 13.

Discussion

Karyotype conservation in the subfamily Phyllomedusinae

The analysis of the chromosomes of the three *Pithecopus* species, presented here, reinforces the conclusion that the macrostructure of the karyotypes of the members of this genus (diploid number and chromosome morphology) is highly conserved (Barth et

al. 2009; Bruschi et al. 2013, Bruschi et al. 2014). The extreme homogeneity of these karyotypes allows for the proposal of a number of different hypotheses on the interspecific chromosomal homologies found in the genus. To begin with, the presence of 26 chromosomes in *Pithecopus* represents the plesiomorphic condition in the subfamily Phyllomedusinae (Schmid et al. 1995, Morand and Hernando 1997, Gruber et al. 2013, Bruschi et al. 2014b, Barth et al. 2014, Schmid et al. 2018). Currently, this subfamily assemble 65 species distributed in eight genus (*Agalychnis* Cope, 1864, *Callimedusa* Duellman, Marion & Hedges, 2016, *Cruziohyla* Faivovich, Haddad, Garcia, Frost, Campbell & Wheeler, 2005, *Hylomantis* Peters, 1873 "1872", *Phasmahyla* Cruz, 1991, *Phrynomedusa* Miranda-Ribeiro, 1923, *Phyllomedusa* Wagler, 1830, *Pithecopus* Cope, 1866) and only 22 species have been karyotyped (Perkins et al. 2019). The karyotype of the phyllomedusines is highly conserved (Barth et al. 2013; Gruber et al. 2013; Bruschi et al. 2014; Schmid et al. 2018). The unique variation in chromosome morphology found in the species of the genus *Phyllomedusa* karyotype, in particular in the *P. tarsius* group (*P. camba* De la Riva, 1999, *P. tarsius* Cope, 1868, *P. neildi* Barrio-Amorós, 2006, and *P. trinitatis* Mertens, 1926), with three telocentric chromosome pairs (pairs 7, 10, and 12), may represent a possible synapomorphy in this group (Bruschi et al. 2014b).

Like the other species of the genus *Pithecopus* (Bruschi et al. 2012, 2013, 2014), the heterochromatin in *P. palliatus* and *P. ayeaye* is found essentially in the centromeric regions of the all chromosomes, with no distinct band or other marking that permits the differentiation of the karyotypes. The only *Pithecopus* species that can be distinguished based on its C-banding pattern is *P. nordestinus*, which is characterized by a considerable accumulation of heterochromatin, primarily in centromeric regions, extending to the pericentromeric portions of both arms of the chromosome 9 (Bruschi et al. 2012), which is a characteristic of this species.

Multiple rDNA sites in the karyotype of Pithecopus

The extreme chromosomal conservation observed in the *Pithecopus* species contrasts with its considerable inter- and intrapopulation variation in the chromosomal pairs that carry the 28S rDNA gene clusters. In the present study, two new cases of multiple NOR sites were recorded in the genus *Pithecopus*, in the karyotypes of *P. ayeaye* and *P. megacephalus*. However, a comparative overview of all the *Pithecopus* karyotypes analyzed up to now indicates the recurrence of the NOR-bearing chromosome pairs, and the position of the NORs on these chromosomes, in particular in pairs 3, 4, 8, 9, 11, and 13. Multiple NORs are common in this genus, and have been recorded in practically all the species (Morand and Hernando 1997, Barth et al. 2009, 2013, Paiva et al. 2009, Bruschi et al. 2012, 2013 and present study). In most cases, the karyotypes shown a NOR-bearing pair (homozygosis), detected in all specimens of population whereas the additional NOR-sites occurred in heterozygous and polymorphic condition (Morand and Hernando 1997; Barth et al. 2009, 2013; Paiva et al. 2009; present study). Although intrapopulation variation in the number of NORs is a frequent

condition in anuran species, the configuration found in *Pithecopus* reflects the unique evolutionary dynamics of this chromosomal marker.

The interesting feature of the genus *Pithecopus* is that when the polymorphic condition is recorded in the different species, it to be located in the same chromosomes and NOR positions. Thus, it is difficult to recognize the phylogenetic signal of this marker for the application of a parsimonious evolutionary analysis. Here, we suggest two possible scenarios to explain this variation: (i) the NOR in pair 9q represents the plesiomorphic condition in *Pithecopus*, with subsequent rearrangements resulting in the repositioning of the NOR to pair 8 in *P. azureus* and in the ancestor of *P. hypochondrialis* + *P. araguaius*, with the NOR in pair 8q also representing an autapomorphy in *P. megacephalus*. Subsequent independent events of the loss or gain of rDNA would have resulted in the appearance of the rDNA sites in chromosomes 3, 4, 7, 11, and 13 in the species with the polymorphic condition. In this context, the NOR in pair 9q should be present in the most recent common ancestor (TMRC) of the *Pithecopus* genus (see Figure 4). Alternatively (ii) an ancestral polymorphism would be the source of the extreme variation in the NOR found in this genus.

While the first of these explanations depends on high rates of loss/gain of copies of the rDNA in the genomes of the species, the second hypothesis would depend on the recurrence of the same pairs as the NOR-bearing chromosomes in the different species in the genus *Pithecopus* (see Fig. 4), which would be consistent with the idea of an ancestral polymorphism as the source of the complex scenario observed in the present day. If this hypothesis is accepted, any attempt to trace an evolutionary pathway from this chromosomal marker will inevitably generate a high degree of homoplasy in the phylogenetic inferences, which is typical of the multiple paralogous copies of this marker in the genome (Robinson et al. 2008).

Assuming the ancestral polymorphism hypothesis, the total reproductive isolation of each evolutionary lineage would have resulted in the fixation of the principal active NOR sites in at least one pair of homologous chromosomes (the homozygous condition), which would permit the degeneration of the other sites, or at least the reduction or silencing of their expression. In *P. nordestinus* and *P. ayaye*, respectively, the position of the active NOR detected by Ag-NOR was confirmed by the FISH using 18S/28S rDNA probes (Barth et al. 2013 and present study), which is consistent with the observation of a homozygous principal pair, together with additional, heterozygous sites, that bear the rDNA gene. While a cell requires at least one cluster of active rDNA to satisfy its demand for ribosomal RNAs, there does not appear to be any restriction on the maximum number of copies in a genome (Cazaux et al. 2011). The case of the species of the genus *Mus* is an example of this, in which 1–21 clusters of the rDNA are found in a given karyotype (Cazaux et al. 2011). Given this, not all rDNA sites are being expressed in the cells, and some may be silenced or even lost during the diversification of the different lineages (e.g., Derjusheva et al. 1998; Santos et al. 2002). The number, chromosomal distribution and inheritance of NOR are an important character to genome comparison in Anuran genomes, as observed in water frogs *Pelophylax lessonae* Camerano, 1882, *Pelophylax ridibundus* Pallas, 1771 and in their

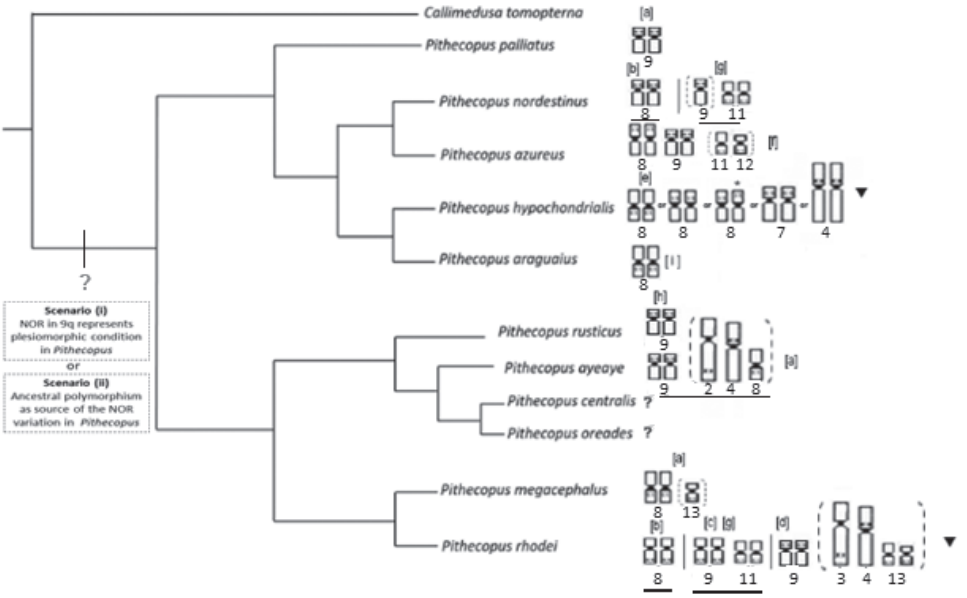


Figure 4. The active NOR-bearing chromosomes found in the karyotypes of the *Pithecopus* species and the broader phylogenetic context of the genus. Two possible scenarios to explain NOR variation are shown in inset (see details in discussion). The phylogenetic arrangement was reconstructed from Duellman et al. (2016) and Haga et al. (2017). Chromosomes within brackets present additional NOR sites in the polymorphic condition within the population. The NOR site of the underlined pairs (black lines) was confirmed by FISH using the rDNA probe. Species with unknown karyotypes are indicated by the "?" symbol. Species suspected to contain cryptic diversity are represented by triangles. The letters within brackets indicate the following references: [a] Present study; [b] Bruschi et al. (2012); [c] Barth et al. (2009); [d] Paiva et al. (2009); [e] Bruschi et al. (2013); [f] Morand and Hernando (1997); [g] Barth et al. (2013); [h] Bruschi et al. (2014a); [i] karyotype described by Bruschi et al. (2013) and recognized as a new species by Haga et al. (2017). The asterisks (*) represent the heteromorphic condition resulting from the paracentric inversion found in the Alta Floresta population by Bruschi et al. (2013).

natural hybrids (*Pelophylax esculentus* Fitzinger, 1843) (Zalesna et al. 2017). In this case, active NOR variability are relationships with ploidy level in hybrids and denote the intragenomic behavior of this chromosomal marker.

One particularly illustrative example of this scenario is the variation in *P. hypochondrialis* found by Bruschi et al. (2013), who detected a pronounced population structure based on the analysis of fragments of mitochondrial and nuclear genes. This study found clear differences among populations, and geographical coherence between the clades recuperated by phylogenetic analysis and the NOR-bearing chromosome, which indicates the possible fixation of distinct chromosomes that bear the transcriptionally-active rDNA genes in populations connected by little gene flow. The principal NOR-bearing chromosomes in this species were pairs 4, 7, and 8, in addition to a polymorphic population with extra sites in pairs 3 and 4. This regional chromosomal variation

may reflect the role of population dynamics in the fixation of the active NOR from the pool of rDNA sites present in the ancestral genome. Once fixed one chromosome pair with NOR site at a population level, the additional copies of rDNA may either (i) become free of selective pressure and degenerate through stochastic events which would account for the absence of hybridization signals in the FISH experiment or (ii) remain silenced in genome and for consequence undetectable by Ag-NOR method. It is important to note here that Bruschi et al. (2013) did not design the experiment to evaluate these specific questions.

The results of the present study also indicate clearly a predominance of rDNA sites located in the pericentromeric and/or subterminal regions of the chromosomes (Fig. 4). Similar results have been obtained for many examples in Anuran karyotypes, as observed in species of the hylid tribe Cophomantini (see Ferro et al. 2018) or in species of the *Agalychnis* Cope, 1864 and *Scinax* Wagner, 1830 genus (Schmid et al. 2018), for example. A number of studies indicate that the NOR-bearing sites in the chromosomes act as hotspots of chromosomal rearrangement (Cazaux et al. 2011). The mechanisms recognized traditionally include the occurrence of unequal crossovers, ectopic recombination, and invasion by mobile genetic elements, all of which have been invoked to account for the observed variation and dispersal of the copies of the NOR in the genome (Poletto et al. 2010; Cazaux et al. 2011; Silva et al. 2013). The evidence points to the possible occurrence of intrachromosomal rearrangements (peri- and paracentric inversions) as the source of the variation in the position of the NOR, such as the distinct positions (8p and 8q) that the rDNA site occupies in the homologs of pair 8 in the different populations of *P. hypochondrialis* (see Fig. 4), for example.

Conclusions

Our findings help to fill the gaps in the knowledge of the karyotype variability of the genus *Pithecopus* and constitute a good example of the complex role of the rDNA genes in karyotype evolution. Ours results reveals that evolutionary dynamics of the NOR sites in genus and its potential as hotspot of chromosomal rearrangements, which implies that it may be a fundamental feature of chromosomal evolution in the genome of *Pithecopus*.

Acknowledgements

We thank the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP 2016/07717-6), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/PROAP – Finance Code 001) for the scholarships provided to JGM, CBG and DYS. We thank the Multi-User Confocal Microscopy Center of the Federal University of Paraná for the capture of the images included in this study.

Reference

Araújo CO, Condez TH, Haddad CFB (2007) Amphibia, Anura, *Phyllomedusa ayeaye* (B. Lutz, 1966): Distribution extension, new state record, and geographic distribution map. Check List 3(2): 156–158. https://doi.org/10.15560/3.2.156

Barth A, Solé M, Costa MA (2009) Chromosome Polymorphism in *Phyllomedusa rohdei* Populations (Anura: Hylidae). Journal of Herpetology 43(4): 676–679. https://doi.org/10.1670/08-210.1

Barth A, Souza VA, Solé M, Costa MA (2013) Molecular cytogenetics of nucleolar organizer regions in *Phyllomedusa* and *Phasmahyla* species (Hylidae, Phyllomedusinae): a cytotaxonomic contribution. Genetics and Molecular Research 12(3): 2400–2408. https://doi.org/10.4238/2013.July.15.3

Barth A, Vences M, Solé M, Costa MA (2014) Molecular cytogenetics and phylogenetic analysis of Brazilian leaf frog species of the genera *Phyllomedusa* and *Phasmahyla* species (Hylidae, Phyllomedusinae). Canadian Journal of Zoology 92(9): 795–802. https://doi.org/10.1139/cjz-2013-0301

Baêta D, Caramaschi U, Cruz CAG, Pombal Jr JP (2009) *Phyllomedusa itacolom*i Caramaschi, Cruz & Feio, 2006, a junior synonym of *Phyllomedusa ayeaye* (B. lutz, 1966) (Hylidae, Phyllomedusinae). Zootaxa 2226: 58–65. https://doi.org/10.11646/zootaxa.2226.1.5

Bruschi DP, Lucas EM, Garcia PCA, Recco-Pimentel S (2014a) Molecular and morphological evidence reveals a new species in the *Phyllomedusa hypochondrialis* group (Hylidae, Phyllomedusinae) from the Atlantic forest of the highlands of southern Brazil. PlosOne 9(8): e105608. https://doi.org/10.1371/journal.pone.0105608

Bruschi DP, Busin CS, Siqueira S, Recco-Pimentel SM (2012) Cytogenetic analysis of two species in the *Phyllomedusa hypochondrialis* group (Anura, Hylidae). Hereditas 149(1):34–30. https://doi.org/10.1111/j.1601-5223.2010.02236.x

Bruschi DP, Rivera M, Lima AP, Zúñiga AB, Recco-Pimentel SM (2014b) Interstitial Telomeric Sequences (ITS) and major rDNA mapping reveal insights into the karyotypical evolution of Neotropical leaf frogs species (Phyllomedusa, Hylidae, Anura). Molecular Cytogenetics 7(22): 1–12. https://doi.org/10.1186/1755-8166-7-22

Bruschi DP, Busin CS, Toledo LF, Vasconcellos GA, Strussmann C, Weber LN, Lima AP, Lima JD, Recco-Pimentel SM (2013) Evaluation of the taxonomic status of populations assigned to *Phyllomedusa hypochondrialis* (Anura, Hylidae, Phyllomedusinae) based on molecular, chromosomal and morphological approach. BMC Genetics 14(70): 1–14. https://doi.org/10.1186/1471-2156-14-70

Caramaschi U, Cruz CAG, Lima R, Brandão R (2016) *Pithecopus ayeaye*. The IUCN Red List of Threatened Species. http://doi.org/10.2305/IUCN.UK.2016-3.RLTS.T55839A107295713.en [Accessed 21 June 2019]

Cazaux B, Catalan J, Veyrunes F, Douzery EJP, Britton-Davidian J (2011) Are ribosomal DNA clusters rearrangement hotspots? A case study in the genus *Mus* (Rodentia, Muridae). BMC Evolutionary Biology 11(124): 1–14. https://doi.org/10.1186/1471-2148-11-124

Derjusheva SE, Loginova JA, Parada R, Chiryaeva OG, Smirnov AF, Jaszczak K (1998) The comparative analysis of NOR polymorphism detected by FISH and Ag-staining on horse chromosomes. Caryologia 51(1): 1–11. https://doi.org/10.1080/00087114.1998.10589115

Duellman WE, Marion AB, Hedges SB (2016) Phylogenetics, classification, and biogeography of the treefrogs (Amphibia: Anura: Arboranae). Zootaxa 4101(1): 1–109. https://doi.org/10.11646/zootaxa.4104.1.1

Faivovich J, Haddad CFB, Baêta D, Jungfer KH, Álvares FR, Brandão RA, Sheil C, Barrientos LS, Barrio-Amorós CL, Cruz CAG, Wheeler WC (2010) The phylogenetic relationships of the charismatic poster frogs *Phyllomedusinae* (Anura, Hylidae). Cladistics 26(3): 227–261. https://doi.org/10.1111/j.1096-0031.2009.00287.x

Ferro JM, Cardozo DE, Suárez P, Boeris JM, Blasco-Zúñiga A, Barbero G, Gomes A, Gazoni T, Costa W, Nagamachi CY, Rivera M, Parise-Maltempi PP, Wiley JE, Pieczarka JC, Haddad CFB, Faivovich J, Baldo D (2018) Chromosome evolution in Cophomantini (Amphibia, Anura, Hylidae). PlosOne 13(2): eO192861. https://doi.org/10.1371/journal.pone.0192861

Frost DR (2019) Amphibian species of the world: an online reference. Version 6.0 American Museum of Natural History, New York, USA. http://research.amnh.org/herpetology/amphibia/index.html [Accessed 18 June, 2019]

Green DM, Sessions SK (1991) Nomenclature for chromosomes. In: Green DM, Sessions SK (Eds) Academic Press. Amphibian cytogenetics and evolution. San Diego, 431–432. https://doi.org/10.1016/B978-0-12-297880-7.50021-4

Gruber SL, Silva ANZ, Haddad CFB, Kasahara S (2013) Cytogenetic analysis of *Phyllomedusa distincta* Lutz, 1950 (2n=2×=26), *P. tetraploidea* Pombal and Haddad, 1992 (2n=4×=52), and their natural triploid hybrids (2n=3×=39) (Anura, Hylidae, Phyllomedusinae). BMC Genetics 14(75): 1–13. https://doi.org/10.1186/1471-2156-14-75

Haga IA, Andrade FS, Bruschi DP, Recco-Pimentel SM, Giaretta AA (2017) Unrevealing the leaf frogs Cerrado diversity: A new species of *Pithecopus* (Anura, Arboranae, Phyllomedusinae) from the Mato Grosso state, Brazil. PlosOne 12(9): e0184631. https://doi.org/10.1371/journal.pone.0184631

Howell WM, Black DA (1980) Controlled silver staining of nucleolar organizer regions with a protective colloidal developer a 1-step method. Experientia 36: 1014–1015. https://doi.org/10.1007/BF01953855

King M, Rofe R (1976) Karyotypic variation in the Australian gekko *Phyllodactylus marmoratus* (Gray) (Gekkonidae: Reptilia). Chromosoma 54: 75–87. https://doi.org/10.1007/BF00331835

Magalhães RF, Rocha PC, Santos FR, Strüssmann C, Giaretta AA (2018) Integrative taxonomy helps to assess the extinction risk of anuran species. Journal for Nature Conservation 45: 1–10. https://doi.org/10.1016/j.jnc.2018.07.001

Morand M, Hernando A (1997) Localización cromossômica de genes ribosomales activos em *Phyllomedusa hypochondrialis* y *P. sauvagii* (Anura: hylidae). Cuadernos de Herpetologia 11(1–2): 31–36. http://sedici.unlp.edu.ar/handle/10915/6276

Oliveira FFR, Nogueira PAG, Eterovick PC (2012) Natural history of *Phyllomedusa megacephala* (Miranda-Ribeiro, 1926) (Anura:Hylidae) in southeastern Brazil, with descriptions of its breeding biology and male territorial behavior. Journal of Natural History 46(1–2): 117–129. https://doi.org/10.1080/00222933.2011.626127

Paiva CR, Nascimento J, Silva APZ, Bernarde OS, Ananias F (2009) Karyotypes and Ag-NORs in *Phyllomedusa camba* De La Riva, and *P. rhodei* Mertens, 1926 (Anura, Hylidae, Phyllomedusinae): cytotaxonomic considerations. Italian Journal of Zoology 77(1): 116–121. https://doi.org/10.1080/11250000903187585

Perkins RD, Gamboa JR, Jonika MM, Lo J, Shum A, Adams RH, Blackmon H (2019) The Amphibian Karyotype Database. Chromosome Research. https://doi.org/10.1007/s10577-019-09613-1

Poletto AB, Ferreira IA, Martins C (2010) The B chromosomes of the African cichlid fish *Haplochromis obliquidens* harbout 18S rRNA gene copies. BMC Genetics 11(1): 1–8. https://doi.org/10.1186/1471-2156-11-1

Ramos EKS, Magalhães RF, Marques NCS, Baêta D, Garcia PCA, Santos FR (2019) Cryptic diversity in Brazilian endemic monkey frogs (Hylidae, Phyllomedusinae, *Pithecopus*) revealed by multispecies coalescents and integrative approaches. Molecular Phylogenetics and Evolution 132: 105–116. https://doi.org/10.1016/j.ympev.2018.11.022

Ramos EKS, Magalhães RF, Sari HER, Rosa AHB, Garcia PCA, Santos FR (2018) Population genetics and distribution data reveal conservation concerns to the sky island. Endemic *Pithecopus megacephalus* (Anura, Phyllomedusidae). Conservation Genetics 19(1): 99–110. https://doi.org/10.1007/s10592-017-1013-z

Robinson TJ, Ruiz-Herrera A, Avise JC (2008) Hemiplasy and homoplasy in the karyotypic phylogenies of mammals. Proceedings of the National Academy of Sciences of the United States of America 105(38): 14477–14481. https://doi.org/10.1073/pnas.0807433105

Santos N, Fagundes V, Yonenaga-Yassuda Y, Souza MJ (2002) Localization of rDNA genes in Phyllostomidae bates reveals silent NORs in *Artibeus cinereus*. Hereditas 136: 137–143. https://doi.org/10.1034/j.1601-5223.2002.1360208.x

Schmid M (1978) Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. Chromosoma 66(1): 361–388. https://doi.org/10.1007/BF00328536

Schmid M, Borgat JP, Hedges SB (2018) The Arboranan frogs: evolution, Biology and Cytogenetics. Cytogenetics Genome Research 155: 1–5. https://doi.org/10.1159/isbn.978-3-318-06405-6

Schmid M, Feichtinger W, Weimer R, Mais C, Bolaños F, Leon P (1995) Chromosome banding in Amphibia. XXI. Inversion polymorphism and multiple nucleolus organizer regions in *Agalychnis callidryas* (Anura, Hylidae). Cytogenetic 69(1–2): 18–26. https://doi.org/10.1159/000133929

Silva DMZA, Pansonato-Alves JC, Utsunomia R, Daniel SN, Hashimoto DT, Oliveira C, Porto-Foresti F, Foresti F (2013) Chromosomal organization of repetitive DNA sequences in *Astyanax bockmanni* (Teleostei, Characiformes): dispersive location, association and co-localization in the genome. Genetica 141(7–9): 329–336. https://doi.org/10.1007/s10709-013-9732-7

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

Viegas-Péquignot E (1992) In situ hybridization to chromosomes with biotinylated probes. In: Willernson D (Ed.) In situ hybridization: a practical approach. Oxford University Press-IRL Press, Oxford, 137–158.

Zalesna A, Florek M, Rybacki M, Ofielska M (2017) Variability of NOR patterns in European water frogs of different genome composition and ploidy level. Comparative Cytogenetic 11(2): 249–266. https://doi.org/10.3897/CompCytogen.v11i2.10804

CompCytogen 13(4): 339–357 (2019)
doi: 10.3897/CompCytogen.v13i4.35572
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Karyotype characteristics, chromosomal polymorphism and gene COI sequences of *Chironomus heteropilicornis* Wülker, 1996 (Diptera, Chironomidae) from the South Caucasus

Mukhamed Kh. Karmokov[1]

1 *Tembotov Institute of Ecology of Mountain territories RAS,I. Armand str., 37a, Nalchik 360051, Russia*

Corresponding author: *Mukhamed Kh. Karmokov* (lacedemon@rambler.ru)

Academic editor: *V. Golygina* | Received 19 April 2019 | Accepted 10 October 2019 | Published 31 October 2019

http://zoobank.org/F0FDE521-5F5D-4013-B713-93B84214C8E1

Citation: Karmokov MK (2019) Karyotype characteristics, chromosomal polymorphism and gene COI sequences of *Chironomus heteropilicornis* Wülker, 1996 (Diptera, Chironomidae) from the South Caucasus. Comparative Cytogenetics 13(4): 339–357. https://doi.org/10.3897/CompCytogen.v13i4.35572

Abstract

The study presents data on the karyotype characteristics, features of chromosomal polymorphism and the gene COI sequences of *Chironomus heteropilicornis* Wülker, 1996 (Diptera, Chironomidae) from the South Caucasus. We found 8 banding sequences in the Caucasian population. Overall, The Caucasian population of the species can be characterized as having a low level of polymorphism. We found one new banding sequence hpiA2 in the banding sequence pool of *Ch. heteropilicornis*. We observed inversion polymorphism only in the arm F. The dendrogram of genetic distances by Nei criteria (1972) shows a clear separation of the Caucasian population from populations of Siberia. At the same time, the distance between populations of Siberia and the population of South Caucasus (0.379–0.445) almost reach the mean distance (0.474 ± 0.314) between subspecies (Gunderina 2001). Due to this, we can assume that the population of South Caucasus separated from Siberian populations at the level of subspecies. Constructed on data for COI gene sequences the phylogenetic tree estimated by the Bayesian inference shows that the sequences of *Ch. heteropilicornis* from the South Caucasus form a separate line in the general branch of *Ch. heteropilicornis* sequences. At the same time, calculated K2P genetic distances between *Ch. heteropilicornis* sequences from Norway and Caucasus (2.0–2.2%) do not exceed the 3% threshold for the genus *Chironomus*.

Keywords

Diptera, Chironomidae, *Chironomus heteropilicornis*, polytene chromosomes, chromosome polymorphism, South Caucasus.

Introduction

Wolgang F. Wülker first described *Chironomus heteropilicornis* Wülker, 1996 from Sweden and Finland. According to the Fauna Europaea web source (Pape and Beuk 2016) the species is known in Europe from Sweden and Finland. However, according to Kiknadze and Istomina (2011) and Kiknadze et al. (2016), the species was also found in North Germany (Aldorf-Markonah, about 40 km south-west of Bremen) and Russia (several sites from the Republic of Sakha (Yakutia)).

The species *Ch. heteropilicornis* belongs to *Ch. pilicornis* group of closely related species. The group was proposed by Wülker (1996) and consists of two species: *Ch. heteropilicornis* and *Ch. pilicornis* Fabricius, 1787.

In the first description of karyotype of *Ch. heteropilicornis* Wülker (1996) presented mapping of arms A, C, D, E, and F made according to mapping system created by Keyl (1962) and Dévai et al. (1989). The description of chromosomal polymorphism was also presented in that study. Almost simultaneously with the work of Prof. Wülker the data on karyotype and chromosomal polymorphism of *Ch. heteropilicornis* from Siberian populations were published by Kiknadze et al. (1996). In this work, the species was described as *Chironomus* sp. *Ya2*, but later was identified as *Ch. heteropilicornis*. Some information on karyotype of *Ch. heteropilicornis* from Germany was presented by Kiknadze et al. (2016). In addition, Kiknadze et al. (2016) revised the mapping of *Ch. heteropilicornis* banding sequences in comparison with mappings of Wülker (1996) and Kiknadze et al. (2004).

The GenBank database does not contain any sequences of the COI gene of *Ch. heteropilicornis*. At the same time, in the BOLD database there are five sequences of the gene of *Ch. heteropilicornis* obtained from an imago collected from Trondheim region in Norway (Ratnasingham and Hebert 2007, accession numbers CHMNO266-15, CHMNO267-15, CHMNO268-15, CHMNO269-15, CHMNO413-15).

The aim of the work was to present the description of karyotype characteristics, chromosomal polymorphism and gene COI sequences of *Ch. heteropilicornis* from the South Caucasus. In addition, it was also very important to compare the chromosomal polymorphism characteristics and DNA data of *Ch. heteropilicornis* from the Caucasus with earlier studies.

Methods

We used fourth instar larvae of *Ch. heteropilicornis* for both DNA and karyological study. We provide the collection sites and abbreviations of earlier studied populations (Kiknadze et al. 1996) in Table 1. We collected larvae from one site of the Republic of Georgia: 18.07.17, 41°38.936'N, 44°12.794'E, Tsalka district in the region of Kvemo Kartli, the lake situated 1.7 km east of Imera settlement, altitude ca 1600 m a.s.l. The lake has a circle shape, max. depth is about 1 m and water salinity is about 40 ppm. The collection site is marked on the map with a dark circle (Fig. 1). The geographic divi-

Table 1. Collection sites and number of analyzed *Ch. heteropilicornis* larvae from Siberian populations (Republic of Sakha (Yakutia)) per Kiknadze et al. (1996).

Localities	Population abbreviation	Collection sites	Collection date	Number of specimens
Siberian populations	VD-BA	Verkhnevilyuysky District, Khoro village, Bakyn lake	18.07.94	48
	NA-LA1	Nyurbinsky District, Antonovka village, lake without name	21.07.94	20
	NA-LA2	Nyurbinsky District, Antonovka village, lake for irrigation	21.07.94	22
	MM-UR	Mirninsky District, Mirny town, Irelyakh river	19.09.94	10

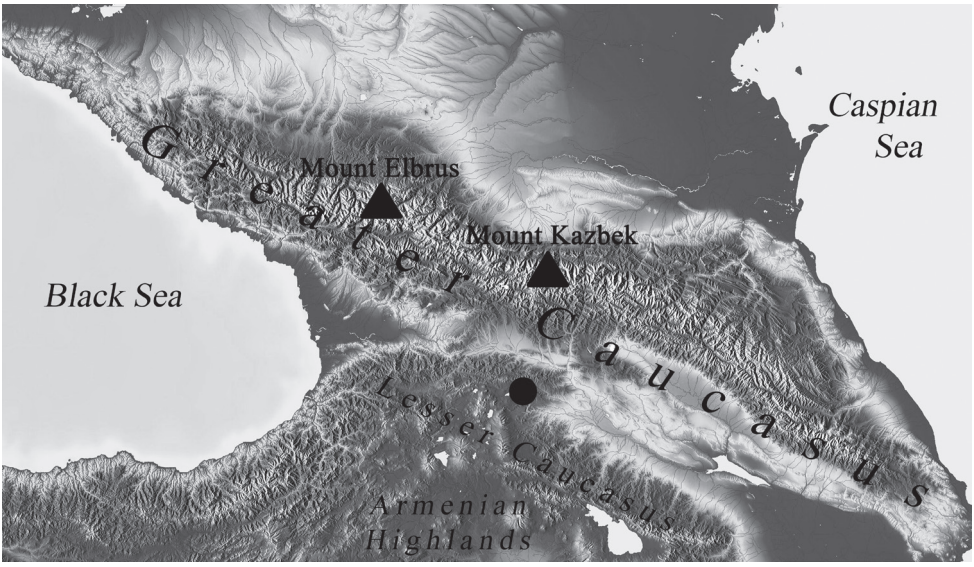


Figure 1. Collection site of *Ch. heteropilicornis* in South Caucasus. Collection site is marked with dark circle.

sion of the Caucasus follows Gvozdetskii (1963). The area to the west of Mount Elbrus considered as the West Caucasus. The area between Mount Elbrus and Mount Kazbek considered as the Central Caucasus, and the area to the east of Mount Kazbek as the East Caucasus. The area that includes the Colchis Lowland, the Kura-Aras Lowland, the Lesser Caucasus, the Talysh Mountains, the Lenkoran Lowland and eastern portion of the Armenian Highlands is considered as the South Caucasus or Transcaucasia.

Thus, the collection site from the Republic of Georgia belongs to the South Caucasus or Transcaucasia. Regarding vertical zonation (Sokolov and Tembotov 1989), the site belongs to the Javakheti-Armenian variant.

The head capsule and body of 10 larvae were slide mounted in Fora-Berlese solution. The specimens have been deposited in the Tembotov Institute of Ecology of Mountain territories RAS in Nalchik, Russia. We studied the karyotype and chromosomal polymorphism in 33 larvae from the Caucasus region.

We fixed the larvae for karyological study in ethanol-glacial acetic acid solution (3:1). The slides of the chromosomes were prepared using the ethanol-orcein technique

(see Dyomin and Iliynskaya 1988, Dyomin and Shobanov 1990). The banding sequences were designated per the accepted convention specifying the abbreviated name of the species, symbol of chromosome arm, and sequence number, as in hpiA1, hpiA2, etc. (Keyl 1962, Wülker and Klötzli 1973).

We performed the identification of chromosome banding sequences for arms A, E and F using the photomaps of Wülker (1996) and Kiknadze et al. (1996, 2016) in the system of Keyl (1962) and chromosome mapping for arms C and D as per Wülker (1996) and Kiknadze et al. (1996, 2016) in the system of Dévai et al. (1989).

We studied the chromosome slides using a Carl Zeiss Axio Imager A2 microscope and performed the statistical data processing using software package STATISTICA 10 (StatSoft).

We used the following parameters of chromosomal polymorphism characteristics for comparison: percentage of heterozygous larvae, average number of heterozygous inversions per larva. We calculated the genetic distances between populations according to Nei criteria (Nei 1972) using Chironomus 1.0 software (Kazakov and Karmokov 2015) based on original data along with Kiknadze et al. (1996).

DNA extraction, amplification and sequencing

We used bodies of five larvae of *Ch. heteropilicornis* previously studied karyologically for further DNA extraction. DNA was extracted from the whole larva body using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for animal tissue. DNA extraction was performed on vacuum-dried samples without prior homogenization. Samples were incubated in lysis buffer for 24 h. After extraction, the head capsules were retrieved for dry mounting. The barcoding region of the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using the Folmer et al. (1994) primers: LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). PCR was performed in a 25-μL reaction volume containing 2.5 mM MgCl2, 0.4 μg BSA, 0.8 mM GeneAmp dNTP Mix (Applied Biosystems), 0.5 μM of each primer, 1U of ABI AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 1X PCR buffer II (Applied Biosystems) and 3 μl template DNA extract.

The amplification profile consisted of an initial step of 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 30 s and 72 °C for 2 min, and finally a 10 min extension step at 72 °C. The PCR products were purified with Illustra ExoStar 1-Step (GE Healthcare).

Purified PCR products were sequenced (in both directions) externally by StarSeq GmbH (Mainz, Germany). The GenBank accession numbers of three sequences obtained in this study (South Caucasus) are provided in Table 2.

Table 2. Collection sites and accession numbers of *Ch. pilicornis* and *Ch. heteropilicornis* nucleotide sequences used in the study.

Species	GenBank and BOLD accession number	Localities
Ch. pilicornis	CNQUF171-14	Canada
	INNV033-08	Canada
	ARCHR033-11	Greenland
	ARCHR026-11	Greenland
	BSCHI735-17	Sweden
	BSCHI736-17	Sweden
Ch. heteropilicornis	CHMNO266-15	Norway
	CHMNO269-15	Norway
	CHMNO268-15	Norway
	CHMNO267-15	Norway
	CHMNO413-15	Norway
	MK795770	South Caucasus
	MK795771	South Caucasus
	MK795772	South Caucasus

Phylogenetic analysis

For the phylogenetic comparison we used DNA data from both GenBank and BOLD databases for the species *Ch. balatonicus* Devai et al., 1983 (JN016826.1, AF192193.1), *Ch. plumosus* (Linnaeus, 1758) (KF278218.1, KF278217.1), *Ch. usenicus* Loginova & Beljanina, 1994 (JN016817.1, JN016806.1), *Ch. entis* Shobanov, 1989 (KM571024.1), *Ch. borokensis* (Kerkis et al., 1988) (AB74026.1), *Ch. muratensis* Ryser, Scholl & Wuelker, 1983 (AF192194.1), *Ch. curabilis* Belyanina, Sigareva & Loginova, 1990 (JN016822.1, JN016810.1), *Ch. nuditarsis* Str. (Keyl, 1961) (KY225345.1), *Ch. dorsalis* Meigen, 1818 (KY838605.1), *Ch. salinarius* Kieffer, 1915 (KR641621.1), *Ch. tentans* (Fabricius), 1805 (AF110157.1), *Ch. pallidivitattus* sensu Edwards, 1929 (AF110165.1), *Ch. dilutus* Shobanov et al., 1999 (JF867805.1), *Ch. nipponensis* Tokunaga, 1940 (LC096172.1), *Ch. cingulatus* Meigen, 1830 (AF192191.1), *Ch.* "annularius" sensu Strenzke (1959) (AF192189.1), *Ch. bernensis* Klotzli, 1973 (AF192188.1), *Ch. commutatus* Keyl, 1960 (AF192187.1), *Ch. novosibiricus* Kiknadze et al., 1993 (AF192197.1), *Ch. tuvanicus* Kiknadze et al., 1993 (AF192196.1), *Ch. whitseli* Sublette & Sublette, 1974 (KR683438.1), *Ch. maturus* Johannsen, 1908 (DQ648204.1), *Ch. acutiventris* Wulker, Ryser & Scroll, 1983 (AF192200.1), *Ch. heterodentatus* Konstantinov, 1956 (AF192199.1), *Ch. melanescens* Keyl, 1961 (MG145351.1), *Ch. aprilinus* Meigen, 1818 (KC250746.1), *Ch. luridus* Strenzke, 1959 (AF192203.1), *Ch. pseudothummi* Strenzke, 1959 (KC250754.1), *Ch. riparius* Meigen, 1804 (KR56187.1), *Ch. piger* Strenzke, 1959 (AF192202.1) and *Drosophila melanogaster* (Meigen, 1830) (BBDEE689-10).

We provide our DNA data for *Ch. pilicornis* and *Ch. heteropilicornis* with corresponding accession numbers and collection sites in Table 2. We conducted the align-

ment of COI nucleotide sequences by MUSCLE with a genetic code of "invertebrate mitochondrial" packaged in MEGA 6 (Tamura et al. 2013). We calculated the pairwise sequence distances (Table 6) consisting of the estimated number of base substitutions per site using MEGA 6 and the K2P model (Kimura 1980). The analysis involved 13 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were in total 614 positions in the final dataset.

We conducted the estimation of phylogenetic relationships by the Bayes algorithm implemented in MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003) for 1,000,000 iterations and 1000 iterations of burn in. We used the GTR with gamma distribution and invariant sites (GTR+I+G) model. We performed the determination of the appropriate model in MEGA 6 (Tamura et al. 2013). Our analysis involved 49 nucleotide sequences. We eliminated all positions with less than 95% site coverage. There were 579 positions in the final dataset. The COI sequence of *Drosophila melanogaster* (BOLD accession number BBDEE689-10) was used as outgroup.

Results

We identified the larvae belonging to the genus *Chironomus* Meigen, 1803 in the studied site as *Ch. heteropilicornis* by both morphological and chromosomal characteristics. The morphological larval characteristics of *Ch. heteropilicornis* from the Caucasian site are similar to those previously described for this species by Wülker (1996) and Kiknadze et al. (1996).

Karyotype of *Ch. heteropilicornis* from the South Caucasus.

The diploid number of chromosomes in *Ch. heteropilicornis* karyotype is 2n = 8, chromosome arm combination is AB, CD, EF, and G (the "thummi" cytocomplex) (Fig. 2). Chromosomes AB and CD are metacentric, EF is submetacentric, and G is telocentric. There are five permanent nucleoli (N) in the karyotype: arms B, D and G contain one nucleolus, arm E has two. There are three Balbiani rings (BR) in the karyotype: two in the arm G and one in the arm B (Fig. 2). The homologues in the arm G lie close to each other or are tightly paired. The centromeric bands are prominent and heterochromatic.

Banding sequences and chromosomal polymorphism of *Ch. heteropilicornis* from the South Caucasus.

Previously, Kiknadze et al. (2016) described 15 banding sequences in *Ch. heteropilicornis* banding sequences pool. In the studied Caucasian population, seven of those sequences are present, and one banding sequence has been found for the first time, providing in total eight banding sequences in the population from Caucasus (Table 3).

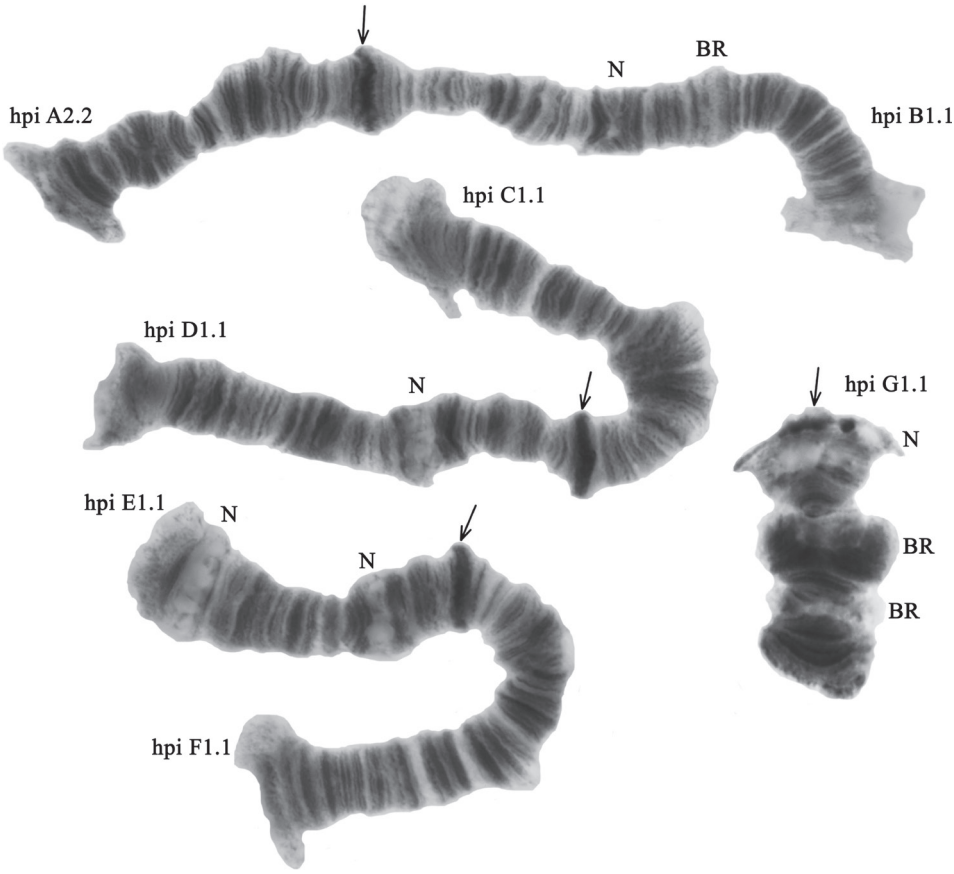


Figure 2. Karyotype of *Ch. heteropilicornis* from the South Caucasus; hpiA2.2, hpiD1.1 etc. – genotypic combinations of banding sequences; BR – Balbiani rings, N – nucleolus. Arrows indicate centromeric bands.

Table 3. Frequencies of banding sequences in different populations of *Ch. heteropilicornis*. N – the number of individuals, * – original data.

Banding sequences	Eastern Siberia (Yakutia)				South Caucasus
	VD-BA N=48	NA-LA1 N=20	NA-LA2 N=32	MM-IR N=10	SC-SJ N=33*
A1	1.000	1.000	0.977	1.000	0
AX	0	0	0.023	0	0
A2	0	0	0	0	1.000
B1	1.000	1.000	1.000	1.000	1.000
C1	0.677	0.800	0.796	0.700	1.000
C2	0.313	0.175	0.204	0.300	0
C3	0.010	0.025	0	0	0
D1	0.167	0	0.068	0.150	1.000
D2	0.833	1.000	0.932	0.850	0
E1	1.000	1.000	1.000	1.000	1.000
F1	0.740	0.775	0.864	0.750	0.955
F2	0.042	0	0.046	0.100	0.045
F3	0.218	0.225	0.090	0.150	0
G1	1.000	1.000	1.000	1.000	1.000

Arm A has one banding sequence, which we designated as hpiA2 (Figs 2, 3, Table 3). The banding sequence hpiA2 is new for the species and described for the first time (Fig. 3, Tables 3, 4). It differs from hpiA1 by one simple inversion step that involves regions **3d-i 6e-4a 13a-14f 7a-9e**:

hpiA2 1a-e 2d-3c **9e-7a 14f-13a 4a-6e 3i-d** 12c-10a 2g-1f 14g-19f C

According to Kiknadze et al. (1996, 2016) in the populations from Yakutia, two banding sequences were present in the arm A: the standard hpiA1 and an inverted one designated by authors as hpiA2. Unfortunately, the latter was not mapped and the chromosome slide containing this banding sequence, as well as its photos, were not preserved (personal communication of Veronika V. Golygina). Due to this, the banding sequence found in Siberia cannot be compared with banding sequence found in Caucasian population, as well as with any other banding sequence that may be found in populations of *Ch. heteropilicornis* in the future. Therefore, we propose to designate Caucasian sequence as hpiA2 and Siberian one as hpiAX.

Arm B was monomorphic with banding sequence hpiB1.1 (Fig. 2, Tables 3, 4).

Arm C was monomorphic with banding sequence hpiC1.1 (Fig. 4, Tables 3, 4).

Arm D also was monomorphic with banding sequence hpiD1.1 (Fig. 5, Tables 3, 4).

Arm E was monomorphic with banding sequence hpiE1.1 (Fig. 6, Tables 3, 4). We found that banding patterns in the arm E from Caucasian population are the same as in the photos from German and Siberian populations (Kiknadze, Istomina 2011; Kiknadze et al. 2016), but in our opinion the photos from the Caucasus has slightly better banding structure. Based on analysis of these new photos we suggest that the mapping of the arm should be revised.

The previous mapping of the arm as per Wülker (1996) and Kiknadze et al. (1996, 2016) was as follows:

hpiE1.1 1a-3e 10b-3f 10c-13g C

We propose a slightly different version of mapping:

hpiE1.1 1a-3e **8d-10d** 8c-3f 10c-13g C

In the new photos, one can clearly see that there is an inversion in the region 10b-8d.

Arm F has two banding sequences: hpiF1 and hpiF2 (Fig. 7). The banding sequence hpiF1 and genotypic combination hpiF1.1 were predominant in the population of South Caucasus (Tables 3, 4). The sequence hpiF2 has been observed only in the heterozygote state (Table 4) and with a rather low frequency (0.091). As with the arm E, the new photos of the arm F from Caucasian population have slightly better banding structure and, as the banding pattern in the arm is the same as in German and

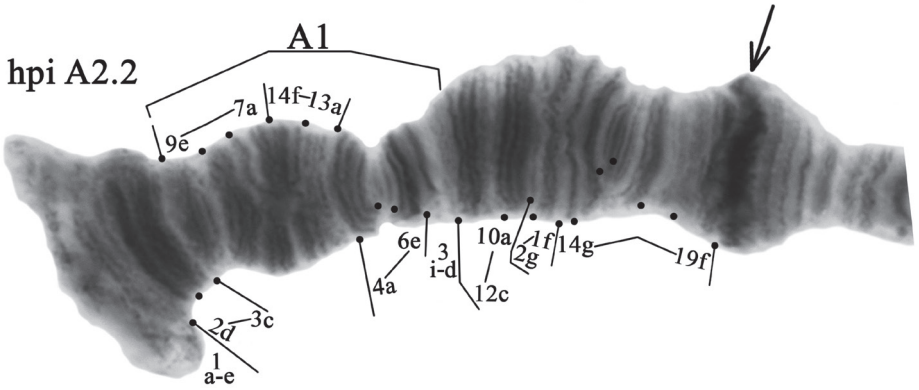


Figure 3. Homozygous genotypic combination hpiA2.2. Designations as in Fig. 2.

Table 4. Frequencies of genotypic combinations in different populations of *Ch. heteropilicornis*. N – the number of individuals, * – original data.

Genotypic combinations	Eastern Siberia (Yakutia)				South Caucasus
	VD-BA N=48	NA-LA1 N=20	NA-LA2 N=32	MM-IR N=10	SC-SJ N=33*
A1.1	1.000	1.000	0.955	1.000	0
A1.X	0	0	0.045	0	0
A2.2	0	0	0	0	1.000
B1.1	1.000	1.000	1.000	1.000	1.000
C1.1	0.438	0.600	0.636	0.500	1.000
C1.2	0.458	0.350	0.318	0.400	0
C2.2	0.083	0	0.046	0.100	0
C1.3	0.021	0.050	0	0	0
D1.1	0	0	0	0	1.000
D1.2	0.333	0	0.137	0.300	0
D2.2	0.667	1.000	0.863	0.700	0
E1.1	1.000	1.000	1.000	1.000	1.000
F1.1	0.500	0.550	0.728	0.500	0.909
F1.2	0.042	0	0.090	0.200	0.091
F2.2	0.021	0	0	0	0
F1.3	0.438	0.450	0.182	0.300	0
G1.1	1.000	1.000	1.000	1.000	1.000
Heterozygous larvae, %	85	75	55	70	9
Average number of heterozygous inversions per larvae	1.3	0.9	0.7	1.2	0.09

Siberian populations (Kiknadze, Istomina 2011), we were able to suggest some correction for mapping of hpiF2.

The previous mapping of the arm as per Kiknadze et al. (2016) was as follows:

hpiF2 1a-9b 12d-15i 9c-10d 17d-16a 12c-a 11ih 19d-18a 11a-g 20a-23f

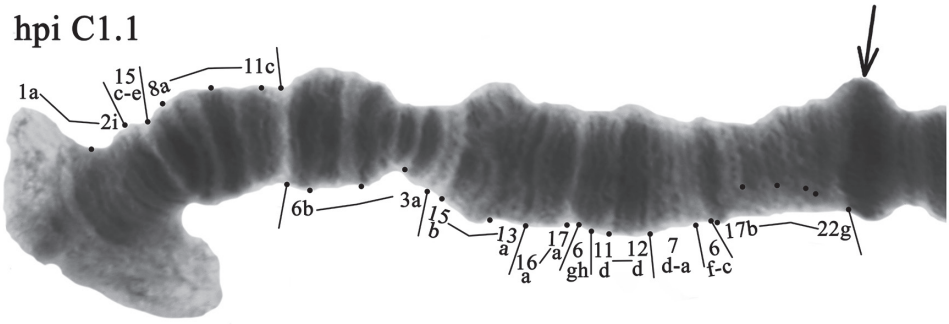


Figure 4. Homozygous genotypic combination hpiC1.1. Designations are as in Fig. 2.

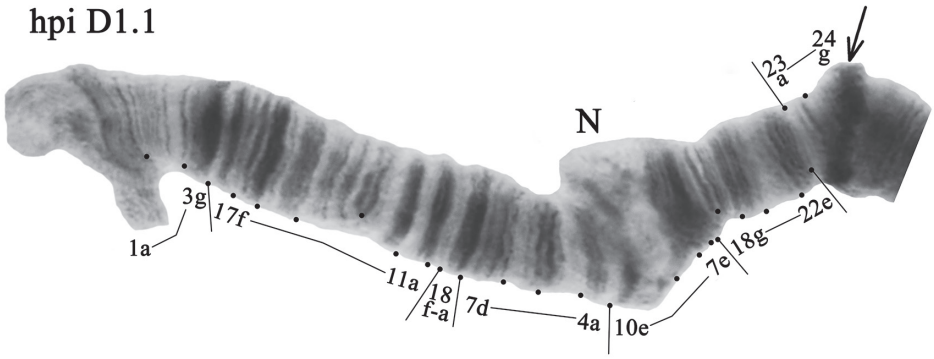


Figure 5. Homozygous genotypic combination hpiD1.1. Designations are as in Fig. 2.

We propose the new version of mapping:

hpiF2 1a-9b 12d-15i 9c-10d 17d-16a 12c-a 11i-f **18e-a 11a-e** 19a-23f

Our mapping shows that the inversion step that differ sequences hpiF1 and hpiF2 was slightly larger than described by Kiknadze et al. (1996), but smaller than described by Kiknadze et al. (2016).

Arm G was monomorphic with banding sequence hpiG1.1 (Fig. 2, Tables 3, 4).

We compared the chromosomal polymorphism of *Ch. heteropilicornis* from the Caucasian population with that of populations from other regions.

Wülker (1996) described nine banding sequences in the populations of Finland and Sweden: hpiA1, hpiB1, hpiC1, hpiC2, hpiD1, hpiE1, hpiF1 (after revision designated as hpiF3), hpiF2 (after revision designated as hpiF1) and hpiG1. Unfortunately, he did not provide the data on frequencies of the banding sequences and genotypic combinations so it was impossible to compare quantitative data.

The data for Siberian populations are available due to Kiknadze et al. (1996).

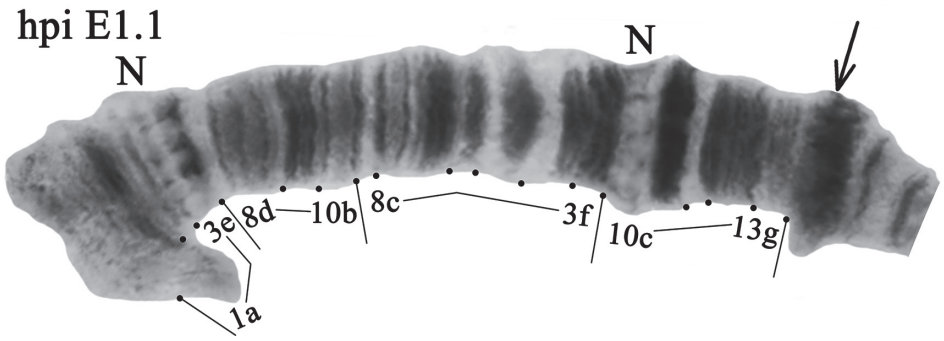


Figure 6. Homozygous genotypic combination hpiE1.1. Designations are as in Fig. 2.

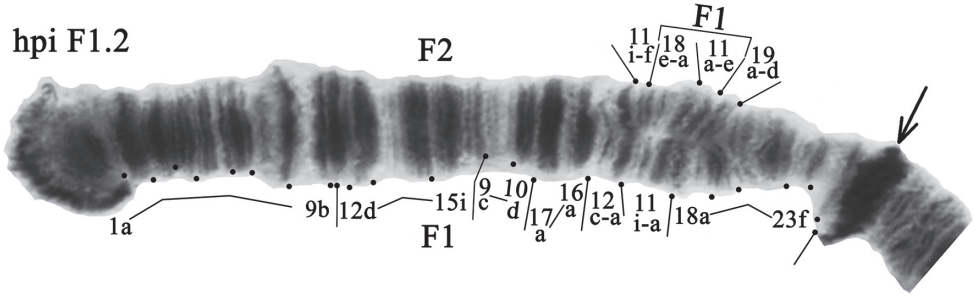


Figure 7. Heterozygous genotypic combination hpiF1.2. Designations as in Fig. 2.

Arm A. In the populations of Finland and Sweden (Wülker 1996) only one banding sequence – hpiA1 – has been observed. Most of Siberian populations (Kiknadze et al. 1996) are also characterized by the presence of the one banding sequence, hpiA1. Only one population contained another banding sequence – hpiAX – in the heterozygote state and with very low frequency (Table 3). In the populations of South Caucasus, only one banding sequence – hpiA2 – has been observed. As it was dominant in Caucasus population and completely absent elsewhere, we believe that it might be endemic to this region (Table 3).

Arm B has been monomorphic in all populations studied up to date, including the population of South Caucasus (Tables 3, 4).

Arm C. In the populations of Finland and Sweden (Wülker 1996) two banding sequence – hpiC1 and hpiC2 – has been observed. The arm also was polymorphic in all the Siberian populations. Out of three banding sequences found in the arm, two – hpiC1 and hpiC2 – were observed both in homo- and heterozygote state with hpiC1 being predominant in all populations, and hpiC3 was found in heterozygote state only (Tables 3, 4). However, the arm was monomorphic in population from South Caucasus with only one genotypic combination – hpiC1.1 – present.

Arm D. In the populations of Finland and Sweden (Wülker 1996) only one banding sequence – hpiD1 – has been observed. The arm was polymorphic in most of the Siberian populations (Table 3). Two banding sequences – hpiD1 and hpiD2 – were found with the former observed in heterozygous state only. The genotypic combination hpiD2.2 was predominant in most of the Siberian population (Table 4) but completely absent in population from South Caucasus where only genotypic combination hpiD1.1 was found.

Arm E has been monomorphic in all studied populations (Tables 3, 4).

Arm F. In the populations of Finland and Sweden (Wülker 1996) two banding sequence – hpiF1 and hpiF3 – has been observed. The arm was also polymorphic both in all Siberian and in South Caucasus populations (Tables 3, 4). Three banding sequences were present in the arm in most of the Siberian populations but only two were found in population from South Caucasus. Banding sequences hpiF1 and hpiF2 were found in both Siberian and South Caucasus populations with hpiF1 predominant in all of them (Table 4). Banding sequence hpiF2 was found mostly as heterozygous combination hpiF1.2 in all populations of Siberia but in one population from Siberia homozygote hpiF2.2 has been observed. Banding sequence hpiF3 was present in populations from Siberia and absent in Caucasian population. It is interesting to note that it showed rather high frequencies yet was found only as heterozygotes with hpiF1.

Arm G was monomorphic in all the studied populations with banding sequence hpiG1 and genotypic combination hpiG1.1 (Tables 3, 4).

The level of inversion polymorphism in Caucasian *Ch. heteropilicornis* population is quite low in comparison with previously studied populations (Table 4). The percentage of heterozygous larvae in the population of the South Caucasus is low (9%), while in the Siberian populations this percentage is much higher and varies from 55% to 85% (Table 4). The average number of heterozygous inversions per larvae is also very low (0.09), while in the Siberian populations this number varies from 0.7 to 1.3 (Table 4).

Kiknadze et al. (1996) found the heterozygosity of centromeric band's size in one site (MM-UR) of Siberia (Table 1). The larvae with heterozygosity of thick and thin centromeric bands in the AB and EF chromosomes were observed. We did not find such type of the chromosomal polymorphism in population of South Caucasus.

According to Kiknadze and Istomina (2011), Siberian and European (Germany) populations differed by very high frequency of the homozygotes hpiD2.2 in Siberia; the sequence hpiD1 were found only as heterozygote hpiD1.2 in Siberia, while it was dominated in Europe as hpiD1.1.

In the dendrogram of genetic distances (Fig. 8), calculated on the basis of frequencies of genotypic combinations in different populations (Table 4) using Nei criteria (Nei 1972), Siberian populations form one clear cluster. The population from the South Caucasus (SC-SJ) does not belong to this cluster. The distance (Table 5) between populations of Siberia (0.005–0.023) is lower than the values that characterize different population of one species (0.136 ± 0.026, Gunderina 2001), so Siberian populations could be considered as truly belonging to one big population. At the same time, the distance between populations of Siberia and the population of South Caucasus (0.379–0.445) is higher than the distance between different population of the

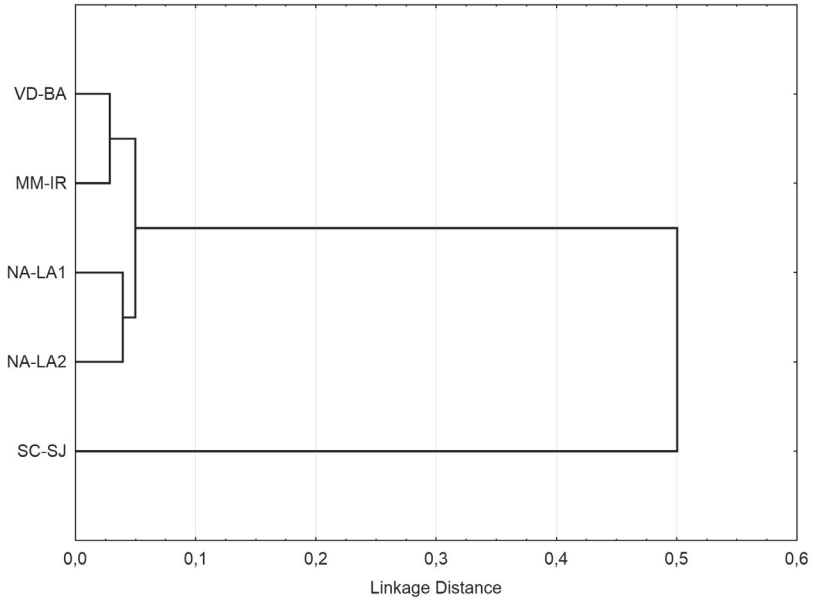


Figure 8. Tree dendrogram for five *Ch. heteropilicornis* populations*, single linkage, Euclidean distances.* For abbreviations of the populations, see Table 1.

Table 5. Values of genetic distances between different populations of *Ch. heteropilicornis* calculated using Nei criteria (Nei 1972).

Population	VD-BA	NA-LA1	NA-LA2	MM-IR	SC-SJ
VD-BA	0				
NA-LA1	0.023	0			
NA-LA2	0.023	0.014	0		
MM-IR	0.005	0.023	0.014	0	
SC-SJ	0.444	0.445	0.376	0.423	0

same species and almost reach the mean distance (0.474 ± 0.314) between subspecies (Gunderina 2001). Due to this, we can assume that the population of the South Caucasus separated from Siberian populations at the level of subspecies.

Overall, we successfully obtained three sequences of *Ch. heteropilicornis* from five Caucasian larvae (Table 2). All three sequences had the same haplotype.

Calculated pairwise sequence distances (Table 6) consisting of the estimated number of base substitutions per site using K2P model (Kimura 1980) show an interesting picture. The distances between Norwegian sequences of *Ch. heteropilicornis* are pretty low and varies from 0% to 0.2%. The distance value between sequences of *Ch. heteropilicornis* of Caucasus is 0 as they all have the same haplotype. The distances between sequences of *Ch. heteropilicornis* from Norway and sequences of *Ch. heteropilicornis* from Caucasus varies from 2.0% to 2.2%. The distances between sequences of *Ch. heteropilicornis* from Norway and *Ch. pilicornis* sequences from Canada-Greenland varies from 5.1% to 5.3%. The distance between sequences of *Ch. heteropilicornis* from

Table 6. Estimates of evolutionary divergence between sequences of *Ch. heteropilicornis* and *Ch. pilicornis*. The number of base substitutions per site (%) from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980).

	***Ch. heteropilicornis*, Norway**					***Ch. heteropilicornis*, Caucasus**			***Ch. pilicornis***				
	1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**Sweden 1**	**Sweden 2**	**Greenland 1**	**Greenland 2**	**Canada**
CHMNO266-15 *Ch. heteropilicornis* Norway_1													
CHMNO267-15 *Ch. heteropilicornis* Norway_2	0.2												
CHMNO268-15 *Ch. heteropilicornis* Norway_3	0.2	0.0											
CHMNO269-15 *Ch. heteropilicornis* Norway_4	0.0	0.2	0.2										
CHMNO413-15 *Ch. heteropilicornis* Norway_5	0.2	0.0	0.0	0.2									
MK795770 *Ch. heteropilicornis* Caucasus_1	2.2	2.0	2.0	2.2	2.0								
MK795771 *Ch. heteropilicornis* Caucasus_2	2.2	2.0	2.0	2.2	2.0	0.0							
MK795772 *Ch. heteropilicornis* Caucasus_3	2.2	2.0	2.0	2.2	2.0	0.0	0.0						
BSCHI736-17 *Ch. pilicornis* Sweden_1	0.2	0.0	0.0	0.2	0.0	2.0	2.0	2.0					
BSCHI735-17 *Ch. pilicornis* Sweden_2	0.2	0.0	0.0	0.2	0.0	2.0	2.0	2.0	0.0				
ARCHR033-11 *Ch. pilicornis* Greenland_1	5.3	5.1	5.1	5.3	5.1	5.6	5.6	5.6	5.1	5.1			
ARCHR026-11 *Ch. pilicornis* Greenland_2	5.3	5.1	5.1	5.3	5.1	5.6	5.6	5.6	5.1	5.1	0.0		
INNV033-08 *Ch. pilicornis* Canada	5.3	5.1	5.1	5.3	5.1	5.6	5.6	5.6	5.1	5.1	0.0	0.0	

Caucasus and *Ch. pilicornis* sequences from Canada-Greenland is the largest among compared populations and reaches 5.6%. The distance between sequences of *Ch. heteropilicornis* from Caucasus and sequences of *Ch. pilicornis* from Sweden reaches 2%. Surprisingly, the distance between sequences of *Ch. heteropilicornis* from Norway and *Ch. pilicornis* from Sweden is pretty low and varies from 0 to 0.2%, that is quite similar to the picture observed in the Norwegian population. The sequences of *Ch. heteropilicornis* from Norway CHMNO267-15, CHMNO268-15, CHMNO413-15 and *Ch. pilicornis* sequences from Sweden BSCHI735-17, BSCHI736-17 have the same haplotype. Finally, the distance between sequences of *Ch. pilicornis* from Canada-Greenland and sequences of *Ch. pilicornis* from Sweden reaches 5.1%.

In the phylogenetic tree of *Chironomus* species, constructed with method of the Bayesian inference (Fig 9), we can see several clear clusters with rather high probabilities that correspond to the groups of closely related species, such as *Ch. plumosus* group, *Ch. nuditarsis* group, *Ch. riihimakiensis* group, *Ch. lacunarius* group, *Ch. obtusidens* group, *Ch. piger* group and *Ch. pilicornis* group. Predictably, the sequences of *Ch. heteropilicornis*

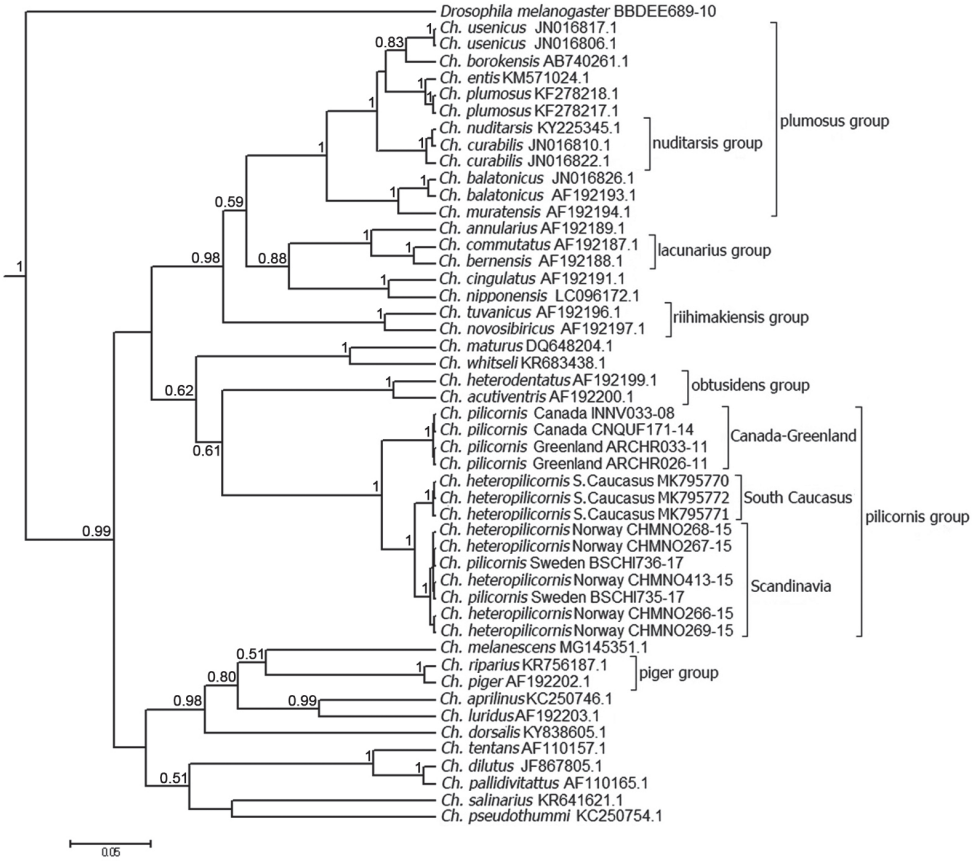


Figure 9. Phylogenetic tree of *Chironomus* species estimated by the Bayesian inference (BA). Support values are given if they exceed 0.5. The numbers at the nodes indicate posterior probabilities.

and *Ch. pilicornis* form clear separate cluster with high support value that corresponds to *Ch. pilicornis* group. At the same time, one can see an interesting picture inside this cluster. There is a separate branch of *Ch. pilicornis* sequences from Canada and Greenland. There is another larger branch of *Ch. heteropilicornis* sequences. Inside this branch, there are two separate lines and the first one is the branch of Caucasian *Ch. heteropilicornis* sequences. The second branch consists of *Ch. heteropilicornis* sequences from Norway and, surprisingly, of *Ch. pilicornis* sequences from Sweden.

Discussion

We found the species *Ch. heteropilicornis* in the South Caucasus for the first time.

Overall, we can characterize the Caucasian population of the species as having a low level of polymorphism. We found one new banding sequence hpiA2 in the banding sequences pool of *Ch. heteropilicornis*. We observed inversion polymorphism only in the arm F.

The dendrogram of genetic distances (Fig. 8) by Nei criteria (Nei 1972), calculated using karyological data, shows a clear separation of the Caucasian population from populations of Siberia. At the same time, the distance between populations of Siberia and the population of South Caucasus (0.379–0.445) almost reach the mean value (0.474 ± 0.314) for the subspecies (Gunderina 2001). Due to this, we can assume that the population of South Caucasus separated from Siberian populations at the level of subspecies.

In the work of Proulx et al. (2013), where genetic, morphological and karyological observations were used to discriminate species of *Chironomus* from Canada, it was shown that intraspecific K2P distance for *Chironomus* species characterized by the COI gene range from zero to 3%. As was noted in that research, these values can be used as a reference in distinguishing *Chironomus* species using this approach, but data on COI gene should not be used without other methods.

Following Proulx et al. (2013) we can conclude that the genetic distances between *Ch. heteropilicornis* sequences from Norway and *Ch. heteropilicornis* sequences from the Caucasus (2.0–2.2%) are lower than the 3% interspecific threshold for genus *Chironomus*. As expected, the genetic distances between *Ch. heteropilicornis* and *Ch. pilicornis* sequences (5.1–5.6%) exceed the 3% range and correspond to separate species. However, the distance values between sequences of *Ch. heteropilicornis* from Norway-Caucasus and *Ch. pilicornis* sequences (BSCHI735-17, BSCHI736-17) from Sweden are lower (0–2.0%) than the interspecific threshold. Moreover, most of *Ch. heteropilicornis* sequences from Norway have the same haplotype as both *Ch. pilicornis* sequences from Sweden. Quite similar picture was observed in the groups of sibling species, such as the *Ch. plumosus* group, and *C. tentans* group (Martin et al. 2002, Guryev and Blinov 2002, Polukonova et al. 2009). If, in this particular case, it is not an error of species identification, which can happen quite often when only morphological methods are used, one of the possible explanations for this picture may be the same as was earlier proposed by Polukonova et al. (2009). It can be a result of

interspecific hybridization with subsequent recurrent crosses resulting in the appearance of mtDNA of one of the parental species in the offsprings. In this case, even an insignificant selective advantage of this mtDNA is able to lead to a rapid fixation of the new haplotype in the population (Powell 1983, Guryev and Blinov 2002). Probably there was an interspecific hybridization event between *Ch. heteropilicornis* (female) and *Ch. pilicornis* (male) in population of Sweden. We suppose that it is quite possible because according to Wülker (1996) both species occurred sympatrically in collection site Kyrkösjärvi, Seinajöki-area (South Ostrobothnia, western Finland). The *heteropilicornis*-like sequences of *Ch. pilicornis* according to BOLD database were obtained from imago collected from Uppland on the eastern coast of Sweden, just north of Stockholm. This collection site is in 450 km south-west of collection site in western Finland, where both species occurred sympatrically. Probably such kind of hybridization events could have occurred more than once. To obtain a clearer picture it would be necessary to conduct simultaneous sequencing of genes of both mitochondrial and nuclear genomes of the same individuals with a preliminary cytogenetic analysis of both species from other sites of Sweden and Scandinavia.

All the obtained data indicate that the studied Caucasian population of *Ch. heteropilicornis* is a separate diverged population of the species on karyological and molecular-biological level. At the same time, the degree of this divergence by DNA data is lower than 3.0% threshold for *Chironomus* species.

Acknowledgments

Molecular lab work for this study was done at the Natural History Museum, University of Oslo, Norway, and supported by SIU grant CPRU-2017/10072 to Vladimir Gusarov.

References

Dévai Gy, Miskolczi M, Wülker W (1989) Standardization of chromosome arms B, C and D in *Chironomus* (Diptera, Chironomidae). Acta Biologica Debricina. Supplementum Oecologica Hungarica 2(1): 79–92.

Dyomin SYu, Iliynskaya NB (1988) Change of the density of polytene chromosomes from different organs of *Chironomus plumosus* larvae. Tsitologiya 30(4): 407–415. [In Russian]

Dyomin SYu, Shobanov NA (1990) Karyotype of *Chironomus entis* from the plumosus group (Diptera, Chironomidae) living in the European part of the Soviet Union. Tsitologiya 32(10): 1046–1054. [In Russian]

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Gunderina LI (2001) Genetic variation and evolution of Chironomids (Diptera, Chironomidae), Doctor of science thesis. Institute of Cytology and Genetics SB RAS. Novosibirsk, 360 pp. [In Russian]

Guryev VP, Blinov AG (2002) Phylogenetic relationships among holarctic populations of *Chironomus entis* and *Chironomus plumosus* in view of possible horizontal transfer of mitochondrial genes. Russian Journal of Genetics 38(3): 310–315.

Gvozdetskii NA (1963) The Caucasus. A Description of Its Nature. State Publishing House of Geographic Literature, Moscow, 261 pp. [In Russian]

Kazakov MA, Karmokov MKh (2015) The method to automate calculation of genetic similarity and genetic distance in chironomids of the genus *Chironomus* by the Nei criterion. Bulletin of the Adygeya State University 3: 142–145. [In Russian with English summary]

Keyl H-G (1962) Chromosomenevolution bei *Chironomus*. II. Chromosomenumbauten und phylogenetische Beziehungen der Arten. Chromosoma 13(4): 464–514. https://doi.org/10.1007/BF00327342

Kiknadze II, Istomina AG, Gunderina LI, Aimanova KG, Salova TA, Savvinov DD (1996) Banding sequence pools of chironomid of Yakutian Permafrost. Tribe Chironomini. Novosibirsk, 166 pp. [In Russian with English summary]

Kiknadze II, Golygina VV, Istomina AG, Gunderina LI (2004) Pattern of chromosomal polymorphism during population and species divergence in *Chironomus* (Diptera, Chironomidae). Siberian Ecological Journal 11(5): 635–652. [In Russian]

Kiknadze II, Istomina AG (2011) Karyological study of *Chironomus* species from the Netherlands. Contemporary Chironomid Studies (pp. 41–72). In: Wang X, Liu W (Eds) Contemporary Chironomid Studies. Proceedings of 17th International Symposium on Chironomidae (6–10 July 2009, Nankai University, China), Nankai, 412 pp.

Kiknadze II, Istomina AV, Golygina VV, Gunderina LI (2016) Karyotypes of Palearctic and Holarctic species of the genus *Chironomus* [Electronic resource]. Russian Academy of Sciences, Siberian Branch, Federal Research Center Institute of Cytology and Genetics. Novosibirsk, 489 pp.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111–120. https://doi.org/10.1007/BF01731581

Martin J, Guryev V, Blinov A (2002) Population variability in *Chironomus* (*Camptochironomus*) species (Diptera, Nematocera) with a Holarctic distribution: evidence of mitochondrial gene flow. Insect Molecular Biology 11: 387–398. https://doi.org/10.1046/j.1365-2583.2002.00348.x

Nei M (1972) The genetic distance between populations. American Naturalist 106: 283–292. https://doi.org/10.1086/282771

Pape P, Beuk Th (2016) Diptera, Chironomidae. Fauna Europaea (version 2017.06.) https://fauna-eu.org

Polukonova NV, Djomin AG, Mugue NS, Shaikevich EV (2009) Comparison of *Chironomus usenicus* and *Chironomus curabilis* with species of the group plumosus (Diptera) inferred from the mitochondrial DNA gene COI and by the polytene chromosomes banding pattern. Russian Journal of Genetics 45(8): 899–905. https://doi.org/10.1134/S102279540908002X

Powell JR (1983) Interspecific Citoplasmatic Gene Flow in the Absence of Nuclear Gene Flow: Evidence from Drosophila. Proceedings of the National Academy of Science of the USA 18: 492–495. https://doi.org/10.1073/pnas.80.2.492

Proulx I, Martin J, Carew M, Hare L (2013) Using various lines of evidence to identify *Chironomus* species (Diptera: Chironomidae) in eastern Canadian lakes. Zootaxa 3741: 401–458. https://doi.org/10.11646/zootaxa.3741.4.1

Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes 7: 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Sokolov VE, Tembotov AK (1989) Mammals of Caucasus: Insectivores. Moscow, 548 pp. [In Russian with English summary]

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729. https://doi.org/10.1093/molbev/mst197

Wülker W, Klötzli AM (1973) Revision der Gattung *Chironomus* Meig. IV. Arten des lacunarius- (commutatus-) complexes. Archiv für Hydrobiologie 72(2): 474–489.

Wülker W (1996) *Chironomus pilicornis* Fabricius, 1787 and *Ch. heteropilicornis* sp. n. (Diptera: Chironomidae) in Fennoscandian reservoirs: Kariosystematics and morphological results. Aquatic Insects 8(4): 209–221. https://doi.org/10.1080/01650429609361624

CompCytogen 13(4): 359–366 (2019)
doi: 10.3897/CompCytogen.v13i4.46897
http://compcytogen.pensoft.net

SHORT COMMUNICATION

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

Karyotype of *Polyommatus* (*Agrodiaetus*) *eriwanensis* Forster, 1960 and taxonomic position of *P.* (*A.*) *interjectus* de Lesse, 1960 (Lepidoptera, Lycaenidae)

Vladimir A. Lukhtanov[1,2], Alexander V. Dantchenko[1,3]

1 *Department of Karyosystematics, Zoological Institute of the Russian Academy of Sciences, Universitetskaya nab. 1, St. Petersburg 199034, Russia* **2** *Department of Entomology, St. Petersburg State University, Universitetskaya nab 7/9, St. Petersburg 199034, Russia* **3** *Faculty of Chemistry, Lomonosov Moscow State University, GSP-1, Leninskiye Gory 1/11, Moscow119991, Russia*

Corresponding author: *Vladimir A. Lukhtanov* (lukhtanov@mail.ru)

Academic editor: *S. Grozeva* | Received 27 September 2019 | Accepted 28 October 2019 | Published 12 November 2019

http://zoobank.org/0A22BB36-510A-4987-BD61-92A7F7E42C7C

Citation: Lukhtanov VA, Dantchenko AV (2019) Karyotype of *Polyommatus* (*Agrodiaetus*) *eriwanensis* Forster, 1960 and taxonomic position of *P.* (*A.*) *interjectus* de Lesse, 1960 (Lepidoptera, Lycaenidae). Comparative Cytogenetics 13(4): 359–366. https://doi.org/10.3897/CompCytogen.v13i4.46897

Abstract

The karyotype of *Polyommatus* (*Agrodiaetus*) *eriwanensis* Forster, 1960 from the type locality ("Eriwan" [Yerevan, Armenia]) and other localities in Armenia was investigated. The number of chromosomal elements (bivalents+ multivalents) observed in male meiosis I was found to vary from 29 to 34. In individuals with n = 34, all observed elements were represented by bivalents. In other specimens, heterozygosity for different number of chromosomal fusions resulted in multivalent formation at MI stage and consequently in a lower number of recognizable chromosomal elements. We show that all karyotype peculiarities of *P.* (*A.*) *interjectus* de Lesse, 1960 (n = 29–32) from Turkey are similar to those in *A. eriwanensis*. The butterflies of these taxa have allopatric distribution and can be considered as conspecific.

Keywords

Armenia, Turkey, biodiversity, chromosome, DNA barcoding, meiosis, karyosystematics

Introduction

Polyommatus (*Agrodiaetus*) *eriwanensis* Forster, 1960 is a little-known taxon that was originally described as a subspecies *Agrodiaetus ripartii eriwanensis* Forster, 1960 from "Eriwan" [Yerevan, Armenia]. According to molecular data, this taxon belongs to the *P. dolus* (Hübner, 1823) clade of the subgenus *Agrodiaetus* Hübner, 1822 (Kandul et al. 2007; Vishnevskaya et al. 2016). Based on the morphological characters, this species belongs to a group of so-called anomalous blue butterflies [known also as the 'brown complex' of the subgenus *Agrodiaetus* and as the *Polyommatus admetus* (Esper, 1783) species complex] (Vishnevskaya et al. 2016). This group is composed of multiple species in which both male and female butterflies have similar brown coloration on the upperside of the wings (Lukhtanov et al. 2003).

The anomalous blue butterflies represent a real stumbling block in *Agrodiaetus* taxonomy (Lukhtanov et al. 2003; Przybyłowicz et al. 2014). Despite morphological similarity, the species of the 'brown complex' demonstrate a high level of differentiation with respect to chromosome number and karyotype structure (de Lesse 1960; Lukhtanov et al. 2015; Vershinina and Lukhtanov 2017; Vishnevskaya et al. 2018). Therefore, as in other *Agrodiaetus* clades (Lukhtanov 2015; Vershinina et al. 2015; Lukhtanov and Dantchenko 2017; Lukhtanov and Shapoval 2017), cytogenetic characters represent the most important tool for solving taxonomic problems.

The karyotype of *P.* (*A.*) *eriwanensis* was studied first by Lukhtanov and Dantchenko (2002) who reported a high level of chromosome number variation in this taxon with haploid chromosome number (n) ranging from n = 28 to n = 35. However, the cytogenetic mechanisms explaining this variation were not studied previously. Here we provide the first detailed analysis of the karyotype of *P.* (*A.*) *eriwanensis* and its comparison with the karyotypes of closely related species.

Material and methods

Fresh (not worn) adult males were used to investigate the karyotypes. After capturing a butterfly in the field, it was placed into a glassine envelope for 1–2 hours to keep it alive until we are ready to process it. Then the butterfly was killed by pinching it firmly on the thorax. Immediately after killing it, the testes were removed from the abdomen and placed into a small 0.5 ml vial with a freshly prepared Carnoy fixative (ethanol and glacial acetic acid, 3:1). Testes were stored in the fixative for 1–12 months at +4 °C. Then the gonads were stained in 2% acetic orcein for 30–60 days at +18–20 °C and analyzed as previously described (Lukhtanov and Dantchenko 2002; Lukhtanov 2017). Haploid chromosome numbers (n) were counted in metaphase I (MI), metaphase II (MII) and anaphase I (AI) cells. The following abbreviation is used in this paper: *ca* is circa, approximately determined chromosome number; n is haploid chromosome number.

Results and discussion

From numerous specimens collected in Armenia in 1996, 1997, 2001 and 2007 only 14 males from four different populations showed metaphase cells which were acceptable for karyotype analysis.

The variable number of distinct chromosome elements was found in MI, MII and AI cells of the studied specimens. In six individuals, 34 chromosome elements were counted (Table 1, Figs 1–6); all observed elements were distinct bivalents in MI and AI cells. Thus, the haploid chromosome number was n = 34 and the diploid number may be calculated as 2n = 68 for these six individuals. In the chromosome set with n = 34, the bivalents were oval or dumb-bell shaped. They were differentiated with respect to their size: the area of the first bivalent was 4–5 times as large as that of the last bivalent (Figs 1–4). In two cells the number of chromosome elements was determined as n = *ca*34 with an approximation due of imperfect spreading of bivalents.

If the number of chromosome elements was lower than 34 (29, *ca*30, *ca*31, *ca*32 or 33), from 1 to 6 V-shaped large elements were observed in MI cells. We treat these V-shaped elements as multivalents. For example, in the specimens KL-1997-6-9, 8 cells showed clearly 32 bivalents + 1 multivalent (possibly a trivalent). A very interesting karyotype was found in the specimen KL-1997-7. In this specimen in all studied MI plates we could find 29 chromosome elements including oval or dumb-bell shaped bivalents and three V-shaped multivalents (Fig. 7). The karyotype contained no exceptionally big or small bivalents or multivalents. We suppose that studied cells were heterozygous for multiple chromosome fusions. Small chromosomes of the standard n = 34 karyotype were probably involved in these rearrangements and were observed as parts of the multivalents. Thus, it is evident that the difference in the number of visible chromatin bodies in MI plates does not reflect the real variation of diploid number. Heterozygosity for chromosome fusion(s) may result in multivalent formation at MI stage and consequently in change of number of recognizable chromosome units.

Thus, the number of chromosome elements (bivalents+multivalents) observed in MI was found to vary from 29 to 34. Similar chromosome numbers were found in the following species of the *P. dolus* clade: n = 29-32 in *P.* (*A.*) *interjectus* (de Lesse, 1960) (de Lesse 1960), n = 38 in *P.* (*A.*) *timfristos* Lukhtanov, Vishnevskaya & Shapoval, 2016 (Vishnevskaya et al. 2016), n = 39 in *P.* (*A.*) *humedasae* (Toso & Balletto, 1976) (Vila et al. 2010), n = 41-42 in *P.* (*A.*) *orphicus* Kolev, 2005 (Kolev 2005), n = 40–42 in *P.* (*A.*) *dantchenkoi* (Lukhtanov & Wiemers, 2003) (Lukhtanov et al. 2003) and n = 43 in *P.* (*A.*) *rjabovianus masul* Lukhtanov, Dantchenko, Vishnevskaya & Saifitdinova, 2015 (Lukhtanov et al. 2015).

We calculated barcoding gaps between these taxa using published *COI* sequences (Lukhtanov et al. 2003; Wiemers 2003; Kandul et al. 2004; Vila et al. 2010; Vishnevskaya et al. 2016) deposited in GenBank (Table 2). As one can see, the species pair *P. eriwanensis* – *P. interjectus* is characterized by a minimum *COI* barcoding gap which is much lower than 'standard' DNA-barcode species threshold (3%) (Hebert et al. 2003;

Table 1. The number of chromosome elements (bivalents+ multivalents) observed in MI cells of the studied specimens of *P.* (*A.*) *eriwanensis.*

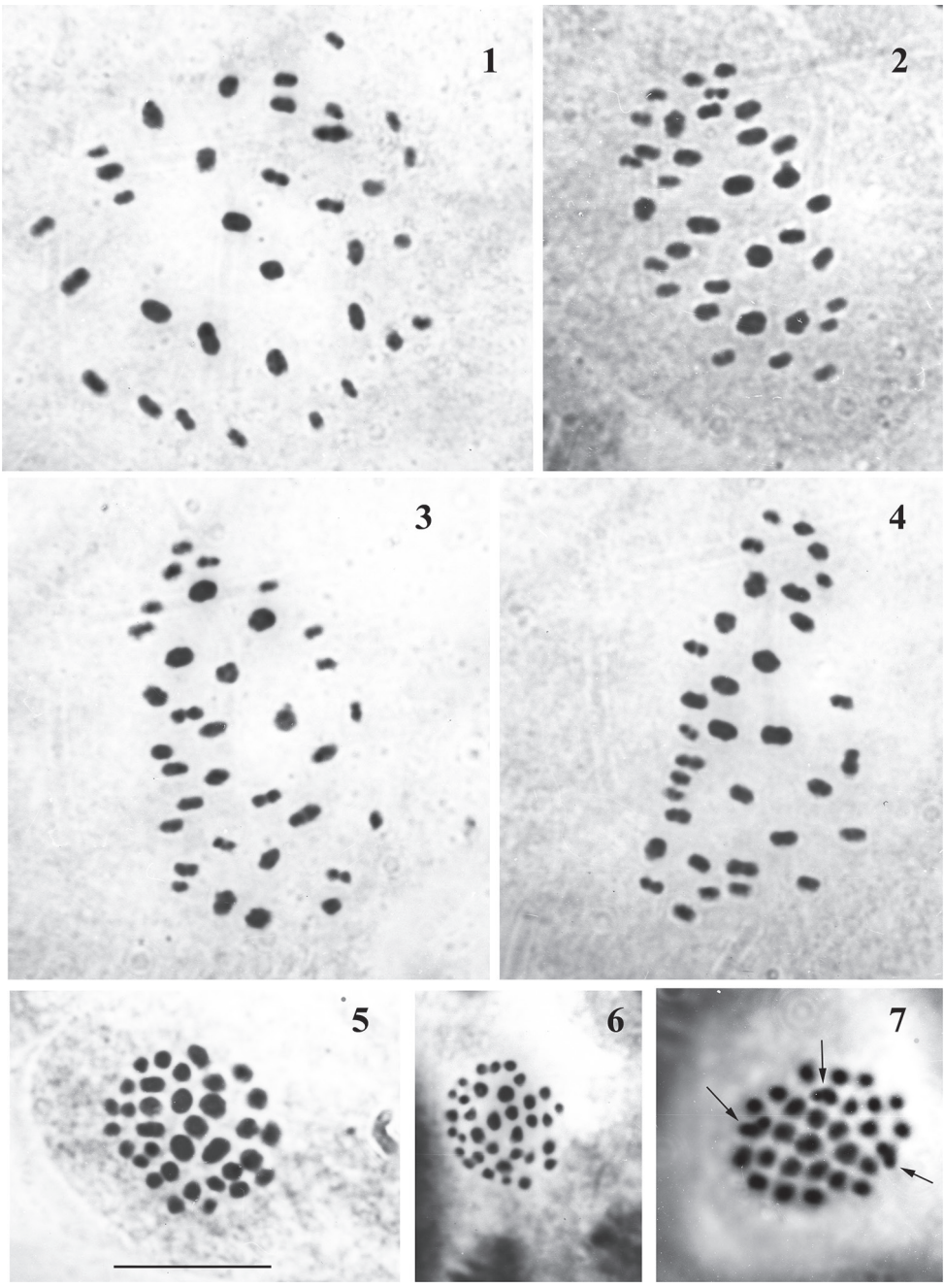
Code number of the specimen	Locality and date of collecting	The number of chromosome elements (bivalents+ multivalents) observed	Number of cells checked
KL-1996-34-1	Armenia, Aragaz Mt, ca30 km NW Yerevan, 14-17.07.1997	*ca*32	3MI
KL-1997-6-1	Armenia, Garny, ca15 km E Yerevan, 02.07.1997	*ca*34	7MI
KL-1997-6-4	Armenia, Garny, ca15 km E Yerevan, 02.07.1997	n = *ca*31	1MI
KL-1997-6-7	Armenia, Garny, ca15 km E Yerevan, 02.07.1997	n = 34	1MI
KL-1997-6-8	Armenia, Garny, ca15 km E Yerevan, 02.07.1997	n = *ca*34	1MI
KL-1997-6-9	Armenia, Garny, ca15 km E Yerevan, 02.07.1997	33	8MI
KL-1997-7	Armenia, Garny, ca15 km E Yerevan, 02.07.1997	29	4MI
KL-1997-76-1	Armenia, Aiodzorsky Range, Gnyshik, ca90 km SE Yerevan, 22.07.1997	n = 34	21MI and AI; 2 MII
AD2001-Nr4	Armenia, Geghadir	n = *ca*30	4MI
AD2001-008	Armenia, Aiodzorsky Range, Gnyshik, ca90 km SE Yerevan, 22.07.1997	n = 34	5MI
001A07	Armenia, Aiodzorsky Range, Gnyshik, ca90 km SE Yerevan, loc. 2, 07.2007	n = 34	4MI
002A07	Armenia, Aiodzorsky Range, Gnyshik, ca90 km SE Yerevan, 07.2007	n = 32	5MI
004A07	Armenia, Aiodzorsky Range, Gnyshik, ca90 km SE Yerevan, loc. 2, 07.2007	n = 32	3MI
004A09	Armenia, Aiodzorsky Range, Gnyshik, ca90 km SE Yerevan, 07.2007	n = *ca*32	2MI

Table 2. The *COI* barcoding gap (i.e. uncorrected p-distance between the two closest sequences found in the studied pair) and chromosome number distance (difference between the two closest chromosome numbers found in the studied pair).

Pair of taxa	Minimal COI p-distance (barcoding gap), %	Chromosome number distance
P. eriwanensis – P. interjectus	0.8	0
P. eriwanensis – P. timfristos	2.0	4
P. eriwanensis – P. humedasae	2.5	5
P. eriwanensis – P. orphicus	2.3	7
P. eriwanensis – P. dantchenkoi	0.8	6
P. eriwanensis – P. rjabovianus masul	2.6	9

Lukhtanov et al. 2016). This pair is also characterized by overlapping numbers of chromosomes. In the MI and MII karyotype of *P.* (*A.*) *interjectus* de Lesse (1960) found from 29 up to 32 chromosome entities with variable number of small and large elements. On the pictures in his paper one can see distinct V-shaped large elements, which are probably multivalents. The chromosome elements were strongly differentiated in *P.* (*A.*) *interjectus* with respect to their size: the area of the largest elements was 4–5 times as large as that of the smallest ones (de Lesse 1960: figs 7 and 8). All these peculiarities are similar to those in *P.* (*A.*) *eriwanensis*. These taxa have allopatric distributions (Fig. 8). Therefore, the taxon studied by de Lesse (1960) can be treated as a subspecies *P.* (*A.*) *eriwanesis interjecus* rather than a distinct species.

Polyommatus eriwanensis is found in southern Armenia (nominotypical subspecies) and in Ezincan, Erzurum, Sivas and Tunceli Provinces in Turkey (*P. eriwanensis*



Figures 1–7. Karyotype in male meiosis of *Polyommatus* (*Agrodiaetus*) *eriwanensis* **1–4** metaphase I of meiosis, squash preparation, n = 34 **5** metaphase I of meiosis, intact (not squashed) preparation, n = 34 **6** metaphase II of meiosis, intact (not squashed) preparation, n = 34 **7** metaphase I of meiosis, intact (not squashed) preparation, n = 29, multivalents are shown by arrows. Scale bar: 10 μm in all figures.

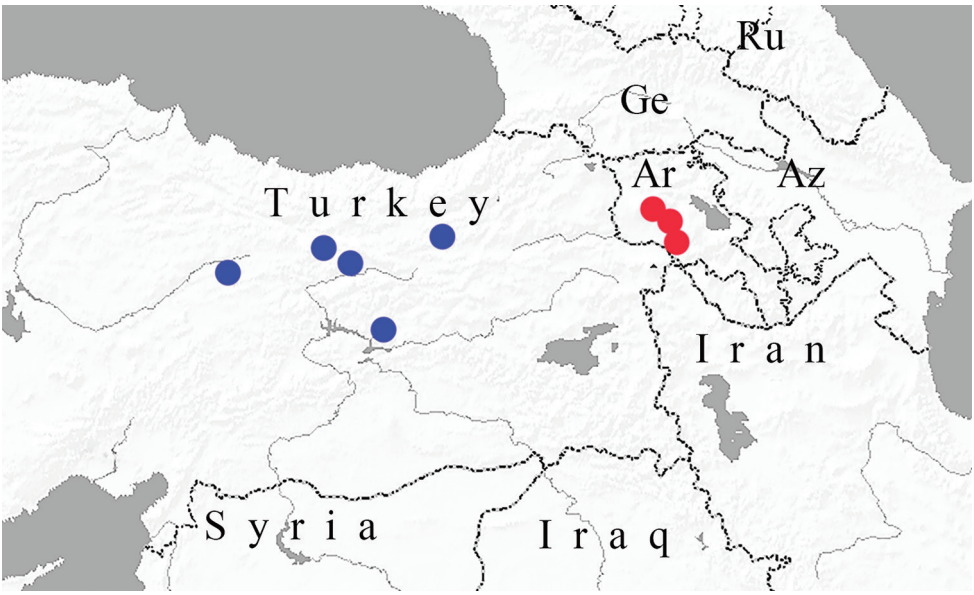


Figure 8. Geographic distribution of *P.* (*A.*) *eriwanensis eriwanensis* (red circles) and *P.* (*A.*) *eriwanensis interjectus* (blue circles) based on information from Hesselbarth et al. (1995) and Lukhtanov et al. (2003). Ar is Armenia, Az is Azerbaijan, Ge is Georgia, Ru is Russia.

interjectus) (Fig. 8). The species is also reported for southern Georgia and western Azerbaijan (Eckweiler and Bozano 2016), as well as for north-western Iran (Tshikolovets et al. 2014). However, the indications for Georgia, Azerbaijan and Iran have not been confirmed by chromosomal or molecular data, and we consider them doubtful.

Acknowledgements

The financial support for this study was provided by the grant N 19-14-00202 from the Russian Science Foundation to the Zoological Institute of the Russian Academy of Sciences. The work was partially performed using equipment of the 'Chromas' Core Facility and the Centre for Molecular and Cell Technologies of the St. Petersburg State University.

References

de Lesse H (1960) Les nombres de chromosomes dans la classification du groupe d'*Agrodiaetus ripartii* FREYER (Lepidoptera, Lycaenidae). Revue Francaise d'Entomologie 27: 240–264.

Eckweiler W, Bozano GC (2016) Guide to the Butterflies of the Palearctic Region. Lycaenidae part IV. Milano, Omnes artes, 132 pp.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences 270: 313–321. https://doi.org/10.1098/rspb.2002.2218

Hesselbarth G, Oorschot H, Wagener S (1995) Die Tagfalter der Türkei unter Berücksichtigung der Angrenzenden Länder (Vol. 1−3). Selbstverlag Siegbert Wagener, Bocholt, 1354 pp.

Kandul NP, Lukhtanov VA, Dantchenko AV, Coleman JWS, Sekercioglu CH, Haig D, Pierce NE (2004) Phylogeny of *Agrodiaetus* Hübner 1822 (Lepidoptera: Lycaenidae) inferred from mtDNA sequences of COI and COII and nuclear sequences of EF1–α: karyotype diversification and species radiation. Systematic Biology 53(2): 278–298. https://doi.org/10.1080/10635150490423692

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

Kolev Z (2005) *Polyommatus dantchenkoi* (Lukhtanov et Wiemers, 2003) tentatively identified as new to Europe, with a description of a new taxon from the Balkan Peninsula (Lycaenidae). Nota Lepidopterologica 28(1): 25–34.

Lukhtanov VA (2015) The blue butterfly *Polyommatus* (*Plebicula*) *atlanticus* (Lepidoptera, Lycaenidae) holds the record of the highest number of chromosomes in the non-polyploid eukaryotic organisms. Comparative Cytogenetics 9(4): 683–690. https://doi.org/10.3897/CompCytogen.v9i4.5760

Lukhtanov VA (2017) A new species of *Melitaea* from Israel, with notes on taxonomy, cytogenetics, phylogeography and interspecific hybridization in the *Melitaea persea* complex (Lepidoptera, Nymphalidae). Comparative Cytogenetics 11(2): 325–357. https://doi.org/10.3897/CompCytogen.v11i2.12370

Lukhtanov VA, Dantchenko AV (2002) Principles of highly ordered metaphase I bivalent arrangement in spermatocytes of *Agrodiaetus* (Lepidoptera). Chromosome Research 10(1): 5–20. https://doi.org/10.1023/A:1014249607796

Lukhtanov VA, Dantchenko AV (2017) A new butterfly species from south Russia revealed through chromosomal and molecular analysis of the *Polyommatus* (*Agrodiaetus*) *damonides* complex (Lepidoptera, Lycaenidae). Comparative Cytogenetics 11(4): 769–795. https://doi.org/10.3897/compcytogen.v11i4.20072

Lukhtanov VA, Shapoval NA (2017) Chromosomal identification of cryptic species sharing their DNA barcodes: *Polyommatus* (*Agrodiaetus*) *antidolus* and *P.* (*A.*) *morgani* in Iran (Lepidoptera, Lycaenidae). Comparative Cytogenetics 11(4): 759–768. https://doi.org/10.3897/compcytogen.v11i4.20876

Lukhtanov VA, Wiemers M, Meusemann K (2003) Description of a new species of the "brown" *Agrodiaetus* complex from South-East Turkey (Lycaenidae). Nota Lepidopterologica 26(1–2): 65–71.

Lukhtanov VA, Dantchenko AV, Vishnevskaya MS, Saifitdinova AF (2015) Detecting cryptic species in sympatry and allopatry: analysis of hidden diversity in *Polyommatus* (*Agrodiaetus*) butterflies (Lepidoptera: Lycaenidae). Biological Journal of the Linnean Society 116: 468–485. https://doi.org/10.1111/bij.12596

Lukhtanov VA, Sourakov A, Zakharov EV (2016) DNA barcodes as a tool in biodiversity research: testing pre-existing taxonomic hypotheses in Delphic Apollo butterflies (Lepidop-

tera, Papilionidae). Systematics and Biodiversity 14(6): 599–613. https://doi.org/10.1080/14772000.2016.1203371

Przybyłowicz Ł, Lukhtanov V, Lachowska-Cierlik D (2014) Towards the understanding of the origin of the Polish remote population of *Polyommatus* (*Agrodiaetus*) *ripartii* (Lepidoptera: Lycaenidae) based on karyology and molecular phylogeny. Journal of Zoological Systematics and Evolutionary Research 52(1): 44–51. https://doi.org/10.1111/jzs.12040

Tshikolovets VV, Naderi A, Eckweiler W (2014) The Butterflies of Iran and Iraq. Tshikolovets Publications, Pardubice, 366 pp.

Vershinina AO, Lukhtanov VA (2017) Evolutionary mechanisms of runaway chromosome number change in *Agrodiaetus* butterflies. Scientific Reports 7: 1–8199. https://doi.org/10.1038/s41598-017-08525-6

Vershinina AO, Anokhin BA, Lukhtanov VA (2015) Ribosomal DNA clusters and telomeric (TTAGG)n repeats in blue butterflies (Lepidoptera, Lycaenidae) with low and high chromosome numbers. Comparative Cytogenetics 9(2): 161–171. https://doi.org/10.3897/CompCytogen.v9i2.4715

Vila R, Lukhtanov VA, Talavera G, Gil-T F, Pierce NE (2010) How common are dot-like distribution ranges? Taxonomical oversplitting in Western European *Agrodiaetus* (Lepidoptera, Lycaenidae) revealed by chromosomal and molecular markers. Biological Journal of the Linnean Society 101: 130–154. https://doi.org/10.1111/j.1095-8312.2010.01481.x

Vishnevskaya MS, Saifitdinova AF, Lukhtanov VA (2016) Karyosystematics and molecular taxonomy of the anomalous blue butterflies (Lepidoptera, Lycaenidae) from the Balkan Peninsula. Comparative Cytogenetics 10(5): 1–85. https://doi.org/10.3897/CompCytogen.v10i5.10944

Vishnevskaya MS, Lukhtanov VA, Dantchenko AV, Saifitdinova AF (2018) Combined analysis of chromosomal and molecular markers reveals cryptic species: karyosystematics of the *Agrodiaetus* Hübner, [1822] blue butterflies. Comparative Cytogenetics 12(3): 325–326. https://doi.org/10.3897/CompCytogen.v12i3.27748

Wiemers M (2003) Chromosome Differentiation and the Radiation of the Butterfly Subgenus *Agrodiaetus* (Lepidoptera: Lycaenidae: *Polyommatus*) a Molecular Phylogenetic Approach. PhD Thesis, University of Bonn, Bonn, 203 pp. http://hss.ulb.uni-bonn.de/2003/0278/0278.htm

CompCytogen 13: 367–382 (2019)
doi: 10.3897/CompCytogen.v13i4.47395
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Description of two new species of the genus *Cacopsylla* Ossiannilsson, 1970 (Hemiptera, Psylloidea) from northern Fennoscandia recognized by morphology, cytogenetic characters and *COI* barcode sequence

Christina Nokkala[1], Valentina G. Kuznetsova[2], Veikko Rinne[3], Seppo Nokkala[1]

1 *Laboratory of Genetics, Department of Biology, University of Turku, FI-20014 Turku, Finland* **2** *Department of Karyosystematics, Zoological Institute, Russian Academy of Sciences, Universitetskaya nab. 1, 199034 Saint Petersburg, Russia* **3** *Zoological Museum, University of Turku, FI-20014 Turku, Finland*

Corresponding author: *Christina Nokkala* (chrinok@utu.fi)

Academic editor: *V. Lukhtanov* | Received 18 October 2019 | Accepted 5 November 2019 | Published 19 November 2019

http://zoobank.org/32E091B4-4FDF-4FCA-8937-3A83AA387DFB

Citation: Nokkala C, Kuznetsova VG, Rinne V, Nokkala S (2019) Description of two new species of the genus *Cacopsylla* Ossiannilsson, 1970 (Hemiptera, Psylloidea) from northern Fennoscandia recognized by morphology, cytogenetic characters and *COI* barcode sequence. Comparative Cytogenetics 13(4): 367–382. https://doi.org/10.3897/CompCytogen.v13i4.47395

Abstract

Based on chromosomal, molecular and morphological analyses, two new *Cacopsylla* Ossiannilsson, 1970 species are described, *C. lapponica* S. Nokkala & Ch. Nokkala, **sp. nov.** and *C. borealis* S. Nokkala et Ch. Nokkala, **sp. nov.** (Hemiptera, Psylloidea). *C. lapponica* is a rare bisexual alpine species living on *Vaccinium uliginosum* Linnaeus, 1753 above tree line on northern hills, where it forms sympatric populations with *C. myrtilli* W. Wagner, 1947. So far, the species has been found in northern Finland, Utsjoki and Kilpisjärvi, and in northern Sweden, Abisko. The chromosome number in males is 2n = 12+X(0), characteristic of psyllids. The species is easily distinguished from *C. myrtilli* by its conspicuously smaller size mainly due to difference in wing size. Additional morphological differences are found in the length of antennae, female genital plates and male parameres. *C. borealis*, in turn, is a relatively common apomictic parthenogenetic species with 5n = 60 + XXXXX living on the same host plant, *Ledum palustre* Linnaeus, 1753, as *C. ledi* (Flor, 1861) and occasionally forming sympatric populations with it. No males have been recorded in *C. borealis*. Its distribution range reaches at least from northern Fennoscandia to Lake Baikal in the East. *C. borealis* can be distinguished from *C. ledi* by differences in the length and width of antennae, dark brown markings on the wing and female terminal structures. For molecular analysis, a 638 bp fragment of the mitochondrial *COI* gene was sequenced. *C. lapponica* differs from the cohabitating *C. myrtilli* by 20 fixed nucleotide substitutions (uncor rected p-distance 3.13 %), while *C. borealis*

differs from *C. ledi* by 21 fixed nucleotide substitutions (uncorrected p-distance 3.29 %). Molecular phylogeny construction (ML and BI) reveals two highly divergent clades, one comprising two bisexual species, *C. lapponica* and *C. fraudatrix* Labina & Kuznetsova, 2012, and the other clade comprising the parthenogenetic species *C. borealis*, *C. myrtilli*, and *C. ledi*. Within this clade, *C. borealis* is more closely associated with *C. myrtilli* than with *C. ledi*.

Keywords

Cacopsylla, *COI*, karyotype, morphology, new species, Northern Europe, bisexual reproduction, parthenogenesis

Introduction

It is well established that in Northern Europe two Holarctic psyllid species, *Cacopsylla myrtilli* (W. Wagner, 1947) and *C. ledi* (Flor, 1861), inhabit the host plants *Vaccinium myrtillus* Linnaeus, 1753 or *V. uliginosum* Linnaeus, 1753 and *Ledum palustre* Linnaeus, 1753, respectively (Ossiannilsson 1992). Both species are widely distributed through the temperate and alpine zones in Fennoscandia, Central Europe and Russia. *C. ledi* is quite evenly distributed throughout the distribution range, while the distribution of *C. myrtilli* is concentrated towards the north and/or high altitudes. Females of both species are triploid and reproduce through apomictic parthenogenesis (Ossiannilsson 1992; Nokkala et al. 2008, 2017). Quite commonly, infrequent males known as rare males exist in their populations. Males are mainly nonfunctional in populations of *C myrtilli* (Nokkala et al. 2013) and functional in *C. ledi* populations (Nokkala et al. 2017). In populations with rare males, infrequent diploid females also exist among the triploids (Nokkala et al. 2015).

So far, bisexual diploid ancestral species from which parthenogenetic *C. myrtilli* and *C. ledi* are evolved are still unknown. Klimaszewski (1971) reported a bisexual *C. myrtilli* population from the Bieszczady Mountains in Poland. However, based on *COI* barcoding DNA sequence and testis structure, Kuznetsova et al. (2012) showed the *Cacopsylla* Ossiannilsson, 1970 species living on *V. myrtillus* in Bieszczady to represent a novel bisexual species, *C. fraudatrix* Labina & Kuznetsova, 2012.

The first observation that new species could also be found in Northern Europe was made in Abisko, northern Sweden. While sampling a *C myrtilli* population near Lapporten at 570 m altitude above the tree line, three males were caught. Unlike in *C. myrtilli* which showed absence of chiasmata in male meiosis (Nokkala et al. 2013), chiasmata were present in meiosis in these males. *COI* sequence of the males was quite different from that found in *C. myrtilli.* Similarly, we found *Cacopsylla* individuals on *L. palustre* displaying *COI* sequence quite different from that of *C. ledi* but utilizing the same host plant.

As a result, we describe two new *Cacopsylla* Ossiannilsson, 1970 species which vary in their external morphology, karyotype and *COI* sequence from *C. myrtilli* and *C. ledi*, respectively.

Material and methods

Sampling

Cacopsylla specimens were collected in various locations in Sweden, Finland and Russia (Table 1). A part of the individuals was brought alive to the laboratory for morphological analysis, while most specimens were stored in alcohol or fixative (see below).

Cytological methods

In most cases whole individuals were fixed immediately after collecting in 3:1 (ethanol: acetic acid) fixative. A part of the insects was dissected, and the abdomen was immersed in fixative for cytological analysis while the head and thorax were stored in ethanol for molecular analysis of the same individual. Cytology was performed as described by Nokkala et al. (2008) with 30 min Schiff staining and 40 min Giemsa staining.

Chromosome preparations were photographed with Nikon DS-Fi3 camera mounted on Nikon Ci-L microscope using NIS Elements software. Final processing of photomicrographs was made with Corel-PhotoPaint 2018 software.

DNA isolation, amplification and sequencing

Genomic DNA was isolated from whole insects or from the head and thorax portion of individuals stored in alcohol using the DNeasy Blood and Tissue Kit (Qiagen) as described previously (Nokkala et al. 2015, 2017). A fragment of the mitochondrial

Table 1. *Cacopsylla* material morphologically analyzed (number of individuals sequenced).

Species	Country	Locality	N		Cooordinates	Altitude (m)	Food plant	Date	Collector
			females	males	Lat. / Long.				
Cacopsylla lapponica sp. nov.	Sweden	Abisko	19 (2)	3 (3)	68°19'14", 18°51'05"	570	*V. uliginosum*	10.8.2012	S. & Ch. Nokkala
	Finland	Utsjoki, Ailigas	8 (1)	7 (1)	69°53'51", 27°03'32"	320	*V. uliginosum*	6.8.2016	S. & Ch. Nokkala
		Kilpisjärvi	5	7	69°03'50", 20°44'20"	620	*V. uliginosum*	27.7.2014	S. & Ch. Nokkala
			3 (2)	3 (1)				5.8.2016	S. & Ch. Nokkala
Cacopsylla borealis sp. nov.	Sweden	Muodoslompolo	97 (4)	0	68°11'13", 23°00'20"		*L. palustre*	9.8.2018	S. & Ch. Nokkala
	Finland	Utsjoki, Hietala	87 (3)	0	69°51'06", 27°00'34"		*L. palustre*	15.8.2017	S. & Ch. Nokkala
		Utsjoki, Ailigas	148 (12)	0	69°53'51", 27°03'32"	320	*L. palustre*	6.8.2016	S. & Ch. Nokkala
			54 (3)	0				15.8.2017	S. & Ch. Nokkala
		Inari, Pitkävuono	117 (5)	0	68°59'56", 26°58'48"		*L. palustre*	16.8.2017	S. & Ch. Nokkala
		Sodankylä, Puisuvanto	165	0	67°46'52", 26°46'09"		*L. palustre*	3.8.2018	S. & Ch. Nokkala
		Salla, Tuntsa	292	0	67°18'11", 29°16'18"		*L. palustre*	1.8.2019	S. & Ch. Nokkala
		Salla, Niemelä	413	0	66°35'48", 28°59'35"		*L. palustre*	2.8.2019	S. & Ch. Nokkala
		Kuusamo, Kantojoki	285	0	66°14'23", 29°09'15"		*L. palustre*	2.8.2019	S. & Ch. Nokkala
		Kuusamo, Sakkojoki	86	0	65°32'02", 29°32'03"		*L. palustre*	3.8.2019	S. & Ch. Nokkala
		Toholampi	19	0	63°45'04", 24°11'58"		*L. palustre*	10.8.2018	S. & Ch. Nokkala
	Russia	Vorkuta	7 (7)	0	67°30'00", 64°02'00"		*L. palustre*	6.8.2013	N. Khabasova
		Baikal	71 (5)	0	51°54'25", 105°04'14"		*L. palustre*	21.7.2007	E. Labina

COI gene was amplified using the primer pair HybCacoCO / HybHCOMod. PCR reactions were carried out in 20 µl volume containing 1 x PCR buffer, 2.0 mM $MgCl_2$, 200 µM dNTP each, 0.5 µM of forward and reverse primers, 0.5 U DreamTaq DNA Polymerase (ThermoFisher Scientific) and 1 µl (ca. 50 ng) of template DNA. Initial denaturation in PCR reaction was 5 min at 95 °C followed by 40 cycles in 95 °C (30 sec), 50 °C (30 sec) and 72 °C (90 sec) with a final extension at 72 °C for 10 min. PCR products were purified with QIAquick PCR Purfication Kit (Qiagen) and sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing. BioEdit 7.2.0 (Hall 1999) software was used to trim the sequences to span a 638 bp fragment of the gene and to align the sequences with related *Cacopsylla* species, *C. fraudatrix* (GenBank accession number JX987970 = h8) (Kuznetsova et al. 2012), *C. myrtilli* (KF494326–KF494332 = h1–h7) (Nokkala et al. 2015), *C. ledi* (MF978762–MF978766 = h9–h11, h13–h14) (Nokkala et al. 2017) and JX987973 = h12 (Kuznetsova et al. 2012). In addition, we sequenced the same gene region from a related bisexual species *C. corcontum* (Šulc, 1909), accession number MK184915 (= h18), used as an outgroup in phylogenetic analysis. Sequences obtained from new species have been deposited in GenBank under the accession numbers MK184912 (= h15) and MK184913 (= h16) for *C. borealis*, and MK184914 (= h17) for *C. lapponica*.

Phylogenetic analysis

Tests of the homogeneity of substitution patterns and estimation of the net composition bias disparity between sequences were carried out with MEGAX software (Kumar et al. 2018). We used two approaches for phylogenetic inference, a maximum likelihood method (ML) and a Bayesian inference method (BI). To find the best substitution model for the current data set, MEGAX software was applied and Tamura 3-parameter + G was selected for the ML analysis and GTR+G+I for the Bayesian analysis. ML phylogenetic reconstruction was carried out using MEGAX software. Branch support was assessed with 1000 bootstrap pseudo-replicates. MrBayes 3.2.6 (Ronquist et al. 2012) was used for Bayesian inference. Two runs of the program for 1 000 000 generation, sampled every 1000 generations, were run with four chains (one cold and three heated, with heating value t = 0.2). The first 250 chains were discarded as burn-in prior to computing the consensus phylogeny and posterior probabilities (burn-infrac = 0.25). Model parameters were treated as unknown and were estimated during analysis.

Morphology

The number of testicular follicles was determined as described by Kuznetsova et al. (2012).The overall length, genital marker qualities and distribution of surface spinules in the c+sc cell of the forewing were determined in females and males (if available). Photomicrographs were taken with a Canon EOS 7D camera attached to an Olym-

pus SZX16 stereomicroscope at the Zoological Museum, University of Turku, Finland. The camera was driven by a QuickPHOTO MICRO 3.1 software. Images were stacked using Zerene Stacker and CombineZP software.

Results

Cytology

In *C. lapponica* sp. nov. populations, males and females were present in equal numbers (Table 1), hence this species reproduces bisexually. Testes in males consisted of two follicles and meiosis was chiasmate showing 12 bivalents and a univalent X chromosome at metaphase I (Fig. 1a). In the populations of *C. borealis* sp. nov. no males were found (Table 1) indicating that females reproduce parthenogenetically. In mature or nearly mature eggs, 65 univalent chromosomes were present at prometaphase, hence, females were apomictic and pentaploid (Fig. 1b).

Phylogenetic analysis

We obtained partial mitochondrial *COI* gene sequences from five females and five males from three populations of *C. lapponica* sp. nov. and from 41 females from seven populations of *C. borealis* sp. nov. (Table 1). From the outgroup species *C. corcontum*, two sequences were obtained. All *C. lapponica* sp. nov. individuals sequenced shared the same haplotype. In *C. borealis* sp. nov. the same haplotype was found in all specimens from six populations, while the sequences obtained from Vorkuta specimens differed from this haplotype by two substitutions. Additional sequences retrieved from the GenBank, all sequences were trimmed to cover a 638 bp fragment of the gene. The alignment thus included 18 sequences. No indels or stop codons were present. In the alignment, a total of 120 variable sites, 39 of them being parsimony informative, were included.

ML and Bayesian reconstructions produced trees with identical topology and similar branch support (Fig. 2). The tree included two highly divergent clades, one consisting of parthenogenetic *C. borealis* sp. nov., *C. myrtilli* and *C. ledi*. The other clade included bisexual *C. lapponica* sp. nov. and *C. fraudatrix*. Although *C. borealis* lives on the same host plant as *C. ledi* and forms mixed populations with it, *C. borealis* is more closely associated with *C. myrtilli.* There are only 9 nucleotide substitutions between *C. borealis* sp. nov. and *C. myrtilli*, uncorrected p-distance being 1.41 %, while 13 substitutions have occurred between *C. lapponica* sp. nov. and *C. fraudatrix*, reflected in an uncorrected p-distance of 2.04% (Table 2). The remaining pairwise substitutions fall between 20–23 and uncorrected p-distances between 3.13–3.61%.

Together with *C. ledi*, the species *C. borealis* sp. nov. and *C. myrtilli* formed a sister group to the [*C. lapponica* sp. nov. + *C. fraudatrix*] clade.

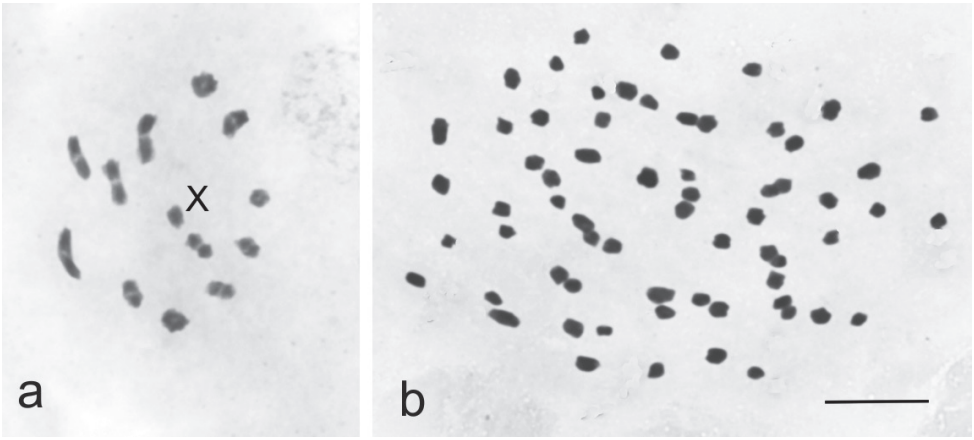


Figure 1. Chromosomes in *C. lapponica* sp. nov. and *C. borealis* sp. nov. **a** Metaphase I in male meiosis in *C. lapponica* sp. nov. showing 12 autosomal bivalents and a univalent X chromosome (2n = 24+X(0)) **b** Late prophase from a mature egg in *C. borealis* sp. nov. with 65 univalent chromosomes (5n = 60+5X). Scale bar: 10 μm.

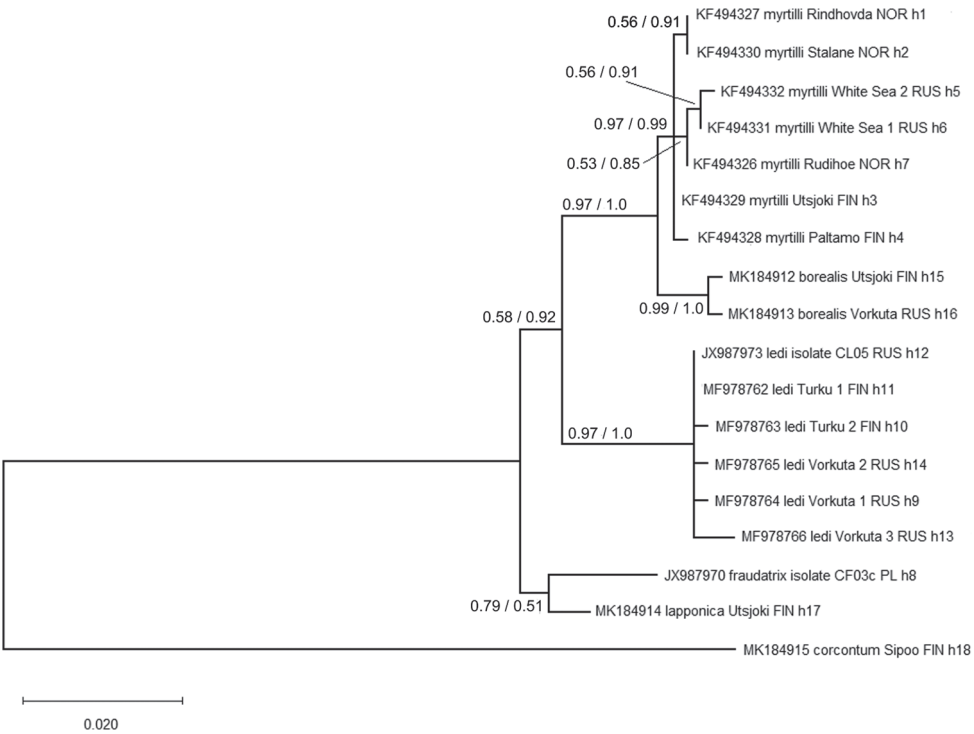


Figure 2. Phylogenetic relationships of *C. lapponica* sp. nov. and *C. borealis* sp. nov. with closely related *Cacopsylla* species. The maximum likelihood bootstrap values /Bayesian posterior probabilities are shown at nodes. Geographical abbreviations: FIN – Finland, NOR – Norway, PL – Poland, RUS – Russia.

Table2. Fixed nucleotide substitutions and uncorrected p-distances between *Cacopsylla* species in the 638 bp mitochondrial *COI* gene fragment. The number of transversions in parentheses ().

	C. borealis		***C. myrtilli***		***C. ledi***		***C. lapponica***	
C. myrtilli	9 (1)	1.41%						
C. ledi	21 (3)	3.29%	20 (4)	3.13%				
C. lapponica	22 (3)	3.44%	20 (4)	3.13%	20 (4)	3.13%		
C. fraudatrix	23 (3)	3.60%	23 (5)	3.61%	23 (4)	3.61%	13 (3)	2.04%

***Cacopsylla lapponica* S. Nokkala & Ch. Nokkala, sp. nov.**
http://zoobank.org/65E15B28-AE24-49A8-9026-88A4C1EBE8EC

Type material. ***Holotype*:** Female; Finland, Utsjoki Ailigas; 69°53'51"N, 27°03'32"E; 320 m; 05 Aug 2016; Seppo & Christina Nokkala leg.; above tree line, host *Vaccinium uliginosum*; http://mus.utu.fi/ZMUT.TYPE794. ***Paratypes*:** 9 females,1male; Finland, Utsjoki Ailigas; 69°53'51"N, 27°03'32"E; 320 m; 05 Aug 2016; Seppo & Christina Nokkala leg.; above tree line, host *Vaccinium uliginosum*; http://mus.utu.fi/ZMUT.TYPE795 – http://mus.utu.fi/ZMUT.TYPE797. The holotype and paratypes are deposited at the Zoological Museum, University of Turku, Finland.

Description. Adult coloration resembles that of cohabitating *C. myrtilli*, but is much paler with dark markings. Wings are very pale yellow and transparent with dark veins (Fig. 3). Adults are clearly smaller in size (Fig. 3), the overall length of males being 1.9–2.1 mm (N = 8) and females 2.3–2.5 mm (N = 10) compared to 2.75–3.25 mm of *C. myrtilli* females (Ossiannilsson 1992).

Diagnosis. The most conspicuous difference in external morphology between *C. myrtilli* and *C. lapponica* is the length of wings. In *C. lapponica*, the wing is much shorter than in *C. myrtilli* (Fig. 4). In *C. lapponica*, the wings are just slightly longer than the abdomen, while in *C. myrtilli* the wings are almost twice as long as the abdomen.

According to the species identification key, the distribution of surface spinules in the s+cs cell in the forewing has been used to separate the closely related species of *C. myrtilli* and *C. ledi* (Ossialnnilsson, 1992). In *C. myrtilli* surface spinules cover the s+cs cell entirely, while in *C. ledi* the spinules are absent in the apical third of the cell. In *C. lapponica* (Fig. 5a), the distribution of spinules is similar to that found in *C. myrtilli*.

Males can also be differentiated by their paramere structure (Fig. 6). In males of *C. lapponica*, the thickest region is in the middle of paramere viewed from behind (Fig. 6a), and a similar region is seen in the apical part of paramere in *C. myrtilli* (Fig. 6b).

Female *C. lapponica* are easily distinguished from *C. myrtilli* females by differences in their terminalia structures (Fig. 7). In *C. lapponica*, the circumanal pore ring complex is symmetric, oval-shaped, and proctiger is sharply pointed (Fig. 7a), whereas in *C. myrtilli*, the same structure is clearly asymmetric and the apical part of proctiger is more rounded (Fig. 7b). As shown below, the subgenital plate evenly decreases in width towards the apex in *C. lapponica* (Fig. 7c), while the width strongly decreased

Figure 3. Adult females of *C. lapponica* sp. nov. (above) and *C. myrtilli* (below) showing conspicuously different sized wings.

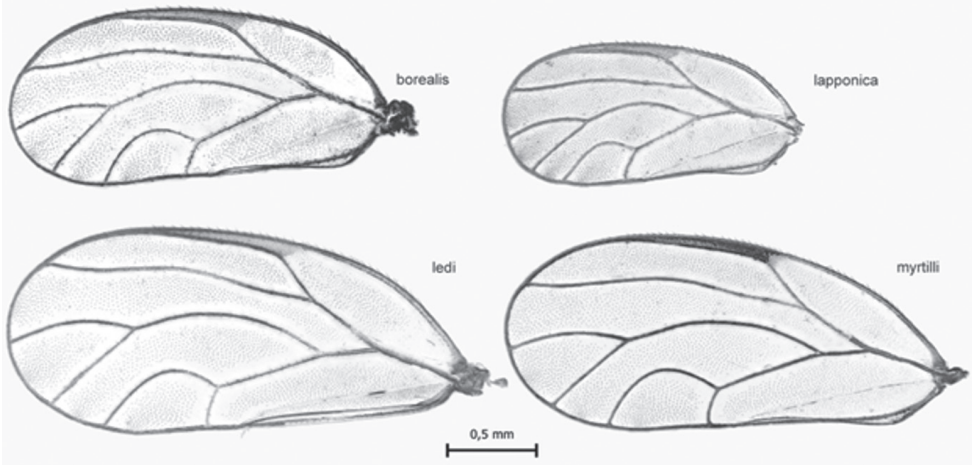


Figure 4. Comparison of forewings in *C. borealis* sp. nov., *C. lapponica* sp. nov., *C. ledi* and *C. myrtilli*.

halfway of the plate in *C. myrtilli* (Fig. 7d). In the side view the subgenital plate differs clearly between the species. In *C. lapponica*, the upper edge runs quite straight and is curved only near the apex (Fig. 7e), while in *C. myrtilli*, there is a strong curve already near the middle of the plate (Fig. 7f).

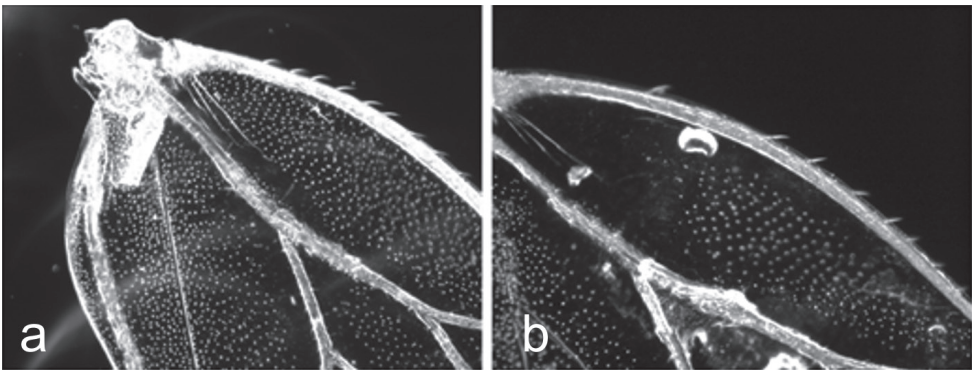


Figure 5. The distribution of surface spinules in the s+cs cell in the forewing **a** *C. lapponica* sp. nov. The distribution of spinules is similar to that of *C. myrtilli* **b** *C. borealis* sp. nov. The distribution of spinules is similar to that of *C. ledi*.

Figure 6. Male parameres from behind **a** *C. lapponica* sp. nov. **b** *C. myrtilli*.

Distribution. Specimens of *C. lapponica* were found in three locations at high altitude above the tree line in northern Sweden and Finland (Table 1). In all these locations, *C. lapponica* coexists with *C. myrtilli* on low growing *V. uliginosum* plants in low numbers. As an example, in a sample collected on 6.8.2016 in Utsojki, Ailigas at 320 m altitude, there were 252 specimens of *C. myrtilli* and among them 15 specimens (8 females and 7 males) of *C. lapponica*, the proportion of *C. lapponica* being 5.6%

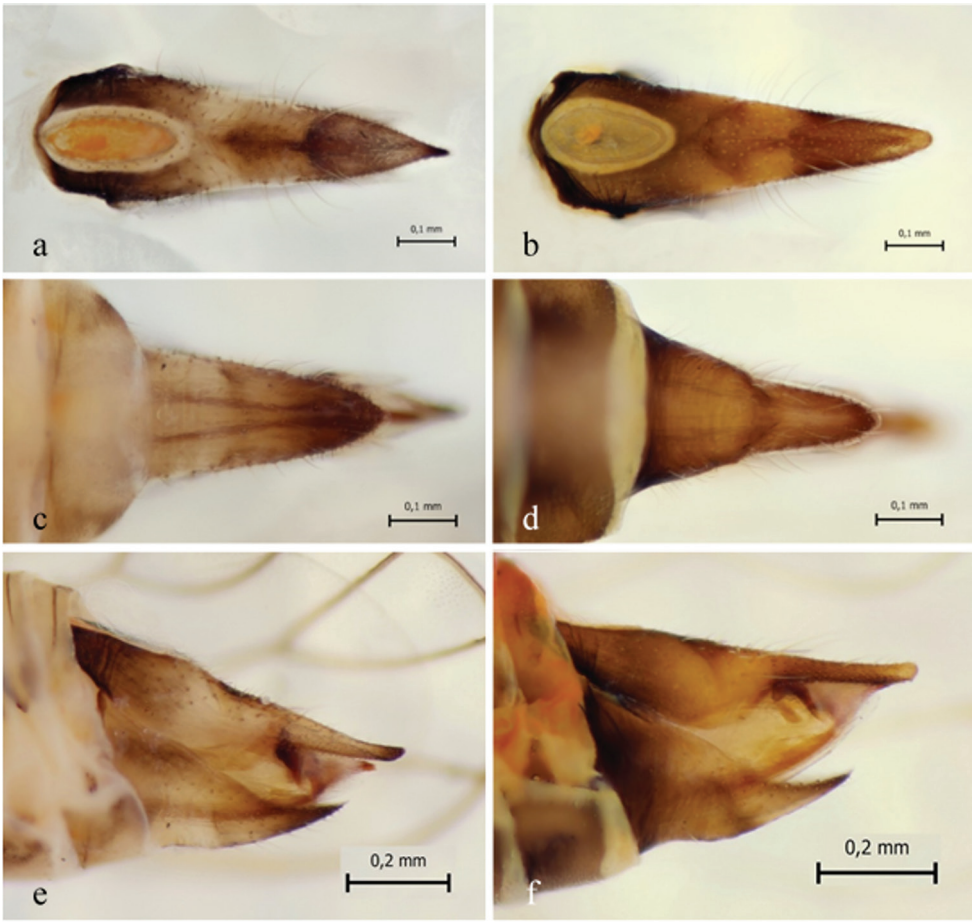


Figure 7. Morphology of female genital structures in *C. lapponica* sp. nov. (**a, c, e**) and *C. myrtilli* (**b, d, f**) **a, b** dorsal plate, proctiger from above **c, d** subgenital plate from below **e, f** genital plates from the left.

of the total. It is obvious, that *C. lapponica* is a rare alpine species restricted to a high-altitude open habitat.

Etymology. The name "lapponica" in Latin means "from Lapponia" or "Lapponian" reflecting the restricted distribution of the species to northern Fennoscandia in locations above the tree line.

***Cacopsylla borealis* S. Nokkala & Ch. Nokkala, sp. nov.**
http://zoobank.org/EB6FC0FC-6BB5-45F4-A4BC-3E88EEAD167A

Type material. *Holotype*: Female; Finland, Salla, Tuntsa; 67°18'11"N, 29°16'18"E; 01. Aug. 2019; Seppo & Christina Nokkala leg.; host *Ledum palustre*; http://mus.utu.fi/ZMUT.TYPE798. ***Paratypes*:** 10 females; Finland, Salla, Tuntsa; 67°18'11"N,

29°16'18"E; 01. Aug. 2019; Seppo & Christina Nokkala leg.; host *Ledum palustre*; http://mus.utu.fi/ZMUT.TYPE799. 5 females; Russia, Baikal; 51°54'25"N, 105°04'14"E; July 2007; E. Labina leg.; host *Ledum palustre*; http://mus.utu.fi/ZMUT.TYPE800. 6 females; Russia, Vorkuta; 67°30'00"N, 64°02'00"E; 6 Aug. 2013; N. Khabasova leg.; host *Ledum palustre*; http://mus.utu.fi/ZMUT.TYPE801. The holotype and paratypes are deposited at the Zoological Museum, University of Turku, Finland.

Description. Adult coloration resembles *C. ledi*, but is more brownish with dark markings. Wings are yellowish and transparent with yellowish veins. Males are unknown. Overall length of females is similar to that of *C. ledi* (2.53–3.04 mm, N = 5).

Diagnosis. The distribution of the surface spinules in the c+sc cell of forewing in *C. borealis* (Fig. 5b) is similar to spinule distribution in *C. ledi*. However, *C. borealis* and *C. ledi* can be indentified reliably by differences in their external morphology (Figs 8–10). A conspicuous difference is found in the forewings, where in clavus, apex and basal wing margin are dark brown in *C. borealis* (Fig. 8). Typically, on mesoscutum there are four highly pigmented longitudinal stripes in *C. borealis*, that are completely absent in *C. ledi* (Fig. 9a–b).

In female terminalia in dorsal view, the circumanal pore ring complex is wider in *C. borealis* than in *C. ledi* (Fig. 9c–d). The ventral subgenital plate seen from below narrows evenly towards the rounded apex in *C. borealis* while in *C. ledi* the structure is more slender and narrows strongly at first and then more evenly towards the apex (Fig. 9e–f). In *C. borealis*, the structure of the female terminalia in side view resembles that found in *C. myrtilli* and is quite different from that found in *C. ledi* (Fig. 9g–h). The antennae in *C. borealis* are thicker and shorter than in *C. ledi* (Fig. 10). Antennae are shortest in *C. lapponica* and thickness is similar compared to that of *C. borealis*, while the antennae have equal length in *C. myrtilli* and *C. ledi*.

Distribution. *C. borealis* forms dense populations in northern Fennoscandia down to latitude 66° (Table 1.) It is also not uncommon to find mixed populations with *C. ledi*. It is easily understood as they are both parthenogenetic and live in those populations strictly reproductively isolated from each other. On the other hand, the wide distribution

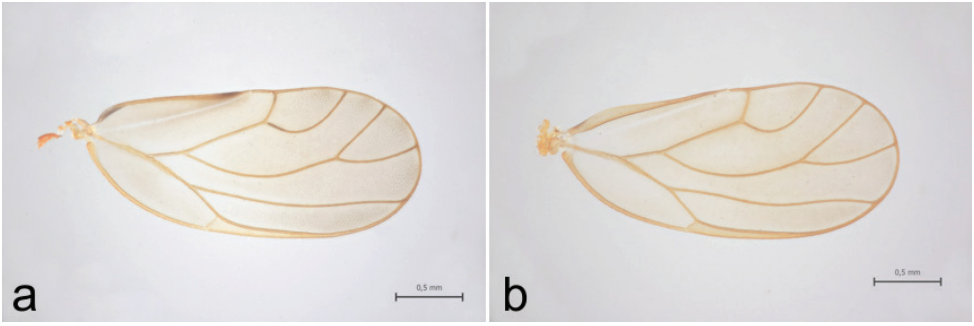


Figure 8. Comparison of forewing coloration in *C. borealis* sp. nov. and *C. ledi* **a** *C. borealis* sp. nov., dark brown apex and basal wing margin in clavus **b** *C. ledi*, clavus without dark brown markings.

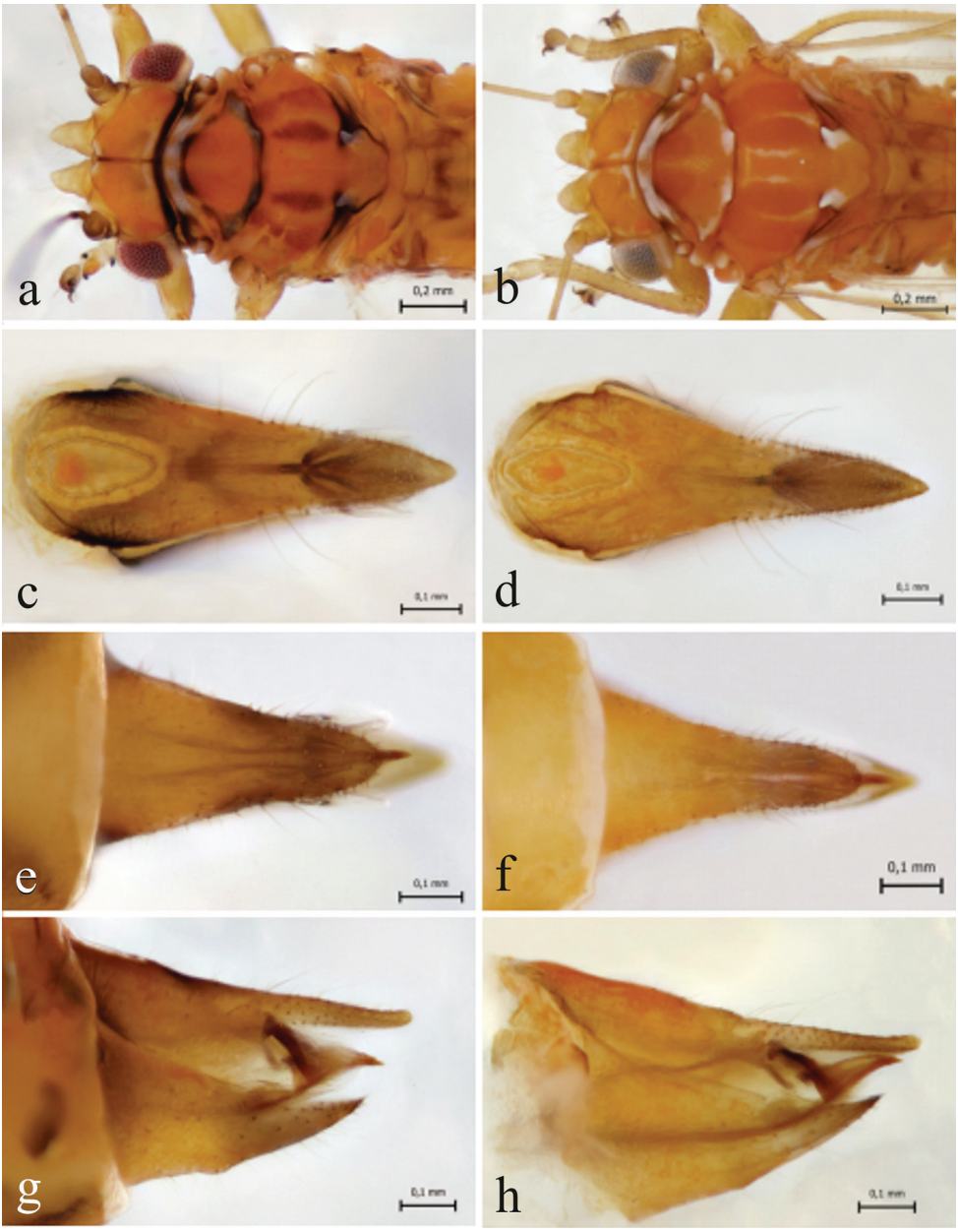


Figure 9. Comparison of morphological details between *C. borealis* sp. nov. (**a, c, e, g**) and *C. ledi* (**b, d, f, h**). Mesoscutum (**a–b**) dorsal plate (proctiger) (**c–d**) subgenital plate, ventral view (**e–f**) female terminalia in side view (**g–h**).

of *C. borealis* from Western Europe to Lake Baikal in the east suggests that *C. borealis* is of old origin. In some locations, most specimens in mixed populations living on *L. palustre* are *C. borealis*, as in Utsjoki, Ailigas (320 m), while in another location, in Utsjoki,

Figure 10. Antennae in *C. borealis* sp. nov., *C. lapponica* sp. nov., *C. ledi* and *C. myrtilli*.

Utsjoki Hietala near the sea level, the proportion of *C. ledi* is close to 20%. However, in several locations, as in Salla, Tuntsa and Salla, Niemelä, as well as in Kuusamo, Kantojoki and Kuusamo, Sakkojoki all individuals collected were *C. borealis*. The species *C. borealis* was found to be quite common in northern Finland. However, the hitherto known distribution of the species in Fennoscandia is restricted to north of latitude 63°.

Etymology. The name "borealis" which means "north" or "northern" in Latin was given because of the wide Palearctic distribution of the species.

Discussion

In the present study, we have described two new psyllid species, *C. lapponica* sp. nov. and *C. borealis* sp. nov. based on *COI* barcoding DNA sequence and uncorrected p-distance differences as well as morphological and cytological characteristics (Tables 2–3).

Table 3. Summary of host plants, type of reproduction and morphological and cytological diagnostic characters among the *Cacopsylla* species studied**.**

Species	Host plant	Type of reproduction	Ploidy level in parthenogenetic females	Karyotype	Type of meiosis in males	Number of follicles per testis	Reference
C. myrtilli	*Vaccinium myrtillus*, *V. uliginosum*	apomictic parthenogenesis (with rare diploid, nonfunctional males)	triploid	3n = 36+XXX	achiasmate	4 (in rare males)	Nokkala et al. 2013
C. borealis	*Ledum palustre*	apomictic parthenogenesis	penta-ploid	5n = 60+XXXXX	–	–	Present study
C. ledi	*L. palustre*	apomictic parthenogenesis (with rare diploid males and females)	triploid	3n = 36+XXX	–	4 (in rare males)	Nokkala et al. 2017
C. lapponica	*V. uliginosum*	bisexual	–	2n = 24+X (males); 24+XX (females)	chiasmate	2	Present study
C. fraudatrix	*V. myrtillus*	bisexual	–	2n = 24+X (males); 24+XX (females)	chiasmate	2	Kuznetsova et al. 2012

The uncorrected p-distance differences between *C. lapponica* sp. nov. and the three species *C. borealis* sp. nov., *C. myrtilli* and *C. ledi* is of the same magnitude (> 3%) as the difference between the recognized species *C. myrtilli* and *C. ledi*. The difference between *C. lapponica* sp. nov. and *C. fraudatrix* is somewhat smaller or 2.04%. *C. lapponica* sp. nov. is a rare, bisexual alpine species with a high-altitude distribution in northern Fennoscandia, utilizing the same food plant and forming sympatric populations with *C. myrtilli*. However, phylogenetically it is associated most closely with another bisexual species, *C. fraudatrix* described from Poland, Bieszczady Mountains (Kuznetsova et al. 2012). Being rare and adapted to an open habitat, *C. lapponica* sp. nov. is very sensitive to exceptional environmental constraints, as dryness or heavy rains. This was realized during 2018, when late summer suffered from extremely warm and dry weather in northern Fennoscandia. Consequently, lower vegetation was completely dried out above tree line in both Utsjoki, Ailigas and Kilpisjärvi. Apparently, for this reason not a single specimen of *C. lapponica* was found in either of these locations. As *C. lapponica* sp. nov. is well adapted to an open habitat and harsh environment, it is plausible that the species might be found in similar habitats in former refugial areas in Central Europe. It is exceptional that a bisexual species, *C. lapponica* sp. nov. has migrated towards the north during recolonization after the last glacial period, while all other related recolonizing species, *C. myrtilli*, *C. ledi* and *C. borealis* sp. nov., are apomictic parthenogens (Nokkala et al. 2008, 2013, 2015, 2017).

On the other hand, *C. borealis* sp. nov. is an abundant pentaploid species with apomictic parthenogenetic reproduction, it has a wide palearctic distribution, may occur alone or form mixed populations with *C. ledi* on *L. palustre*. It is phylogenetically tightly associated with another parthenogen, *C. myrtilli*. In *COI* phylogeny, the parthenogenetic species *C. ledi* and *C. borealis* sp. nov. + *C. myrtilli* form a monophyletic sister clade to the clade consisting of bisexual *C. lapponica* sp. nov. + *C. fraudatrix*. All parthenognetic species form abundant populations north of latitude 63° in Fennoscandia. *C. borealis* sp. nov. has not been recorded south of this, populations of *C. myrtilli* are sparse, while *C. ledi* is relatively abundant, at least in southern parts of Finland around latitude 60° (Nokkala et al. 2017) and is found as south as Germany

(Ossiannilsson 1992). It is remarkable, that the bisexual species *C. lapponica* sp. nov. has an extremely narrow distribution and habitat range compared to the three parthenogenetic species subjected in this study. In fact, these observations on the distribution ranges are well in accordance with the hypothesis of geographic parthenogenesis stating that parthenogenetic species have a wider ecological adaptation ability than their sexual progenitors (Kearney 2005).

In the present study, the two novel species, *C. lapponica* sp. nov. and *C. borealis* sp. nov., were found by chance while sampling the known species of *C. myrtilli* and *C. ledi*. It is plausible that there still exist undescribed species in northern high-altitude habitats waiting to be discovered. As climate change proceeds, northern habitats will experience substantial changes. This may lead to a situation, where an undescribed rare species with restricted habitat requirements will become extinct before it is found. Considering the species in the present study, *C. lapponica* sp. nov. is an example of a species endangered to become extinct, because of its narrow habitat requirements. Therefore, it is evident, that there is an urgent need to look systematically for undescribed species in northern high-altitude habitats.

Conclusions

In the present study we have described two new psyllid species in the genus *Cacopsylla* Ossiannilssion, 1970, *C lapponica* sp. nov. and *C. borealis* sp. nov., based on chromosomal analyses, *COI* haplotype analysis and morphological characters. *C. lapponica* sp. nov. is bisexual and *C. borealis* sp. nov. a pentaploid parthenogenetic species. They may form sympatric populations with *C. myrtilli* and *C. ledi*, respectively. In *COI* phylogeny, *C. lapponica* sp. nov. is associated with another bisexual species, *C. fraudatrix*, while *C. borealis* sp. nov. associates with parthenogenetic *C. myrtilli* and *C. ledi*.

The authors have declared that no competing interests exist.

Acknowledgements

We thank N. Khabasova and E. Labina for collecting specimens from Vorkuta (Republic of Komi) and Lake Baikal, respectively. We also wish to thank Dr. Anna Namyatova and another anonymous reviewer for valuable suggestions on the manuscript. The present study was financially supported by research grant from the Russian Science Foundation no. 19-14-00202 (to VGK).

References

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Kearney M (2005) Hybridization, glaciation and geographical parthenogenesis. Trends in Ecology and Evolution 20 (9): 495–502. https://doi.org/10.1016/j.tree.2005.06.005

Klimaszewski SM (1971) Koliszki (Homoptera, Psyllodea) Bieszczadów. Fragmenta Faunistica 17: 151–178. https://doi.org/10.3161/00159301FF1971.17.7.161

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular Biology and Evolution 35(6): 1547–1549. https://doi.org/10.1093/molbev/msy096

Kuznetsova VG, Labina ES, Shapoval NA, Maryańska-Nadachowska A, Lukhtanov VA (2012) Cacopsylla fraudatrix sp.n. (Hemiptera: Psylloidea) recognised from testis structure and mitochondrial gene COI. Zootaxa 3547(1): 55–63. https://doi.org/10.11646/zootaxa.3547.1.5

Nokkala C, Kuznetsova VG, Nokkala S (2015) Rare diploid females coexist with rare males: a novel finding in triploid parthenogenetic populations in the psyllid *Cacopsylla myrtilli* (W. Wagner, 1947) (Hemiptera, Psylloidea) in northern Europe. Genetica 143(5): 589–595. https://doi.org/10.1007/s10709-015-9858-x

Nokkala C, Kuznetsova VG, Nokkala S (2013) Meiosis in rare males in parthenogenetic *Cacopsylla myrtilli* (Wagner, 1947) (Hemiptera, Psyllidae) populations from northern Europe. Comparative Cytogenetics 7(3): 241–251. https://doi.org/10.3897/compcytogen.v7i3.6126

Nokkala S, Kuznetsova VG, Nokkala C (2017) Characteristics of parthenogenesis in *Cacopsylla ledi* (Flor, 1861) (Hemiptera, Sternorryncha, Psylloidea): cytological and molecular approaches. Comparative Cytogenetics 11(4): 807–817. https://doi.org/10.3897/compcytogen.v11i4.21362

Nokkala S, Maryańska-Nadachowska A, Kuznetsova VG (2008) First evidence of polyploidy in Psylloidea (Homoptera, Sternorrhyncha): a parthenogenetic population of *Cacopsylla myrtilli* (W. Wagner, 1947) from northeast Finland is apomictic and triploid. Genetica 133(2): 201–205. https://doi.org/10.1007/s10709-007-9200-3

Ossiannilsson F (1992) The Psylloidea (Homoptera) of Fennoskandia and Denmark. Fauna Entomologica Scandinavica 26: 1–346.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systymatc Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

CompCytogen 13(4): 383–388 (2019)
doi: 10.3897/CompCytogen.v13i4.47302
http://compcytogen.pensoft.net

SHORT COMMUNICATION

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

Description of the karyotype of *Sphyracephala detrahens* (Diptera, Diopsidae)

Ayumi Kudo[1]

1 *Department of Biological Sciences School of Science, Tokyo Metropolitan University, Minamiohsawa 1-1, Hachiohji-shi, Tokyo 192-0397, Japan*

Corresponding author: *Ayumi Kudo* (kudokudo1103@gmail.com)

Academic editor: *I. Sharakhov* | Received 14 October 2019 | Accepted 31 October 2019 | Published 3 December 2019

http://zoobank.org/022BF5FE-4994-474F-94D3-40AFFF0090AA

Citation: Kudo A (2019) Description of the karyotype of *Sphyracephala detrahens* (Diptera, Diopsidae). Comparative Cytogenetics 13(4): 383–388. https://doi.org/10.3897/CompCytogen.v13i4.47302

Abstract

The eye stalks in Diopsidae (Bilberg, 1820) have been widely examined, but the evolutionary origin of this unique trait remains unclear. Thus, further studies of *Sphiracephala* (Say, 1828), the extant genus forming a basal branch of Diopsinae, are needed. The present study aimed to identify the karyotype of *Sphyracephala detrahens* (Walker, 1860) with conventional Giemsa staining. Cytogenetic analysis revealed a diploid number of 2n = 10 including two pairs of metacentric chromosomes, a pair of telocentric chromosomes, a pair of dot-like chromosomes, and a pair of sex chromosomes in *S. detrahens*. The congener *Sphyracephala brevicornis*(Say, 1817) has been reported to have the same diploid number, 2n = 10, but different chromosome formula. These results demonstrate that chromosome rearrangements often occur in the genus *Sphyracephala*.

Keywords

Cytogenetics, chromosomes, karyology

Introduction

Nearly all species of Diopsidae (Bilberg, 1820) are well-known for their exaggerated eye stalks (Shillito 1971). There are approximately 160–8000 species and 10–15 genera containing stalk-eyed flies in the family Diopsidae (Shillito 1971; Steyskal 1972; Carr et al. 2006; Ovtshinnikova and Galinskaya 2016; Roskov et al. 2019). Although

both males and females in Diopsinae have eyes that are laterally displaced from the central head, the level of sexual dimorphism varies between and within species (Burkhardt and de la Motte 1985; Wilkinson and Dodson 1997; Meier and Hilger 2000). Some species of stalk-eyed flies with extreme sexual dimorphism are used as model organisms to study the evolution of sexually selected traits (Wilkinson et al. 1998; Carr et al. 2005; Husak and Swallow 2011; Knell et al. 2013). For example, in *Teleopsisdalmanni* (Wiedemann, 1830), morphology, sexual behavior, development, and cytology has been widely studied (Wilkinson and Reillo 1994; Presgraves et al. 1997; Hurley et al. 2002; Egge et al. 2011; Worthington et al. 2012; Cotton et al. 2015; Meade et al. 2019). However, limited information regarding the ecology, biology, and cytology of most stalk-eyed fly species is available, particularly for monomorphic species and primitive groups such as Sphyracepalini.

Sphyracephala shows the most likely ancestral state of extant Diopsinae (Kotrba 2004). *Sphyracephala detrahens* (Walker, 1860) is distributed in Taiwan, China, the Philippines, Indonesia, Papua New Guinea, and the southern islands of Japan (Ohara 1993). A few studies have examined the ecology and morphology of Japanese populations, and found the length of eye stalks less or not sexually dimorphic (Ohara 1993, 1997).

Although Baker and Wilkinson (2001) suggested that ancestral species in Diopsinae share monomorphic eye stalks, Kotrba (2004) used cladistics analysis that included the extinct species of *Prosphyracephala* to predict that sexual dimorphic eye stalks evolved in early Diopsinae. To reveal the origin of eye stalks in Diopsinae, basic studies including cytogenetic analysis of the species in Sphyracephalini need to be performed. The current study aimed to describe the karyotype of *S. detrahens* using standard chromosome staining.

Material and methods

S. detrahens was collected from Iriomote Island, Okinawa, Japan in April 2019 by A. Kudo (Fig. 1).All flies were maintained on organic media with yeast at 25 °C in a 14-h light:10-h dark cycle.

Metaphase chromosomes were obtained from cerebral ganglia of 3rd instar larvae as described by Imai et al. (1988) without colchicine treatment. The chromosome preparations were stained with 5% Giemsa solution. The preparations were observed under a Keyence BZ-X700 fluorescence microscope (Osaka, Japan) equipped with a Nikon Plan Apo100×/1.45 oil objective and Nikon immersion Oil Type NF (Tokyo, Japan). Twenty metaphase cells with well-spread chromosomes were selected and photographed using Keyence BZ-X Analyzer software, and then processed in GIMP ver. 2. 10. 12. Fifteen individuals including 10 females and 5 males were successfully karyotyped. The length of the long and short chromosome arm was measured with Image J software ver. 1.52a (NIH, Bethesda, MD, USA). These data were used to calculate the chromosome index and arm ratio, following which chromosome classification and idiogram construction were performed as described by Levan et al. (1964).

Figure 1. Stalk-eyed flies *Sphyracephala detrahens*.

Table 1. Morphometric parameters of *Sphyracephala detrahens* chromosomes from mitotic metaphase plates.

Chromosome	Length of short arm (mean ± SE µm)	Length of long arm (mean ± SE µm)	Total length of Chromosome (mean ± SE µm)	Arm ratio†	Centromeric index‡	Chromosome classification§
1	4.11 ± 0.15	4.45 ± 0.15	8.56 ± 0.29	1.08	48.0	m
2	2.49 ± 0.09	2.94 ± 0.10	5.43 ± 0.18	1.18	45.9	m
3	–	–	3.95 ± 0.14	–	–	t
4	–	–	0.66 ± 0.02	–	–	d
X	1.63 ± 0.06	3.56 ± 0.11	5.19 ± 0.17	2.18	31.5	sm
Y	1.80 ± 0.18	2.13 ± 0.24	3.93 ± 0.41	1.18	45.9	m

† Arm ratio = length of long arm/length of short arm;
‡ Centromeric index = length of short arm/total length of chromosome;
§ Chromosome classification; m: metacentric chromosome; sm: submetacentric chromosome; t: telocentric chromosome; d: dot-like chromosome.

Results and discussion

This is the first study to reveal that the chromosome number of *S. detrahens* was 2n = 10 (Fig. 2). The karyotype of *S. detrahens* consisted of two pairs of metacentric chromosomes, a pair of rod-shaped telocentric chromosomes, a pair of dot-like microchromosomes, and a pair of sex chromosomes (Figs 2, 3). In the female cerebral ganglia cells, a homomorphic sex chromosome pair was formed by the two submetacentric X-chromosomes (Fig. 2A). In the male cerebral ganglia cells, a heteromorphic pair of sex chromosomes was formed by the X-chromosome and metacentric Y-chromosome (Fig. 2B). The Y-chromosome was slightly stained and was shorter than the X-chromosome (Fig. 2).

Although a congener, *S. brevicornis*, had the same diploid chromosome number 2n = 10, the karyograms of *S. brevicornis* differed from that of *S. detrahens* (Fig. 3); the karyotype of *S. brevicornis* consisted of two pairs of metacentric chromosomes, two pairs of telocentric chromosomes, and a pair of small telocentric XY pair (Jan 1966).

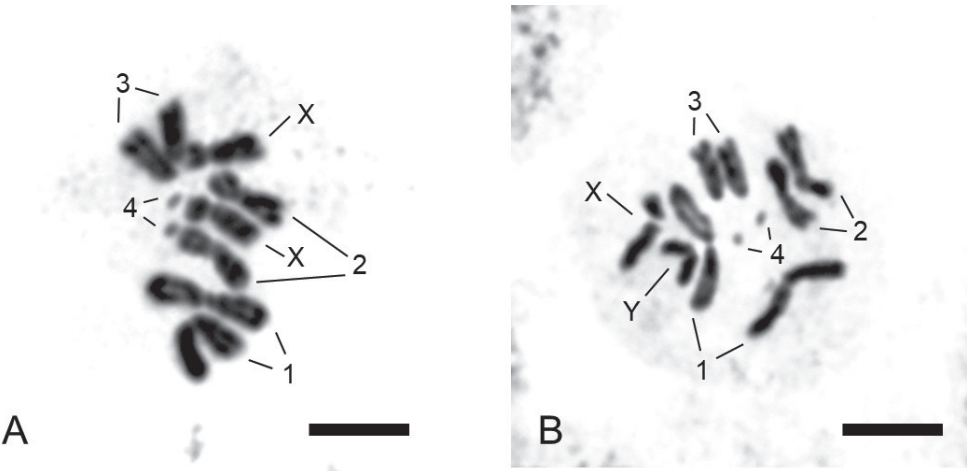


Figure 2. Mitotic metaphase of *Sphyracephala detrahens* with 2n = 10 chromosomes **A** female **B** male. Scale bars: 5μm.

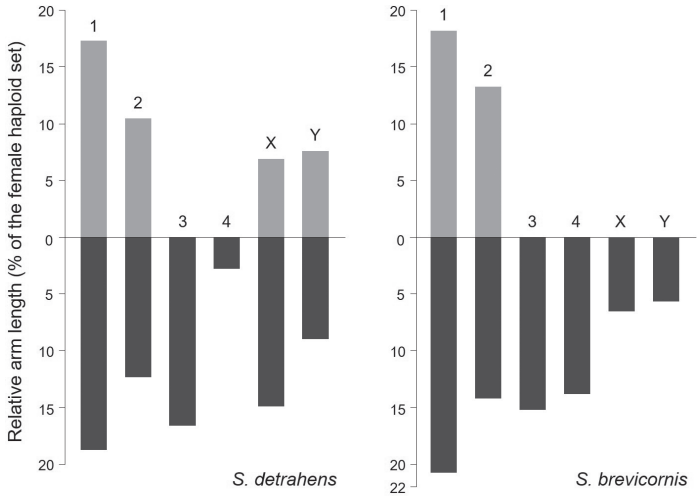


Figure 3. Idiograms of *Sphyracephala detrahens* and *Sphyracephala brevicornis*. The numbers above each bar indicate chromosome numbers. The light and dark regions represent short arms and long arms, respectively. Idiograms of *S. brevicornis* were modified and redrawn from Idiogram 1 of *S. brevicornis* (Jan 1966).

The sex chromosomes showed the greatest differences between the two species. Both the X and Y chromosomes in *S.detrahens* were bi-armed and larger compared to those in *S. brevicornis*. Thus, chromosomal rearrangements occurred in these two species and their relatives. Information about the phylogenetic relationships between *S. detrahens* and its congeners has been never analyzed. Further investigations into phylogenetic relationships will aid in the understanding of differences in karyograms between *S. detrahens* and *S. brevicornis*. Despite the lack of karyological information in Diopsinae, comparative cytogenetic analyses using related species will lead to a greater understanding of chromosomal evolution in stalk-eyed flies.

Acknowledgements

I would like to thank Haruna Fujioka, Wataru Kojima and Yasukazu Okada for collecting flies; Keiko Sakiyama for fly maintenance; Kazuyuki Hirai for his technical advice on chromosomal preparation; and Masafumi Nozawa for comments on karyotyping. I also thank Igor Sharakhov for comments on this manuscript.

References

Baker RH, Wilkinson GS (2001) Phylogenetic analysis of sexual dimorphism and eyespan allometry in stalk-eyed flies (Diopsidae). Evolution 55: 1373–1385. https://doi.org/10.1111/j.0014-3820.2001.tb00659.x

Burkhardt D, de la Motte I (1985) Selective pressures, variability, and sexual dimorphism in stalk-eyed flies (Diopsidae). Naturwissenschaften 72: 204–206. https://doi.org/10.1007/BF01195763

Carr M, Cotton S, Foldvari M, Kotrba M (2006) A description of a new species of *Diasemopsis* (Diptera, Diopsidae) from the Comoro Islands with morphological, molecular and allometric data. Zootaxa 1211: 1–19. https://doi.org/10.11646/zootaxa.1211.1.1

Carr M, Hurley I, Fowler K, Pomiankowski A, Smith HK (2005) Expression of *defective proventriculus* during head capsule development is conserved in *Drosophila* and stalk-eyed flies (Diopsidae). Development Genes and Evolution 215: 402–409. https://doi.org/10.1007/s00427-005-0488-7

Cotton AJ, Cotton S, Small J, Pomiankowski (2015) Male mate preference for female eyespan and fecundity in the stalk-eyed fly, *Teleopsisdalmanni*. Behavioral Ecology 26: 376–385. https://doi.org/10.1093/beheco/aru192

Egge AR, Brandt Y, Swallow JG (2011) Sequential analysis of aggressive interactions in the stalk-eyed fly *Teleopsisdalmanni*. Behavioral Ecology and Sociobiology 65: 369–379. https://doi.org/10.1007/s00265-010-1054-5

Hurley I, Pomiankowski A, Fowler K, Smith H (2002) Fate map of the eye-antennal imaginal disc in the stalk-eyed fly *Cyrtodiopsis dalmanni*. Development Genes and Evolution 212: 38–42. https://doi.org/10.1007/s00427-001-0206-z

Husak JF, Swallow JG (2011) Compensatory traits and the evolution of male ornaments. Behaviour 148: 1–29. https://doi.org/10.1163/000579510X541265

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

Jan KY (1966) Cytotaxonomical studies of some Schizophorous diptera. PhD Thesis, McGill University, 205 pp. http://digitool.Library.McGill.CA:80/R/-?func=dbin-jump-full&object_id=43809&silo_library=GEN01

Knell RJ, Naish D, Tomkins JL, Hone DWE (2013) Sexual selection in prehistoric animals: detection and implications. Trends in Ecology & Evolution 28: 38–47. https://doi.org/10.1016/j.tree.2012.07.015

Kotrba M (2004) Baltic amber fossils reveal early evolution of sexual dimorphism in stalk-eyed flies (Diptera: Diopsidae). Organisms, Diversity & Evolution 4: 265–275. https://doi.org/10.1016/j.ode.2004.02.005

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

Meade LC, Dinneen D, Kad R, Lynch DM, Fowler K, Pomiankowski A (2019) Ejaculate sperm number compensation in stalk-eyed flies carrying a selfish meiotic drive element. Heredity 122: 916–926. https://doi.org/10.1038/s41437-018-0166-y

Meier R, Hilger S (2000) On the egg morphology and phylogenetic relationships of Diopsidae (Diptera: Schizophora). Journal of Zoological Systematics and Evolutionary Research 38: 1–36. https://doi.org/10.1046/j.1439-0469.2000.381128.x

Ohara K (1993) First record of the family Diopsidae from Japan (Diptera, Brachycera). Bulletin of the Tokushima Prefectural Museum 3: 39–47. [In Japanese]

Ohara K (1997) *Sphyracephala detrahens* (Diptera, Diopsidae) in the Yaeyama Islands, Okinawa, Japan. Bulletin of the Tokushima Prefectural Museum 7: 25–37. [In Japanese]

Ovtshinnikova OG, Galinskaya TV (2016) Musculature of the male genitalia of a member of the genus *Teleopsis* Rondani, 1875 (Diopsidae, Diptera). Oriental Insects 50: 61–68. https://doi.org/10.1080/00305316.2016.1171174

Presgraves DC, Severance E, Wilkinson GS (1997) Sex chromosome meiotic drive in stalk-eyed flies. Genetics 147: 1169–1180.

Roskov Y, Ower G, Orrell T, Nicolson D, Bailly N, Kirk PM, Bourgoin T, DeWalt RE, Decock W, Nieukerken E van, Zarucchi J, Penev L (Eds) (2019) Species 2000 & ITIS Catalogue of Life, 2019 Annual Checklist. Digital resource at www.catalogueoflife.org/annual-checklist/2019. Species 2000: Naturalis, Leiden, the Netherlands. ISSN 2405-884X.

Shillito JF (1971) The genera of Diopsidae (Insecta: Diptera). Zoological Journal of the Linnean Society 50: 287–295. https://doi.org/10.1111/j.1096-3642.1971.tb00763.x

Steyskal GC (1972) A Catalogue of Species and Key to the Genera of the Family Diopsidae (Diptera: Acalyptratae). Stuttgarter Beiträgezur Naturkunde 234: 1–19. https://www.biodiversitylibrary.org/page/33729331#page/177/mode/1up

Wilkinson GS, Dodson GN (1997) Function and Evolution of Antlers and Eye Stalks in Flies. The Evolution of Mating Systems in Insects and Arachnids. Cambridge University Press, Cambridge, 310–328. https://doi.org/10.1017/CBO9780511721946.019

Wilkinson GS, Presgraves DC, Crymes L (1998) Male eye span in stalk-eyed flies indicates genetic quality by meiotic drive suppression. Nature 391: 276–279. https://doi.org/10.1038/34640

Wilkinson GS, Reillo PR (1994) Female choice response to artificial selection on an exaggerated male trait in a stalk-eyed fly. Proceedings of the Royal Society B: Biological Sciences 255: 1–6. https://doi.org/10.1098/rspb.1994.0001

Worthington AM, Berns CM, Swallow JG (2012) Size matters, but so does shape: quantifying complex shape changes in a sexually selected trait in stalk-eyed flies (Diptera: Diopsidae). Biological Journal of the Linnean Society 106: 104–113. https://doi.org/10.1111/j.1095-8312.2011.01841.x

CompCytogen 13(4): 389–402 (2019)
doi: 10.3897/CompCytogen.v13i4.36879
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Molecular-cytogenetic analysis of diploid wheatgrass *Thinopyrum bessarabicum* (Savul. and Rayss) A. Löve

Ekaterina D. Badaeva[1,2], Sergei A. Surzhikov[2], Alexander V. Agafonov[3]

1 *N.I. Vavilov Institute of General Genetics, Russian Academy of Sciences. Gubkina str. 3, Moscow 117333, Russia* **2** *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences. Vavilova str. 34, Moscow 117334, Russia* **3** *Central Siberian Botanical Garden, Russian Academy of Sciences, Siberian Branch, Zolotodolinskaya st., 101, Novosibirsk 630090, Russia*

Corresponding author: *Ekaterina D. Badaeva* (katerinabadaeva@gmail.com)

Academic editor: *Manoj Dhar* | Received 6 June 2019 | Accepted 9 November 2019 | Published 3 December 2019

http://zoobank.org/8B0EA133-1EC0-4C81-A87B-6CD275877833

Citation: Badaeva ED, Surzhikov SA, Agafonov AV (2019) Molecular-Cytogenetic analysis of diploid wheatgrass *Thinopyrum bessarabicum* (Savul. and Rayss) A. Löve. Comparative Cytogenetics 13(4): 389–402. https://doi.org/10.3897/CompCytogen.v13i4.36879

Abstract

Thinopyrum bessarabicum (T. Săvulescu & T. Rayss, 1923) A. Löve, 1980 is diploid (2n=2x=14, JJ or E^bE^b), perennial self-fertilizing rhizomatous maritime beach grass, which is phylogenetically close to another diploid wheatgrass species, *Agropyron elongatum* (N. Host, 1797) P. de Beauvois, 1812. The detailed karyotype of *Th. bessarabicum* was constructed based on FISH with six DNA probes representing 5S and 45S rRNA gene families and four tandem repeats. We found that the combination of pAesp_SAT86 (= pTa-713) probe with pSc119.2 or pAs1/ pTa-535 allows the precise identification of all J-genome chromosomes. Comparison of our data with the results of other authors showed that karyotypically *Th. bessarabicum* is distinct from *A. elongatum*. On the other hand, differences between the J-genome chromosomes of *Th. bessarabicum* and the chromosomes of hexaploid *Th. intermedium* (N. Host, 1797) M. Barkworth & D.R. Dewey, 1985 and decaploid *Th. ponticum* (J. Podpěra, 1902) Z.–W. Liu & R.–C. Wang, 1993 in the distribution of rDNA loci and hybridization patterns of pSc119.2 and pAs1 probes could be an indicative of (1) this diploid species was probably not involved in the origin of these polyploids or (2) it could has contributed the J-genome to *Th. intermedium* and *Th. ponticum*, but it was substantially modified over the course of speciation

Keywords

Chromosome, evolution, *FISH*-karyotyping, J genome, rRNA gene distribution, *Thinopyrum bessarabicum*

Introduction

Thinopyrum bessarabicum (T. Săvulescu & T. Rayss, 1923) A. Löve 1980 (syn. *Agropyron bessarabicum* T. Săvulescu & T. Rayss, 1923 or *A. junceum* (K. Linnaeus, 1753) P. de Beauvois, 1812) is a diploid (2n = 2x = 14, JJ or E^bE^b), perennial self-fertilizing rhizomatous maritime beach grass (Dewey 1984; Wang 2011). Phylogenetically it is closely related to another diploid wheatgrass species, *A. elongatum* (N. Host, 1797) P. de Beauvois 1812 (2n = 2x = 14, EE or J^eJ^e), and in some taxonomical systems they are assigned to a common genomic group (Dvořák 1981; Dewey 1984; Wang and Lu 2014). Other authors, however, showed that genomes of these species are genetically distinct (Wang 1985; Jauhar 1988; Forster and Miller 1989; Moustakas 1991; Linc et al. 2017) and differ from each other in a number of species-specific chromosome rearrangements (Gaál et al. 2018; Grewal et al. 2018). *Th. bessarabicum* is thought to be the parental form of many polyploidy *Thinopyrum* Á. Löve, 1980 species including tetraploid *Th. distichum* (C.P. Thunberg, 1794) Á. Löve 1980, *Th. sartorii* (P.E. Boissier & T. von Heldreich, 1859) Á. Löve 1980 and *Th. junceiforme* (Á. Löve & D. Löve, 1948) Á. Löve 1980 with the genome constitution JJEE or $E^bE^bE^eE^e$, and hexaploid *Th. intermedium* (N. Host, 1797) M. Barkworth and D.R. Dewey 1985 (2n = 6x = 42, $EEE^{st}E^{st}StSt$) and *Th. junceum* (K. Linnaeus, 1753) Á. Löve 1980 (syn. *Elymus farctus* (D. Viviani, 1808) Runemark ex Melderis 1978) with the genome constitution 2n = 6x = 42, JJJJEE or $E^bE^bE^bE^bE^eE^e$ (Dewey 1984; Charpentier 1992; Liu and Wang 1993; Chen et al. 1998; Tang et al. 2000; Wang et al. 2010; Wang 2011; Kruppa and Molnar-Lang 2016). Genomes related to the J-genome of *Th. bessarabicum* could also present in decaploid *Th. ponticum* (J. Podpěra, 1902) Z.-W. Liu and R.-C. Wang 1993 (2n = 10x = 70, $EEEEEEE^{st}E^{st}E^{st}E^{st}$ or EEEEEEStStStSt (Chen et al. 1998).

The natural distribution range of *Th. bessarabicum* spans along Black sea shore from southeastern and eastern Europe to Turkey (Wang 2011). Because of high tolerance to soil salinity (Gorham et al. 1985; Forster et al. 1987; King et al. 1997; Ceoloni et al. 2015) and pest resistance (Zhang et al. 2002; Xu et al. 2009; Zheng et al. 2014; Grewal et al. 2018), this species is considered as valuable source of useful genes for wheat improvement (William and Mujeeb-Kazi 1993). A number of common wheat-*Th. bessarabicum* amphiploids, disomic addition, substitution, and recombinant lines were produced and characterized using molecular, genetic and cytogenetic methods (William and Mujeeb-Kazi 1993; Zhang et al. 2002; Qi et al. 2010; Patokar et al. 2016; Du et al. 2017; Grewal et al. 2018; Hamdani et al. 2018). As a result of analysis of wheat-*Th. bessarabicum* recombinant lines using a combination of cytogenetic technique with high-throughput genotyping, the homoeologous relationships of all individual *Th. bessarabicum* chromosomes with common wheat chromosomes were established (Grewal et al. 2018). A significant syntenic relationship between the seven linkage groups of *Th. bessarabicum* and their orthologous chromosomes from A, B and D genomes of *Triticum aestivum* K. Linnaeus, 1753 was shown. As a diploid wheat, *Th. bessarabicum* carries a species-specific translocation between 4J and 5J chromosomes, but it possesses additional centomeric translocation between 2J and 5J and a paracentric inversion of 7JS chromosome (Grewal et al. 2018).

Th. bessarabicum is characterized by symmetric karyotype consisting of metacentric and submetacentric chromosomes. Four chromosomes carry satellites (SAT) on their short arms. Due to similarity of size and morphological parameters of the J-genome chromosomes, additional methods are necessary for their identification.

The C-banding technique, which was broadly used at the end of XXth for chromosome identification in wheat and related species, was also employed for the analysis of *Th. bessarabicum* chromosomes (Endo and Gill 1984; William and Mujeeb-Kazi 1993; Mirzaghaderi et al. 2010). These studies showed that the J-genome chromosomes possess Giemsa C-bands in subtelomeric regions of either one or both chromosome arms, and small intercalary heterochromatin blocks appear in perinucleolar regions of the SAT chromosomes (Endo and Gill 1984; William and Mujeeb-Kazi 1993). The lack of diagnostic intercalary C-bands restricts applicability of this method for *Th. bessarabicum* chromosome identification.

Fluorescence *in situ* hybridization or FISH provides a broad prospective for plant chromosome analysis. This approach has already been applied for *Th. bessarabicum*, and a standard set of probes – 45S rDNA, pSc119.2, or pAs1 was used for chromosome identification (Du et al. 2017; Linc et al. 2017; Grewal et al. 2018). Besides them, Du et al. (2017) developed several novel J-genome specific oligo-probes with predominantly subtelomeric location for the detection of alien chromatin in wheat-*Th. bessarabicum* introgression lines.

In a current study we mapped six "classical" DNA probes, including 45S and 5S rDNAs (Gerlach and Bedbrook 1979, Gerlach and Dyer 1980), pSc119.2 (Bedbrook et al. 1980), pAs1 (Rayburn and Gill 1986) together with two recently isolated DNA sequences pTa-535 (Komuro et al. 2013) and pAesp_SAT86 (Badaeva et al. 2015) on chromosomes of diploid *Th. bessarabicum* to develop molecular karyotype of this species. Two polyploid *Thinopyrum* species – *Th. intermedium* and *Th. ponticum*, which presumably contain the J-genome, were included in the investigation in order to verify the relationships between species.

Material and methods

Thinopyrum accessions used in analyses, their origin and genome constitution are given in Table 1.

Fixation of the material, slide preparation and fluorescence *in situ* hybridization (FISH) were carried out as described earlier (Badaeva et al. 2017). The oligo-probes pSc119.2, pAs1-1, and pTa-535-1 labelled at the 5' end with fluorescein (pSc119.2, pAs1) or with Cy-3 (pAs1 and pTa-535) were synthesized in the Laboratory of Biological Microchips of the Engelhardt Institute of Molecular Biology RAS (Moscow, Russia) according to Tang et al. (2014). The probes pTa71, pTa794, and pAesp_SAT86 were prepared by labeling plasmid DNA with fluorescein-12 dUTP or biotin-16-dUTP (Roche, Germany) using nick-translation kit (Roche, Germany). The slides were analyzed on a Zeiss Imager D1 microscope. Metaphase plates were photographed at magnification 100× with a black and white digital camera Axiocam HRm using a software AxioVision, release 4.6. The images were processed using Adobe Photoshop, version 7.0.

Table 1. List of materials studied and their origin.

No	Species	Accession #	2*n*	Ploidy level	Genome composition (per 1*n*)*	Origin	Donor name
1	*Thinopyrum bessarabicum*	W6 10232	14	2×	J or E^b	Russia, Crimea	USDA-ARS (U.S.A.)
2	*Th. bessarabicum*	PI 531711	14	2×	J or E^b	Russia, Crimea	USDA-ARS (U.S.A.)
3	*Th. intermedium*	–	42	6×	$E^{st}E^{st}$St or EtEst(V-J-R)	Russia, unknown	obtained from collection of Moscow Scientific-Research Agricultural Institute of Nonchernozem Zone "Nemchinovka"
4	*Th. ponticum*	–	70	10×	$EEEE^{st}E^{st}$ or EEEStSt	Russia, on a sea shore of the island Sergeevskyi, White sea	collected by Dr. A.A. Pomortsev, Vavilov Institute of General Genetics RAS, Moscow, Russia

* – Genome symbols are given according to Wang (2011).

Results

FISH with pTa71 probe revealed four prominent 45S rDNA signals in the regions of secondary constrictions of two pairs of *Th. bessarabicum* chromosomes (Fig. 1a). Two large pTa794 (5S rDNA) sites were found on a chromosome pair carrying large satellites. They were located on satellites, distally to NORs, which is typical for the genetic group 1 of the Triticeae. Very tiny 5S rDNA signals appeared occasionally in the middle of short arm of the second pair of SAT chromosomes. As far as signals were observed in some, but not all cells, they were not considered in the analysis.

Hybridization pattern of oligo-pAs1 and oligo-pSc119.2 probes obtained in a current study (Fig. 1b) corresponded to those published earlier by Grewal et al. (2018), which allowed us to classify the J-genome chromosomes according to genetic nomenclature reported in this paper. Unequal pSc119.2-sites were present in subterminal regions of either both (1J, 3J, 4J, 6J) or only one chromosome arm (2JS, 5JS, 7JS). The largest pSc119.2 signals were observed on 2JS, 4J, and 6J, whereas chromosome 5J had the smallest signals (Figs 1b–d, 2).

Hybridization with pAs1 probe resulted in fuzzy labelling of distal chromosome halves; signal intensities varied from medium to relatively high depending on a chromosome and fluorochrome used (signals generated by Fluorescein-labelled pAs1 probe (Fig. 2, lanes D, E) were always weaker than signals of the same probe labelled with Cy3 or TAMRA (Fig. 2, lanes B, G), and only strongest FITC-signals were visualized by FISH). Most intense pAs1-signals were found on 5JL, 6JS, and in the distal and median regions of the 7J short arm (Figs 1b, 2). Labelling patterns of pTa-535 probe (Figs 1c, 2) were similar to those of pAs1, although pTa-535 signals on 3JL were significantly stronger, while those on 1J – slightly weaker compared to pAs1.

Hybridization with the pAesp_SAT86 probe produced sharp, large diagnostic signals on four (1J, 4J, 5J, and 6J) out of seven pairs of *Th. bessarabicum* chromosomes (Figs 1d, 2). Labelling patterns were identical in both *Th. bessarabicum* accessions and, in combination with either pSc119.2 or pAs1/ pTa-535, allowed the precise identification of all J-genome chromosomes. The chromosome 1J was characterized by bright double signals in the middle of long arm, and 5J contained diagnostic prominent sig-

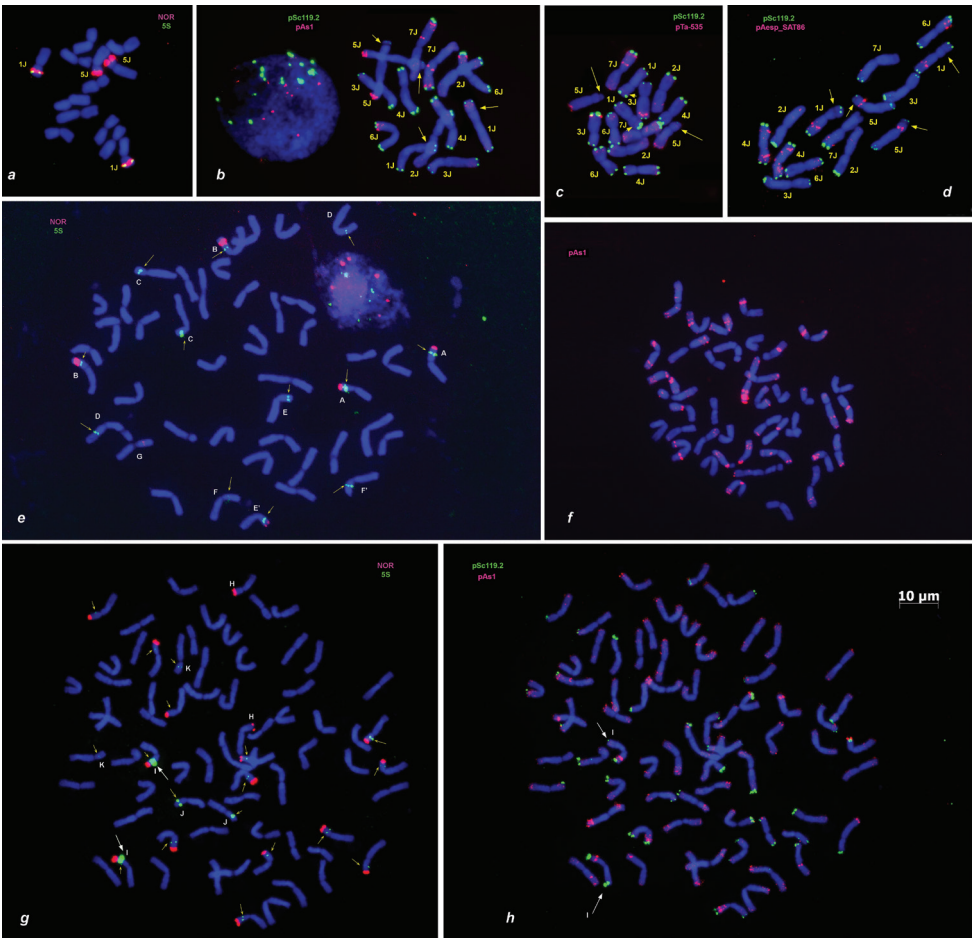


Figure 1. Distribution of rDNA probes and different tandem repeats on metaphase chromosomes of perennial grass species: *Th. bessarabicum* W6 10232 (**a–c**) and PI 531711 (**d**), *Th. intermedium* (**e, f**); and *Th. ponticum* (**g, h**). Probe combination in **a, e, g** pTa71, red + pTa794, green **b, h** pSc119.2, green + pAs1, red **c** pSc119.2, green + pTa-535, red **d** pSc119.2, green + pAesp_SAT86, red **f** pAs1, red. The letters from A to K designate pairs of homologous chromosomes identified in *Th. intermedium* (**e**) or *Th. ponticum* (**g**) mitotic cells based on characteristic patterns of 5S and/or 45S rDNA probes. Yellow arrows (**b–d**) show position of secondary constrictions on *Th. bessarabicum* chromosomes. 5S rDNA sites on *Th. intermedium* (**e**) or *Th. ponticum* (**g**) chromosomes are indicated with small arrows. White arrows (**h**) show homologous *Th. ponticum* chromosomes with contrasting pSc119.2 patterns. Scale bar: 10 µm.

nal in the short arm, adjacent to the centromere (Figs 1d, 2). Chromosomes 4J and 6J, which were hardly distinguishable based on pSc119.2 and pAs1 labelling patterns, were easily discriminated using the pAesp_SAT86 probe. Chromosome 4J carried two prominent signals in the short and two in the long arm, while the chromosome 6J was characterized by double pAesp_SAT86 sites in a distal part of the short arm (Figs 1d, 2).

Relatively faint pAesp_SAT86 signals were detected on chromosomes 2J and 7J, which both carried sharp pSc119.2 sites in their short arms (Fig. 2). A single pAesp_

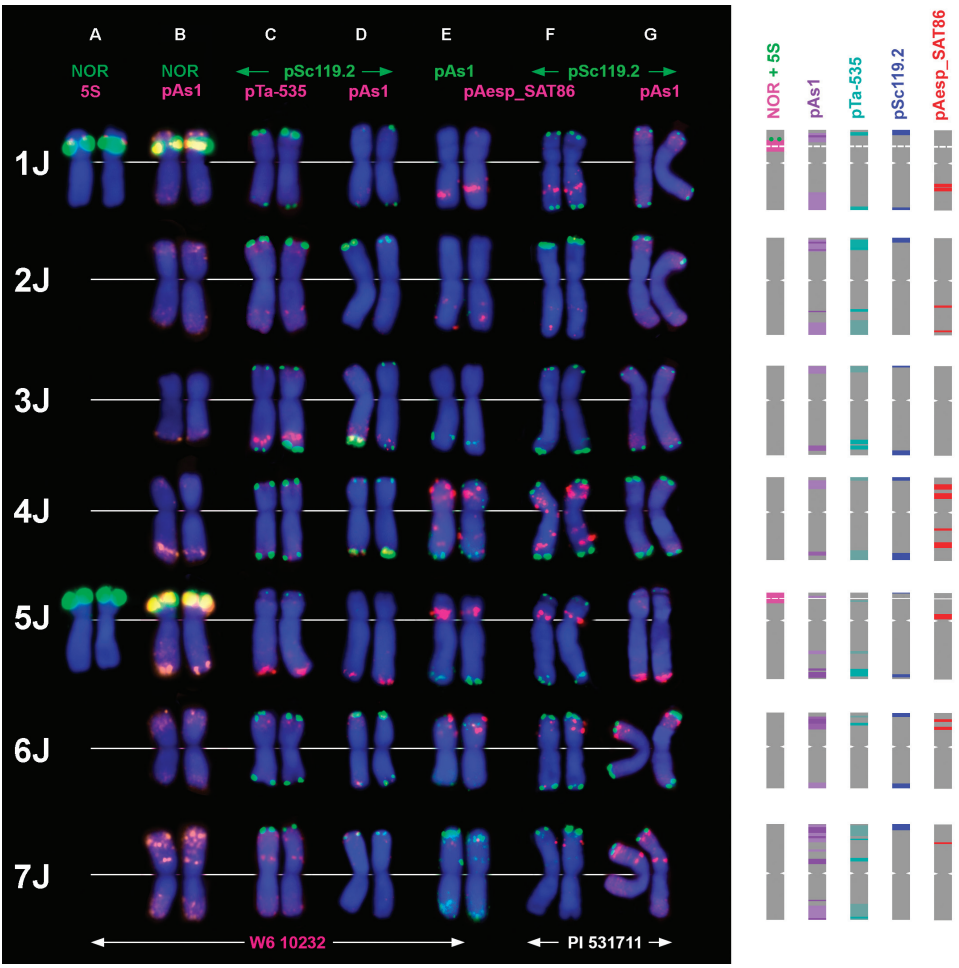


Figure 2. Distribution of different tandem repeats on *Th. bessarabicum* chromosomes; their idiograms are given on the right. The probe combinations are shown on the top, probe color corresponds to signal color. 1 – 7 – genetic groups. The pAs1 probe on lanes B, and G was labelled with Cy-3/TAMRA, while on lanes D and E with fluorescein resulting in lower pAs-1 signal intensities**.**

SAT86 signal was found on the short arm of 7J (containing pSc119.2 site), whereas two wheak signals appeared on the long arm of 2J (lacking pSc119.2 site). No pAesp_ SAT86 hybridization sites were detected on the chromosome 3J.

FISH with pTa71 and pTa794 probes on hexaploid *Th. intermedium* revealed twelve 5S rDNA signals (Fig. 1e, arrowed), five of which were co-localized with NORs (chromosomes A, B, E/E'), which were found in subterminal regions of the same chromosome arms. The remaining 5S sites were distributed among seven other chromosomes (designated C-F on Fig. 1e) in either distal or proximal position of the arm. In addition, a weak 45S rDNA signal was detected approximately in the middle of short arm of a single chromosome designated G. Such asymmetric distribution of rDNA

clusters among *Th. intermedium* chromosomes can be the consequence of unbalanced translocations that could have occurred in the genome of this perennial, vegetatively propagated plant and then maintained in a progeny over years or even decades. High number of unbalanced translocations was also detected by FISH with pAs1 probe in another *Th. intermedium* genotype (Fig. 1f): at least eighteen out of 21 homologous chromosomes pairs exhibited different labeling patterns, which significantly complicated their identification.

Eighteen chromosomes of decaploid *Th. ponticum* possessed 5S rDNA clusters of variable sizes (Fig. 1g, indicated with small arrows), fourteen of them also carried terminal NORs. Only one chromosome pair designated I, can be distinguished from others based on the extremely large pTa794 (5S rDNA) signals. Two different chromosome pairs lacking NORs contained 5S rDNA loci significantly different in size (chromosomes J and K, Fig. 1g), while another chromosome pair – H, possesses only terminal large 45S rDNA signals, like the chromosome 5J of *Th. bessarabicum*. Subsequent hybridization of pSc119.2 and pAs1 probes on the same metaphase cell revealed distinct pSc119.2 sites in subtelomeric regions of one or both arms nearly in a half of *Th. ponticum* chromosomes (Fig. 1h). Polymorphism of hybridization patterns was observed between homologous chromosomes (Fig. 1h, chr. I, shown with white arrows). The pAs1 signals were located in distal regions of nearly all chromosomes, however, owing to high ploidy level, similar location and high polymorphism, pAs1-labelling patterns did not allow identification of all *Th. ponticum* chromosomes.

Discussion

Diploid *Th. bessarabicum* is considered as one of genome donors to *Th. intermedium* (Chen et al. 1998; Liu and Wang 1993; Wang et al. 2010) and *Th. ponticum* (Chen et al. 1998). The molecular karyotype of intermediate wheatgrass has been recently constructed by Cui et al. (2018) and Yu et al. (2019) based on tandemly repeated DNA. In addition, the 5S and 45S rDNA probes were mapped on chromosomes of several *Th. intermedium* genotypes by Mahelka et al. (2013) and Yu et al. (2019). Molecular karyotypes were developed for other diploid and polyploid wheatgrass species (Brasileiro-Vidal et al. 2003; Linc et al. 2012, 2017; Li et al. 2016a, b, 2018; Said et al. 2018), thus permitting their comparison to assess genome relationships.

The distribution of rDNA loci is often used in phylogenetic studies of plants. In the Triticinae, major NORs can be located on group 1, 5 and 6 chromosomes (Appels et al. 1980), whereas the 5S rDNA loci appear on group 1 and 5 chromosomes (Dvořák et al. 1989). The number and relative position of 45S and 5S rDNA clusters on chromosomes of diploid *Aegilops* K. Linnaeus, 1753 (Badaeva et al. 1996) or *Hordeum*, K. Linnaeus, 1753, species (Taketa et al. 2001) is found to be highly diverse, but conservative for each genomic group. Genome-specific patterns of rRNA gene probes were also reported for several diploid wheatgrass species – *Agropyron elongatum*, *A. cristatum* (K. Linnaeus, 1753) J. Gaertner 1770, *Th. bessarabicum*, *Dasypyrum villosum* (K. Linnaeus, 1753) T. Candargy 1901 and *D. breviaristatum* (H. Lindberg, 1932)

Frederiksen 1991, with genome constitutions E, P, J, V and V^b respectively (Dvořák et al. 1984; Liu et al. 2010; Linc et al. 2012, 2017; Zhang et al. 2013; Li et al. 2016a, 2018; Said et al. 2018).

Earlier Linc et al. (2017) revealed two pairs of major NORs in karyotypes of the three diploid wheatgrass species, *Th. bessarabicum*, *A. elongatum*, and *Pseudoroegneria spicata* (F.T. Pursh, 1813) Á. Löve 1980. The SAT chromosomes of *Th. bessarabicum* were assigned to homoeologous groups 5 and 6 by analogy with *A. elongatum*, which carries NORs on chromosomes 5E and 6E (Dvořák et al. 1984; Linc et al. 2012; Li et al. 2018). Based on relative position of 5S and 45S rDNA loci and taking into consideration the similarity of pAs1 and pSc119.2-labelling patterns with chromosomes 1J and 5J reported by Grewal et al. (2018), we concluded that the SAT chromosomes of *Th. bessarabicum* belong to genetic groups 1 and 5.

Both *Th. bessarabicum* and *A. elongatum* contain a pair of 5S rDNA loci on group 1 chromosomes. Major clusters of 45S rDNA probe are located on group 1 and 5 chromosomes of *Th. bessarabicum* (Grewal et al. 2018), but on chromosomes 5E and 6E of *A. elongatum* (Dvořák et al. 1984; Linc et al. 2012; Li et al. 2018), which contains additional minor NORs on 1ES (Li et al. 2018). Based on dissimilarity of rDNA probe distribution we conclude that the J-genome of *Th. bessarabicum* is genetically distinct from the E-genome of *A. elongatum*.

Interestingly, polyploid *Thinopyrum* possess higher number of 5S rDNA loci per 1x compared to diploids species. Thus, we detected twelve pTa794 sites (two per 1x) in hexaploid *Th. intermedium* (Fig. 1e, indicated with small arrows), five of them were co-localized with NORs. From nine to ten 5S rDNA signals (1.5–1.67 per 1x) were revealed in four *Th. intermedium* genotypes by Mahelka et al. (2013). Yu et al. (2019) found twelve 5S and six 45 rDNA loci in intermediate wheatgrass; two chromosome pairs from the J-genome and one pair from St genome showed hybridization sites of both probes. In all cases the chromosomes carrying clusters of both rDNA families, displayed an identical signal arrangement: the 5S rDNA site was always located proximally to NOR.

We found similar pattern in decaploid *Th. ponticum* (Fig. 1f). Earlier Brasileiro-Vidal et al. (2003) reported that 17 chromosomes of *Th. ponticum* possessed both 45S and 5S rDNA sites, and the 5S rDNA sites were located proximally to NORs. Li and Zhang (2002) suggested that exclusively terminal position of 45S rDNA clusters is a secondary trait that has emerged during evolution of polyploid species. However, such arrangement of ribosomal probes was found only in diploid wheats (Dubcovsky and Dvořák 1995, Badaeva et al. 2015), but it was not observed in *Aegilops* (Badaeva et al. 1996), or the J-genome of *Th. bessarabicum* (Fig. 1a). Therefore, *Th. bessarabicum* was probably not involved in the origin of these polyploids or the J-genome was significantly modified during speciation.

The karyotype of *Th. bessarabicum* shared many common features with karyotypes of other diploid grasses. These are distinct pSc119.2 sites in subtelomeric chromosome regions and high amount of pAs1 repeat, which is accumulated predominantly in the distal chromosome halves (Zhang et al. 2013; Li et al. 2016a, 2018; Du et al. 2017; Linc et al. 2017; Grewal et al. 2018; Said et al. 2018). This or related repeats belong-

ing to the same *Afa*-family are highly abundant in the D-genome of *Aegilops tauschii* Cosson, 1850, in the A-genome of diploid wheat (Megyeri et al. 2012), the I-genome *Hordeum* species (Taketa et al. 2000), diploid and polyploid species from *Elymus* K. Linnaeus, 1753, *Leymus* C.F.F. Hochstetter, 1848, and *Psathyrostachys* S.A. Nevsky, 1933 genera (Nagaki et al. 1999; Dvořák 2009). *Th. bessarabicum* is similar to *Ae. tauschii* and diploid wheat also in a high amount of pTa-535 repeat, which is detected in genomes of *D. breviaristatum* (Li et al. 2016a), *Th. elongatum* (Li et al. 2018) and in the J and J^s genomes of intermediate wheatgrass (Yu et al. 2019).

As was shown in a current study, the sequence pAesp_SAT86 (= pTa-713) hybridizes specifically to six out of seven *Th. bessarabicum* chromosomes. Probe distribution is species-specific, because it differs from the pTa-713 labeling patterns of wheat (Komuro et al. 2013; Badaeva et al. 2015), *Aegilops* (Ruban and Badaeva 2018) or *A. elongatum* (Li et al. 2018) chromosomes. The pTa-713 signals are detected on chromosomes 1E, 4E, 5E and 7E of *A. elongatum* (Li et al. 2018). Orthologous chromosomes of *Th. bessarabicum* and *A. elongatum* belonging to group 4 and 5 display similar, while of other groups – different patterns. This can be due to site-specific sequence amplification/ elimination or species-specific chromosomal rearrangements identified in both species (Gaál et al. 2018; Grewal et al. 2018), which further confirms the distinctness of their genomes.

Conclusion

A detailed karyotype of *Th. bessarabicum* was constructed using FISH with six DNA probes representing 5S and 45S rDNAs and four tandem repeats belonging to different families. A combination of pAesp_SAT86 (= pTa-713) probe with either pSc119.2 or pAs1/ pTa-535 was found to be most effective for the identification of J-genome chromosomes. Comparison of our results with data available from literature showed that the J-genome of *Th. bessarabicum* is distinct from genomes of other diploid wheatgass species. Differences between chromosomes of *Th. bessarabitum*, on one hand, and *Th. intermedium* and *Th. ponticum*, on the other hand, indicate that probably *Th. bessarabitum* did not contribute genome to these polyploid species. Alternatively, the J-genome could be present in polyploid wheatgrasses, but in significantly rearranged form.

All authors declare that there is no conflict of interests exists. All of the authors have contributed substantially to the manuscript and approved the submission.

Acknowledgements

We thank Dr. A.A. Pomortsev (Vavilov Institute of General Genetics Russian Academy of Sciences) and Dr. Dave Stout (USDA-ARS, WRPIS Washington State University, U.S.A.) for providing the material. This work was supported by State Budgetary Project No 0112-2019-0002 and by the grant from Russian Stated Foundation for Basic Research 17-04-00087.

References

Appels R, Gerlach WL, Dennis ES, Swift H, Peacock WJ (1980) Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. Chromosoma 78(3): 293–311. https://doi.org/10.1007/BF00327389

Badaeva ED, Friebe B, Gill BS (1996) Genome differentiation in *Aegilops*. 2. Physical mapping of 5S and 18S-26S ribosomal RNA gene families in diploid species. Genome 39(6): 1150–1158. https://doi.org/10.1139/g96-145

Badaeva ED, Ruban AS, Aliyeva-Schnorr L, Municio C, Hesse S, Houben A (2017) In situ hybridization to plant chromosomes. In: Liehr T (Ed) Fluorescence In Situ Hybridization (FISH) Application Guide. Springer, Berlin, 477–494. https://doi.org/10.1007/978-3-662-52959-1

Badaeva ED, Amosova AV, Goncharov NP, Macas J, Ruban AS, Grechishnikova IV, Zoshchuk SA, Houben A (2015) A set of cytogenetic markers allows the precise identification of all A-genome chromosomes in diploid and polyploid wheat. Cytogenetics and Genome Research 146(1): 71–79. https://doi.org/10.1159/000433458

Bedbrook RJ, Jones J, O'Dell M, Thompson RJ, Flavell RB (1980) A molecular description of telomeric heterochromatin in *Secale* species. Cell 19(2): 545–560. https://doi.org/10.1016/0092-8674(80)90529-2

Brasileiro-Vidal AC, Cuadrado A, Brammer SP, Zanatta ACA, Prestes AM, Moraes-Fernandes MIB, Guerra M (2003) Chromosome characterization in *Thinopyrum ponticum* (Triticeae, Poaceae) using in situ hybridization with different DNA sequences. Genetics and Molecular Biology 26(4): 505–510. https://doi.org/10.1590/S1415-47572003000400014

Charpentier A (1992) Production of disomic addition lines and partial amphiploids of *Thinopyrum junceum* on wheat. Comptes Rendus de l'Académie des Sciences (Paris) 315 (13): 551–557.

Chen Q, Conner RL, Laroche A, Thomas JB (1998) Genome analysis of *Thinopyrum intermedium* and *Thinoryrum ponticum* using genomic in situ hybridization Genome 41(4): 580–586. https://doi.org/10.1139/g99-090

Ceoloni C, Kuzmanovic L, Forte P, Virili ME, Bitti A (2015) Wheat-perennial Triticeae introgressions: major achievements and prospects. In: Molnár-Láng M, Ceoloni C, Doležel J (Eds) Alien Introgression in Wheat Cytogenetics, Molecular Biology, and Genomics. Springer International Publishing, 273–313. https://doi.org/10.1007/978-3-319-23494-6_11

Cui Y, Zhang Y, Qi J, Wang H, Wang RRC, Bao Y, Li X (2018) Identification of chromosomes in *Thinopyrum intermedium* and wheat *Th. intermedium* amphiploids based on multiplex oligonucleotide probes. Genome 61(7): 515–521. https://doi.org/10.1139/gen-2018-0019

Dewey DR (1984) The genomic system of classification as a guide to intergeneric hybridization with the perennial Triticeae In: Gustafson JP (Ed.) Gene Manipulation in Plant Improvement. New York, 209–280. https://doi.org/10.1007/978-1-4613-2429-4_9

Du P, Zhuang LF, Wang YZ, Yuan L, Wang Q, Wang DR, Dawadondup, Tan LJ, Shen J, Xu HB, Zhao H, Chu CG, Qi ZJ (2017) Development of oligonucleotides and multiplex probes for quick and accurate identification of wheat and *Thinopyrum bessarabicum* chromosomes. Genome 60(2): 93–103. https://doi.org/10.1139/gen-2016-0095

Dubcovsky J, Dvořák J (1995) Ribosomal RNA multigene loci: nomads of the Triticeae genomes. Genetics 140(4): 1367–1377. http://www.genetics.org/cgi/content/abstract/140/4/1367

Dvořák J (1981) Genome relationships among *Elytrigia* (=*Agropyron*) *elongata*, *E. stipifolia*, «*E. elongata* 4x,» *E. caespitosa*, *E. intermedia*, and «*E. elongata* 10x». Canadian Journal of Genetics and Cytology 23 (3): 481–492. https://doi.org/10.1139/g81-053

Dvořák J (2009) Triticeae genome structure and evolution. In: Muehlbauer GJ, Feuillet C (Eds) Genetics and Genomics of the Triticeae, First edition. New York, 685–711. https://doi.org/10.1007/978-0-387-77489-3_23

Dvořák J, Lassner MW, Kota RS, Chen KC (1984) The distribution of the ribosomal RNA genes in the *Triticum speltoides* and *Elytrigia elongata* genomes. Canadian Journal of Genetics and Cytology 62(5): 628–632. https://doi.org/10.1139/g84-097

Dvořák J, Zhang H-B, Kota RS, Lassner M (1989) Organization and evolution of the 5S ribosomal RNA gene family in wheat and related species. Genome 32(6): 1003–1016. https://doi.org/10.1139/g89-545

Endo TR, Gill BS (1984) The heterochromatin distribution and genome evolution in diploid species of *Elymus* and *Agropyron*. Canadian Journal of Genetics and Cytology 26(6): 669–678. https://doi.org/10.1139/g84-106

Forster BP, Gorham J, Miller TE (1987) Salt tolerance of an amphiploid between *Triticum aestivum* and *Agropyron junceum*. Plant Breeding 98(1): 1–8. https://doi.org/10.1111/j.1439-0523.1987.tb01083.x

Forster BP, Miller TE (1989) Genome relationship between *Thinopyrum bessarabicum* and *Thinopyrum elongatum*. Genome 32(5): 930–931. https://doi.org/10.1139/g89-532

Gaál E, Valárik M, Molnár I, Farkas A, Linc G (2018) Identification of COS markers specific for *Thinopyrum elongatum* chromosomes preliminary revealed high level of macrosyntenic relationship between the wheat and *Th. elongatum* genomes. PLoS ONE 13: e0208840. https://doi.org/10.1371/journal.pone.0208840

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Research 7(7): 1869–1885. https://doi.org/10.1093/nar/7.7.1869

Gerlach WL, Dyer TA (1980) Sequence organization of the repeated units in the nucleus of wheat which contains 5S-rRNA genes. Nucleic Acids Research 8(21): 4851–4865. https://doi.org/10.1093/nar/8.21.4851

Gorham J, McDonnelL E, Budrewicz E, Wyn Jones RG (1985) Salt tolerance in the Triticeae: Growth and solute accumulation in leaves of *Thinopyrum bessarabicum*. Journal of Experimental Botany 36(7): 1021–1031. https://doi.org/10.1093/jxb/36.7.1021

Grewal S, Yang C, Edwards SH, Scholefield D, Ashling S, Burridge AJ, King IP, King J (2018) Characterisation of *Thinopyrum bessarabicum* chromosomes through genome-wide introgressions into wheat. Theoretical and Applied Genetics 131(2): 389–406. https://doi.org/10.1007/s00122-017-3009-y

Hamdani A, Gul A, Bux H, Qureshi ST, Mujeeb-Kazi A (2018) Cytological and phenological characterization of adapted genetic stocks derived from *Triticum aestivum/Thinopyrum bessarabicum*. Pakistan Journal of Agricultural Science 55(2):257–262. https://doi.org/10.21162/PAKJAS/18.4266

Jauhar PP (1988) A reassessment of genome relationships between *Thinopyrum bessarabicum* and *T. elongatum* of the Triticeae. Genome 30(6): 903–914. https://doi.org/10.1139/g88-146

Komuro S, Endo R, Shikata K, Kato A (2013) Genomic and chromosomal distribution patterns of various repeated DNA sequences in wheat revealed by a fluorescence in situ hybridization procedure. Genome 56(3): 131–137. https://doi.org/10.1139/gen-2013-0003

King IP, Forster BP, Law CC, Cant KA, Orford SE, Gorham J, Reader S, Miller TE (1997) Introgression of salt-tolerance genes from *Thinopyrum bessarabicum* into wheat. New Phytologist 137(1): 75–81. https://doi.org/10.1046/j.1469-8137.1997.00828.x

Kruppa K, Molnar-Lang M (2016) Simultaneous visualization of different genomes (J, J^{St} and St) in a *Thinopyrum intermedium* × *Thinopyrum ponticum* synthetic hybrid (Poaceae) and in its parental species by multicolour genomic in situ hybridization (mcGISH). Comparative Cytogenetics 10: 283–293. https://doi.org/10.3897/CompCytogen.v10i2.7305

Li D, Zhang X (2002) Physical localization of the 18S-5·8S-26S rDNA and sequence analysis of ITS regions in *Thinopyrum ponticum* (*Poaceae*: *Triticeae*): Implications for concerted evolution. Annals of Botany 90(4): 445–452. https://doi.org/10.1093/aob/mcf213

Li G, Gao D, Zhang H, Li J, Wang H, La S, Ma J, Yang Z (2016a) Molecular cytogenetic characterization of *Dasypyrum breviaristatum* chromosomes in wheat background revealing the genomic divergence between *Dasypyrum* species. Molecular Cytogenetics 9: 1–6. https://doi.org/10.1186/s13039-016-0217-0

Li G, Wang H, Lang T, Li J, La S, Yang E, Yang Z (2016b) New molecular markers and cytogenetic probes enable chromosome identification of wheat-*Thinopyrum intermedium* introgression lines for improving protein and gluten contents. Planta 244(4): 865–876. https://doi.org/10.1007/s00425-016-2554-y

Li D, Li T, Wu Y, Zhang X, Zhu W, Wang Y, Zeng J, Xu L, Fan X, Sha L, Zhang H, Zhou Y, Kang H (2018) FISH-based markers enable identification of chromosomes derived from tetraploid *Thinopyrum elongatum* in hybrid lines. Frontiers in Plant Sciences 526(9): 1–12. https://doi.org/10.3389/fpls.2018.00526

Linc G, Sepsi A, Molnar-Lang M (2012) A FISH karyotype to study chromosome polymorphisms for the *Elytrigia elongata* E genome. Cytogenetic and Genome Research 136(2): 138–144. https://doi.org/10.1159/000334835

Linc G, Gaál E, Molnár I, Icsó D, Badaeva E, Molnár-Láng M (2017) Molecular cytogenetic (FISH) and genome analysis of diploid wheatgrasses and their phylogenetic relationship. PLoS ONE 12(3): e0173623. https://doi.org/10.1371/journal.pone.0173623

Liu Z-W, Wang RRC (1993) Genome analysis of *Elytrigia caespitosa*, *Lophopyrum nodosum*, *Pseudoroegneria geniculata* ssp. *scythica*, and *Thinopyrum intermedium* (*Triticeae*: *Gramineae*). Genome 36(1): 102–111. https://doi.org/10.1139/g93-014

Liu C, Li G-R, Sehgal SK, Jia J-Q, Yang Z-J, Friebe B, Gill B (2010) Genome relationships in the genus *Dasypyrum*: evidence from molecular phylogenetic analysis and in situ hybridization. Plant Systematics and Evolution 288(3–4): 149–156. https://doi.org/10.1007/s00606-010-0319-9

Mahelka V, Kopecky D, Baum BR (2013) Contrasting patterns of evolution of 45S and 5S rDNA families uncover new aspects in the genome constitution of the agronomically important grass *Thinopyrum intermedium* (Triticeae). Molecular Biology and Evolution 30(9): 2065–2086. https://doi.org/10.1093/molbev/mst106

Megyeri M, Farkas A, Varga M, Kovács G, Molnár-Láng M, Molnár I (2012) Karyotypic analysis of *Triticum monococcum* using standard repetitive DNA probes and simple se-

quence repeats. Acta Agronomica Hungarica 60(2): 87–95. https://doi.org/10.1556/AAgr.60.2012.2.1

Mirzaghaderi G, Shahsevand Hassani H, Karimzadeh G (2010) C-banded karyotype of *Thinopyrum bessarabicum* and identification of its chromosomes in wheat background. Genetic Resources and Crop Evolution 57(3): 319–324. https://doi.org/10.1007/s10722-009-9509-0

Moustakas M (1991) Further evidence of the genome relationships between *Thinopyrum bessarabicum* and *T. elongatum*. Cytobios 68: 197–206.

Nagaki K, Kishii M, Tsujimoto H, Sasakuma T (1999) Tandem repetitive Afa-family sequences from *Leymus racemosa* and *Psathyrostachys juncea* (Poaceae). Genome 42(6): 1258–1260. https://doi.org/10.1139/gen-42-6-1258

Patokar C, Sepsi A, Schwarzacher T, Kishii M, Heslop-Harrison JS (2016) Molecular cytogenetic characterization of novel wheat-*Thinopyrum bessarabicum* recombinant lines carrying intercalary translocations. Chromosoma 125(1): 163–172. https://doi.org/10.1007/s00412-015-0537-6

Qi Z, Du P, Qian B, Zhuang L, Chen H, Chen T, Shen J, Guo J, Feng Y, Pei Z (2010) Characterization of a wheat-*Thinopyrum bessarabicum* (T2JS-2BS·2BL) translocation line. Theoretical and Applied Genetics 121(3): 589–597. https://doi.org/10.1007/s00122-010-1332-7

Rayburn AL, Gill BS (1986) Isolation of a D-genome specific repeated DNA sequence from *Aegilops squarrosa*. Plant Molecular Biology Reporter 4(2): 102–109. https://doi.org/10.1007/BF02732107

Ruban AS, Badaeva ED (2018) Evolution of the S-genomes in *Triticum-Aegilops* alliance: Evidences from chromosome analysis. Frontiers in Plant Science 9(1756): 1–25. https://doi.org/10.3389/fpls.2018.01756

Said M, Hřibová E, Danilova TV, Karafiátová M, Čížková J, Friebe B, Doležel J, Gill BS, Vrána J (2018) The *Agropyron cristatum* karyotype, chromosome structure and cross-genome homoeology as revealed by fluorescence *in situ* hybridization with tandem repeats and wheat single-gene probes. Theoretical and Applied Genetics 131(10): 2213–2227. https://doi.org/10.1007/s00122-018-3148-9

Taketa S, Ando H, Takeda K, Von Bothmer R (2001) Physical locations of 5S and 18S-25S rDNA in Asian and American diploid *Hordeum* species with the I genome. Heredity 86(5): 522–530. https://doi.org/10.1046/j.1365-2540.2001.00768.x

Taketa S, Ando H, Takeda K, Harrison GE, Heslop-Harrison JS (2000) The distribution, organization and evolution of two abundant and widespread repetitive DNA sequences in the genus *Hordeum*. Theoretical and Applied Genetics 100 (2): 169–176. https://doi.org/10.1007/s001220050023

Tang S, Li Z, Jia X, Larkin PJ (2000) Genomic in situ hybridization (GISH) analyses of *Thinopyrum intermedium*, its partial amphiploid Zhong 5, and disease-resistant derivatives in wheat. Theoretical and Applied Genetics 100(3–4): 344–352. https://doi.org/10.1007/s001220050045

Tang Z, Yang Z, Fu S (2014) Oligonucleotides replacing the roles of repetitive sequences pAs1, pSc119.2, pTa-535, pTa71, CCS1, and pAWRC.1 for FISH analysis. Journal of Applied Genetics 55(3): 313–318. https://doi.org/10.1007/s13353-014-0215-z

Wang RR-C (1985) Genome analysis of *Thinopyrum bessarabicum* and *T. elongatum*. Canadian Journal of Genetics and Cytology 27(6): 722–728. https://doi.org/10.1139/g85-108

Wang RR-C (2011) *Agropyron* and *Psathyrostachys*. In: Kole C (Ed.) Wild Crop Relatives: Genomic and Breeding Resources. Cereals, Springer-Verlag, Berlin, 77–108. https://doi.org/10.1007/978-3-642-14228-4_2

Wang RR-C, Lu B (2014) Biosystematics and evolutionary relationships of perennial Triticeae species revealed by genomic analyses. Journal of Systematics and Evolution 52(6): 697–705. https://doi.org/10.1111/jse.12084

Wang RR-C, Larson SR, Jensen KB (2010) Analyses of *Thinopyrum bessarabicum*, *T. elongatum*, and *T. junceum* chromosomes using EST-SSR markers. Genome 53(12): 1083–1089. https://doi.org/10.1139/G10-088

William MDHM, Mujeeb-Kazi A (1993) *Thinopyrum bessarabicum*: biochemical and cytological markers for the detection of genetic introgression in its hybrid derivatives with *Triticum aestivum* L. Theoretical and Applied Genetics 86(2): 365–370. https://doi.org/10.1007/BF00222103

Xu SS, Jin Y, Klindworth DL, Wang RR-C, Cai X (2009) Evaluation and characterization of seedling resistances to stem rust *Ug99* races in wheat-alien species derivatives. Crop Science 49(6): 2167–2175. https://doi.org/10.2135/cropsci2009.02.0074

Yu Z, Wang H, Xu Y, Li Y, Lang T, Yang Z, Li G (2019) Characterization of chromosomal rearrangement in new wheat–*Thinopyrum intermedium* addition lines carrying *Thinopyrum*–specific grain hardness genes. Agronomy 9(1): 1–18. https://doi.org/10.3390/agronomy9010018

Zhang JY, Li X-M, Wang RR-C, Cortes A, Rosas V, Mujeeb-Kazi A (2002) Molecular cytogenetic characterization of E^b-genome chromosomes in *Thinopyrum bessarabicum* disomic addition lines of bread wheat. International Journal of Plant Sciences 163(1): 167–174. https://doi.org/10.1086/324531

Zheng Q, Klindworth DL, Friesen TL, Liu A-F, Li Z-S, Zhong S, Jin Y, Xu SS (2014) Characterization of *Thinopyrum* species for wheat stem rust resistance and ploidy level. Crop Science 54(6): 2663–2672. https://doi.org/10.2135/cropsci2014.02.0093

Zhang W, Zhang R, Feng Y, Bie T, Chen P (2013) Distribution of highly repeated DNA sequences in *Haynaldia villosa* and its application in the identification of alien chromatin. Chinese Science Bulletin 58(8): 890–897. https://doi.org/10.1007/s11434-012-5598-9

CompCytogen 13(4): 403–410 (2019)
doi: 10.3897/CompCytogen.v13i4.48891
http://compcytogen.pensoft.net

SHORT COMMUNICATION

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

Comparative FISH mapping of ribosomal DNA clusters and TTAGG telomeric sequences to holokinetic chromosomes of eight species of the insect order Psocoptera

Natalia Golub[1], Boris Anokhin[1], Valentina Kuznetsova[1]

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

Corresponding author: *Natalia Golub* (nvgolub@mail.ru)

Academic editor: *V. Gokhman* | Received 28 November 2019 | Accepted 28 November 2019 | Published 6 December 2019

http://zoobank.org/EEB9D81A-FBC1-4312-8157-07B468F8E87A

Citation: Golub N, Anokhin B, Kuznetsova V (2019) Comparative FISH mapping of ribosomal DNA clusters and TTAGG telomeric sequences to holokinetic chromosomes of eight species of the insect order Psocoptera. Comparative Cytogenetics 13(4): 403–410. https://doi.org/10.3897/CompCytogen.v13i4.48891

Abstract

Repetitive DNAs are the main components of eukaryotic genome. We mapped the 18S rDNA and TTAGG telomeric probe sequences by FISH to meiotic chromosomes of eight species of the order Psocoptera considered a basal taxon of Paraneoptera: *Valenzuela burmeisteri* (Brauer, 1876), *Stenopsocus lachlani* Kolbe, 1960, *Graphopsocus cruciatus* (Linnaeus, 1768), *Peripsocus phaeopterus* (Stephens, 1836), *Philotarsus picicornis* (Fabricius, 1793), *Amphigerontia bifasciata* (Latreille, 1799), *Psococerastis gibbosa* (Sulzer, 1766), and *Metylophorus nebulosus* (Stephens, 1836). These species belong to five distantly related families of the largest psocid suborder Psocomorpha: Caeciliusidae, Stenopsocidae, Peripsocidae, Philotarsidae, and Psocidae. We show that all the examined species share a similar location of 18S rDNA on a medium-sized pair of autosomes. This is the first study of rDNA clusters in the order Psocoptera using FISH. We also demonstrate that these species have the classical insect $(TTAGG)_n$ telomere organization. Our results provide a foundation for further cytogenetic characterization and chromosome evolution studies in Psocoptera.

Keywords

Insecta, psocids, Psocomorpha, meiosis, holokinetic chromosomes, $(TTAGG)_n$, 18S rDNA, FISH

Introduction

Psocoptera (booklice and barklice) are a small insect order considered a basal taxon of Paraneoptera (Yoshizawa and Saigusa 2001). The order includes 5941 species in 485 genera, 41 families and 3 suborders: Trogiomorpha, Troctomorpha and Psocomorpha (Mockford 2018). To date, 90 psocopteran species (51 genera, 21 families) have been cytogenetically studied, most of them (80 species, 43 genera, 16 families) belonging to the largest suborder Psocomorpha (reviewed in Golub and Nokkala 2009). In this suborder, the majority of species (71 species from 36 genera and 15 families) display 2n = 16 + XX/X(0) indicative of a particular karyotype conservatism of the group. This karyotype is considered modal and ancestral both for Psocomorpha and for the order Psocoptera in general (Wong and Thornton 1966, Golub 1999, Golub and Nokkala 2009). To date, different derived karyotypes have been reported only for nine species: *Amphipsocus japonicus* Enderlein, 1906 and *Kolbia quisquiliarum* Bertkau, 1882 from the family Amphipsocidae (2n = 14 + XX/neo-XY); *Elipsocus moebiusi* Tetens, 1891 and *Loensia variegata* (Latreilee, 1799) from the families Elipsocidae and Psocidae, respectively (2n = 12 +XX/X(0); *Neopsocopsis hitricornis* (Reuter, 1893), *Metylophorus nebulosus* (Stephens, 1836), and *Amphigerontia jezoensis* Okamoto, 1907 from the family Psocidae (2n = 14 +XX/X(0); *Stenopsocus lachlani* Kolbe, 1960 and *Stenopsocus aphidiformis* Enderlein, 1906 from the family Stenopsocidae (2n = 22 +XX/X(0)).

Psocoptera are characterized by holokinetic chromosomes (Meinander et al. 1974, Golub and Nokkala 2009), which are known to lack such physical landmarks as primary constrictions (the centromeres) and, thus, show no distinguishable markers that could be studied by conventional techniques. In the majority of psocid species, the chromosomes are small and of similar size, making it impossible to identify individual chromosomes. Although karyotypes have been described for many psocid species, individual chromosomes were not identified in most of these reported karyotypes. Such chromosome sets, therefore, are not comparable among related species and cannot be used for evolutionary studies.

The application of banding techniques to chromosome studies of Psocoptera is scarce (Golub and Nokkala 2001, Golub et al. 2004). Golub et al. (2004) used C-banding, silver impregnation and sequence-specific fluorochromes CMA_3 and DAPI to study male meiotic karyotypes of *Psococerastis gibbosa* (Sulzer, 1766) with 2n = 16 + X(0), *Blaste conspurcata* (Rambur, 1842) with 2n = 16 + X(0), and *Amphipsocus japonicus* with 2n = 14 + neo-XY. Based on the results obtained, the authors had concluded that NORs (nucleolus organizer regions) were located differently in these species: on an autosomal bivalent, on the X chromosome, and on the neo-XY bivalent, respectively. We believe however that additional studies are needed to confirm the precise localization of NORs in the above species. Using C-banding, the authors found minor interspecific differences in amount, molecular composition and localization of C-heterochromatin as well as some analogous differences between various chromosomes of a particular species.

Our knowledge of karyotype structure and evolution in Psocoptera could be improved by the implementation of molecular cytogenetic approaches. Fluorescence

in situ hybridization (FISH) has become the most important technique for tracing individual chromosomes in holokinetic insects (e.g., Panzera et al. 2012, 2015, Maryańska-Nadachowska et al. 2013, 2018, Mandrioli et al. 2014, Kuznetsova et al. 2015, Anjos et al. 2016, Golub et al. 2017, Salanitro et al. 2017, Grozeva et al. 2019). It was shown in some case studies that species with the same chromosome complement differ in the number and location of rDNA sites (Panzera et al. 2012, 2015, Maryańska-Nadachowska et al. 2013, Golub et al. 2017). Moreover, some higher insect taxa were shown to differ in respect to the presence/absence of the insect-type telomere motif $(TTAGG)_n$. Specifically, such variation has been demonstrated for some Paraneoptera, e.g. Hemiptera, where more basal taxa appear to have the ancestral insect telomere motif $(TTAGG)_n$ while more advanced taxa have lost this telomeric sequence (reviewed in Kuznetsova et al. 2019). The only psocid species studied so far by FISH, *Stenopsocus lachlani* (Psocomorpha, Stenopsocidae), was documented to have the $(TTAGG)_n$ telomere motif (Frydrychová et al. 2004).

Here, we used FISH with the telomeric TTAGG and 18S rDNA probes to study male meiotic chromosomes of *Valenzuela burmeisteri* (Brauer, 1876), *Stenopsocus lachlani*, *Graphopsocus cruciatus* (Linnaeus, 1768), *Peripsocus phaeopterus* (Stephens, 1836), *Philotarsus picicornis* (Fabricius, 1793), *Amphigerontia bifasciata* (Latreille, 1799), *Psococerastis gibbosa*, and *Metylophorus nebulosus*. The standard karyotypes of these species were previously reported (reviewed in Golub and Nokkala 2009). We demonstrate that the above species, belonging to five different families of the largest suborder Psocomorpha (Caeciliusidae, Stenopsocidae, Peripsocidae, Philotarsidae, and Psocidae), are characterized by conserved karyotypes in respect to telomere composition and rDNA location. This is the first study of rDNA clusters in the order Psocoptera using FISH.

Material and methods

The information on the localities where the specimens were collected and on the number of specimens/nuclei examined is presented in Table 1. Only male adult specimens were analyzed. Males were fixed in the Carnoy fixative (3:1; 96% ethanol and glacial acetic acid) and stored at 4 °C. Testes were dissected out in a drop of 45% acetic acid and squashed. The cover slips were removed using dry ice. Prior to staining, the preparations were examined by phase contrast microscopy.

Fluorescence in situ hybridization was performed according to the published protocol (Grozeva et al. 2015) with minor modifications. The target 18S rDNA probe (about 1200 bp fragment) was PCR amplified and labelled with biotin-11-dUTP (Fermentas, EU) using primers: 18SrRNA_F 5'-GATCCTGCCAGTAGTCATATG-3', 18SrRNA_R 5'-GAGTCAAATTAAGCCGCAGG-3' (Anokhin et al. 2010). Genomic DNA was extracted from the true bug *Pyrrhocoris apterus* (Linnaeus, 1758). An initial denaturation period of 3 min at 94 °C was followed by 35 cycles of 30 s at 94 °C, annealing for 30 s at 55.5 °C and extension for 1.5 min at 72 °C, with a final extension step of 3 min at 72 °C. The telomere probe $(TTAGG)_n$ was amplified by

Table 1. Material studied.

Species	Collection date and localities	Number of studied males / nuclei
Fam. Caeciliusidae		
Valenzuela burmeisteri	Russia, the Altai Republic, Artybash vic., 51°47'28"N, 87°15'21"W, July, 2019	4/12
Fam. Stenopsocidae		
Stenopsocus lachlani	Russia, the Altai Republic, Artybash vic., 51°47'28"N, 87°15'21"W, July, 2019	2/22
Graphopsocus cruciatus	Russia, Voronezh region, Maklok vic., 51°48'42"N, 39°24'51"W, August, 2018	4/18
Fam. Peripsocidae		
Peripsocus phaeopterus	Russia, the Altai Republic, Artybash vic., 51°47'28"N, 87°15'21"W, July, 2019	6/30
Fam. Philotarsidae		
Philotarsus picicornis	Russia, the Altai Republic, Artybash vic., 51°47'28"N, 87°15'21"W, July, 2019	6/42
Fam. Psocidae		
Amphigerontia bifasciata	Russia, Karachay-Cherkess Republic, Teberda vic., 43°27'00"N, 41°45'00"W, July, 2017	2/10
Metylophorus nebulosus	Russia, the Altai Republic, Artybash vic., 51°47'28"N, 87°15'21"W, July, 2019	5/28
Psococerastis gibbosa	Russia, the Altai Republic, Artybash vic., 51°47'28"N, 87°15'21"W, July, 2019	4/46

PCR and labeled with rhodamine-5-dUTP (GeneCraft, Köln, Germany) using primers: TTAGG_F 5'-TAACCTAACCTAACCTAACCTAA-3' and TTAGG_R 5'-GGTTAGGTTAGGTTAGGTTAGG-3' (Grozeva et al. 2011). An initial denaturation period of 3 min at 94 °C was followed by 30 cycles of 45 s at 94 °C, annealing for 30 s at 50 °C and extension for 50 s at 72 °C, with a final extension step of 3 min at 72 °C. The 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 in the hybridization mixture containing labelled 18S rDNA and $(TTAGG)_n$ probes with an addition of salmon sperm blocking reagent and then hybridized for 42 h at 37 °C. 18S rDNA probes were detected with NeutrAvidin-Fluorescein conjugate (Invitrogen, Karlsbad, CA, USA). The chromosomes were mounted in an antifade medium (ProLong Gold antifade reagent with DAPI, Invitrogen) and covered with a glass coverslip.

Results and discussion

Standard karyotypes

All chromosome numbers fully correspond to the previously published karyotype data for all studied species (reviewed in Golub and Nokkala 2009). Males of *Valenzuela burmeisteri*, *Graphopsocus cruciatus*, *Peripsocus phaeopterus*, *Philotarsus picicornis*, *Amphigerontia bifasciata*, and *Psococerastis gibbosa* were confirmed to have 2n = 16 + X(0),

the chromosome complement known to be the most characteristic and presumably ancestral for Psocoptera (Wong and Thornton 1966, Golub 1999, Golub and Nokkala 2009). Males of *Stenopsocus lachlani* and *Metylophorus nebulosus* were confirmed to have 2n = 22 + X(0) and 2n = 14 + X(0), respectively. Thus, even though the sex chromosome system is the same among all studied species, the number of autosomes differs considerably between them. Based on the meiotic figures, we can infer that the karyotype structure of these species is uniform: all the bivalents constitute a decreasing size series, which makes identifying individual bivalents almost impossible. The only exception to this rule is the karyotype of *M. nebulosus*. In this species, metaphase I nuclei were shown to include seven bivalents with a particular element being significantly larger than the other ones (Meinander et al. 1974, Golub 1999, present study).

FISH mapping of 18S rDNA repeats

In each of the species studied, FISH mapping with the 18S rDNA probe revealed two large clusters located in a sub-terminal position on the homologues of a medium-sized bivalent (Fig. 1a–h). The signals could be observed either in the chiasmate or the opposite region of the rDNA-carrying bivalent, thus suggesting that the same homologue is able to orient differently within the bivalent. Following current knowledge, all the studied species are suggested to share a similar chromosomal location of the rRNA genes on the same pair of autosomes. However, this speculation is premature, since the precise identification of particular bivalents in psocid karyotypes is currently impossible due to the absence of additional differential chromosomal landmarks.

FISH mapping of TTAGG telomeric repeats

In each of the species studied, FISH mapping with TTAGG repeats revealed signals located in a telomeric position on the chromosomes. The signals were visible in most but not all terminal regions of meiotic chromosomes. Moreover, in some species, the signals were bright (Fig. 1b, e, g, h), whereas in other species they were not so clearly defined (Fig. 1a, c, d, f).

A previous investigation by Frydrychová et al. (2004) documented presence of the $(TTAGG)_n$ telomere motif in *S. lachlani* (Stenopsocidae). Despite the variability in the signal intensity, the currently existing data on eight genera from five different families lead to the conclusion that psocids, at least those from the suborder Psocomorpha, share the telomere structure $(TTAGG)_n$ known to be characteristic of the majority of insect orders and considered ancestral for the class Insecta in general (Kuznetsova et al. 2019).

In conclusion, the present study contributes to the understanding of the chromosome structure of Psocoptera and provides a foundation for further cytogenetic characterization and chromosome evolution studies in this group.

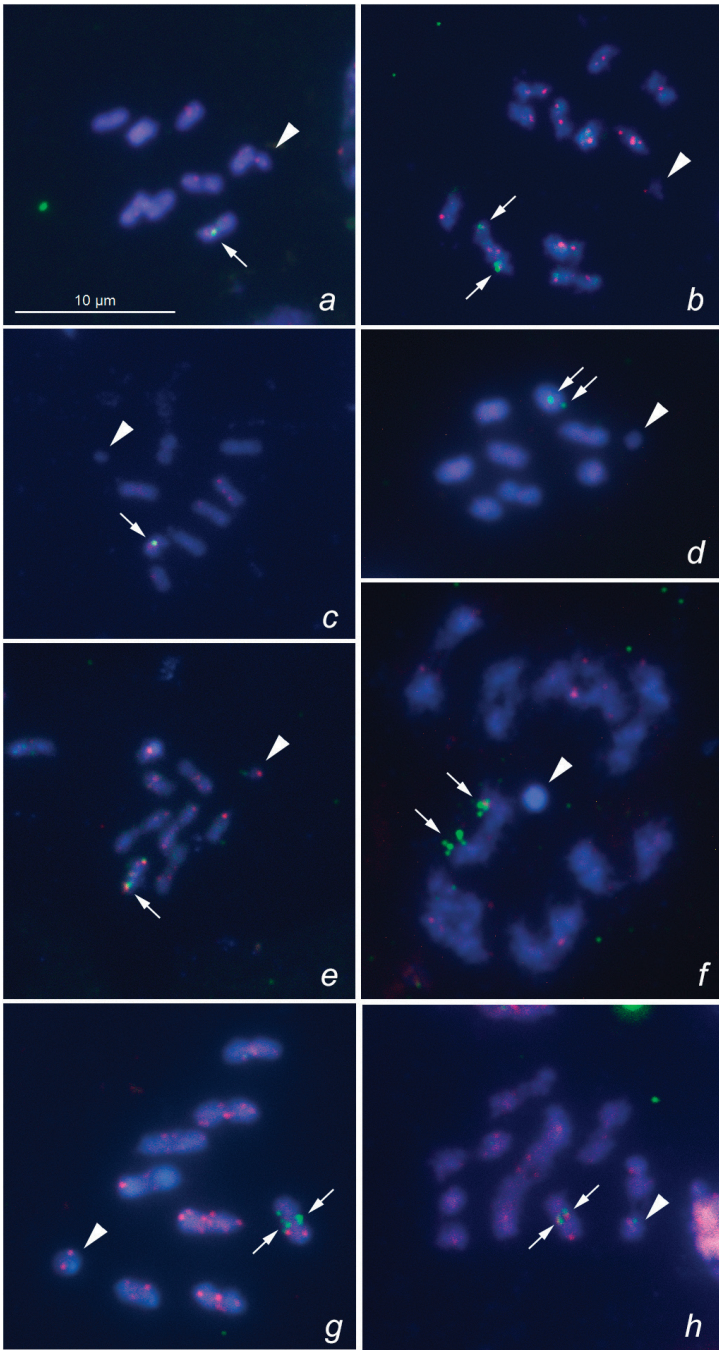


Figure 1. FISH mapping of TTAGG telomeric sequences (red signals) and 18S rDNA (green signals) to meiotic chromosomes of Psocoptera **a** *Valenzuela burmeisteri*, MI, n = 8 + X **b** *Stenopsocus lachlani*, MI, n = 11 + X **c** *Graphopsocus cruciatus*, MI, n = 8 + X **d** *Peripsocus phaeopterus*, MI, n = 8 + X **e** *Philotarsus picicornis*, MI, n = 8 + X **f** *Amphigerontia bifasciata*, diakinesis, n = 8 + X **g** *Psococerastis gibbosa*, MI, n = 8 + X **h** *Metylophorus nebulosus*, MI, n = 7 + X. Arrowheads and arrows indicate sex chromosomes and 18S rDNA signals, respectively. Scale bar: 10 µm.

Acknowledgements

The present study was supported by the research project no. AAAA-A19-119020790106-0, by the research grants from the Russian Foundation for Basic Research Nos 17-04-00828 and 19-54-18002, and by the Presidium of the RAS, Program No. 41 "Biodiversity of natural systems and biological resources of Russia".

References

Anjos A, Rocha GC, Paladini A, Mariguela TC, Cabral-de-Mello DC (2016) Karyotypes and repetitive DNA evolution in six species of the genus *Mahanarva* (Auchenorrhyncha: Cercopidae). Cytogenetic and Genome Research 149: 321–327. https://doi.org/10.1159/000450730

Anokhin B, Hemmrich-Stanisak G, Bosch TCG (2010) Karyotyping and single-gene detection using fluorescence in situ hybridization on chromosomes of *Hydra magnipapillata*. Comparative Cytogenetics 4(2): 97–110. https://doi.org/10.3897/compcytogen.v4i2.41

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

Golub NV (1999) Review of karyological data on psocids (Psocoptera) and description of karyotypes of 13 species. Entomologicheskoe Obozrenie 78(2): 376–390. [In Russian, with English translation in Entomological Review 79: 412–424]

Golub NV, Nokkala S (2001) The karyotypes of two bark-lice species (Psocoptera, Psocomorpha, Amphipsocidae): the first description of the Neo-XY sex chromosome system in Psocoptera. Folia biologica (Kraków) 49(3–4): 153–156.

Golub NV, Nokkala S (2009) Chromosome numbers in eight species of Palaearctic Psocoptera (Insecta). Comparative Cytogenetics 3(1): 33–41. https://doi.org/10.3897/compcytogen.v3i1.6

Golub NV, Nokkala S, Kuznetsova VG (2004) Holocentric chromosomes of psocids (Insecta, Psocoptera) analysed by C-banding, silver impregnation and sequence specific fluorochromes CMA_3 and DAPI. Folia biologica (Kraków) 52(3–4): 143–149. https://doi.org/10.3409/1734916044527476

Golub NV, Golub VB, Kuznetsova VG (2017) Distribution of the major rDNA loci among four hemipteran species of the family Tingidae (Heteroptera, Cimicomorpha). Folia Biologica (Kraków) 65(3): 155–158. https://doi.org/10.3409/fb65_3.155

Grozeva S, Kuznetsova VG, Anokhin BA (2011) Karyotypes, male meiosis and comparative FISH mapping of 18S ribosomal DNA and telomeric $(TTAGG)_n$ repeat in eight species of true bugs (Hemiptera, Heteroptera). Comparative Cytogenetics 5(4): 355–374. https://doi.org/10.3897/compcytogen.v5i4.2307

Grozeva S, Anokhin B, Kuznetsova VG (2015) Bed bugs (Hemiptera). In: Sharakhov I (Ed.) Protocols for Cytogenetic Mapping of Arthropod Genomes. CRC press, Taylor & Francis, Boca Raton, 285–326. https://doi.org/10.1201/b17450-9

Grozeva S, Anokhin BA, Simov N, Kuznetsova VG (2019) New evidence for the presence of the telomere motif (TTAGG)n in the family Reduviidae and its absence in the families Nabidae and Miridae (Hemiptera, Cimicomorpha). Comparative Cytogenetics 13(3): 283–295. https://doi.org/10.3897/CompCytogen.v13i3.36676

Kuznetsova VG, Grozeva S, Gokhman VE (2019) Telomere structure in insects: A review. Journal of Zoological Systematics and Evolutionary Research 19 November, 2019. https://doi.org/10.1111/jzs.12332

Kuznetsova VG, Grozeva SM, Hartung V, Anokhin BA (2015) First evidence for $(TTAGG)_n$ telomeric sequence and sex chromosome post-reduction in Coleorrhyncha (Insecta, Hemiptera). Comparative Cytogenetics 9(4): 523–532. https://doi.org/10.3897/CompCytogen.v9i4.5609

Mandrioli M, Zanasi F, Manicardi GC (2014) Karyotype rearrangements and telomere analysis in *Myzus persicae* (Hemiptera, Aphididae) strains collected on *Lavandula* sp. plants. Comparative Cytogenetics 8(4): 259–274. https://doi.org/10.3897/CompCytogen.v8i4.8568

Maryańska-Nadachowska A, Kuznetsova VG, Karamysheva TV (2013) Chromosomal location of rDNA clusters and TTAGG telomeric repeats in eight species of the spittlebug genus *Philaenus* (Hemiptera: Auchenorrhyncha: Aphrophoridae). European Journal of Entomology 110(3): 411–418. https://doi.org/10.14411/eje.2013.055

Maryańska-Nadachowska A, Kuznetsova VG, Golub NV, Anokhin BA (2018) Detection of telomeric sequences and ribosomal RNA genes in holokinetic chromosomes of five jumping plant-lice species: First data on the superfamily Psylloidea (Hemiptera: Sternorrhyncha). European Journal of Entomology 115: 632–640. https://doi.org/10.14411/eje.2018.061

Meinander M, Halkka O, Söderlund V (1974) Chromosomal evolution in the Psocoptera. Notulae Entomologicae 54: 81–84.

Mockford EL (2018) Biodiversity of Psocoptera. In: Foottit RG, Adler PH (Eds) Insect Biodiversity: Science and Society. Wiley Online Library, 417–456. https://doi.org/10.1002/9781118945582.ch16

Panzera Y, Pita S, Ferreiro MJ, Ferrandis I, Lages C, Pérez R, Silva AE, Guerra F, Panzera F (2012) High dynamics of rDNA cluster location in kissing bug holocentric chromosomes (Triatominae, Heteroptera). Cytogenetic and Genome Research 138: 56–67. https://doi.org/10.1159/000341888

Panzera F, Pita S, Nattero J, Panzera Y, Galvão C, Chavez T, Arias AR, Téllez LC, Noireau F (2015) Cryptic speciation in the *Triatoma sordida* subcomplex (Hemiptera, Reduviidae) revealed by chromosomal markers. Parasites and Vectors 8: 495. https://doi.org/10.1186/s13071-015-1109-6

Salanitro LB, Massaccesi AC, Urbisaglia S, Bressa MJ, Chirino MG (2017) A karyotype comparison between two species of bordered plant bugs (Hemiptera, Heteroptera, Largidae) by conventional chromosome staining, C-banding and rDNA-FISH. Comparative Cytogenetics 11(2): 239–248. https://doi.org/10.3897/CompCytogen.v11i2.11683

Yoshizawa K, Saigusa T (2001) Phylogenetic analysis of paraneopteran orders (Insecta: Neoptera) based on forewing base structure, with comments on monophyly of Auchenorrhyncha (Hemiptera). Systematic Entomology 26: 1–13. https://doi.org/10.1046/j.1365-3113.2001.00133.x

Wong SK, Thornton IWB (1966) Chromosome numbers of some Psocid genera (Psocoptera). Nature 211: 214–215. https://doi.org/10.1038/211214a0

CompCytogen 13(4): 411–422 (2019)
doi: 10.3897/CompCytogen.v13i4.36205
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Cytogenetic characterization of *Hypostomus soniae* Hollanda-Carvalho & Weber, 2004 from the Teles Pires River, southern Amazon basin: evidence of an early stage of an XX/XY sex chromosome system

Luciene Castuera de Oliveira[1], Marcos Otávio Ribeiro[2], Gerlane de Medeiros Costa[1], Cláudio Henrique Zawadzki[3], Ana Camila Prizon-Nakajima[2], Luciana Andreia Borin-Carvalho[2], Isabel Cristina Martins-Santos[2], Ana Luiza de Brito Portela-Castro[2]

1 *Faculdade de Ciências Biológicas e Agrárias, Universidade do Estado de Mato Grosso, Alta Floresta, Mato Grosso, Brazil* **2** *Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá, Maringá, Paraná, Brazil* **3** *Departamento de Biologia, Acc., Universidade Estadual de Maringá, Maringá, Paraná, Brazil*

Corresponding author: *Luciana Andreia Borin-Carvalho* (labcarvalho@uem.br)

Academic editor: *N. Bogutskaya* | Received 15 May 2019 | Accepted 11 November 2019 | Published 11 December 2019

http://zoobank.org/C94FF4A6-86DE-45EF-9014-C17E6086C408

Citation: de Oliveira LC, Ribeiro MO, Costa GM, Zawadzki CH, Prizon-Nakajima AC, Borin-Carvalho LA, Martins-Santos IC, Portela-Castro ALB (2019) Cytogenetic characterization of *Hypostomus soniae* Hollanda-Carvalho & Weber, 2004 from the Teles Pires River, southern Amazon basin: evidence of an early stage of an XX/XY sex chromosome system. Comparative Cytogenetics 13(4): 411–422. https://doi.org/10.3897/CompCytogen.v13i4.36205

Abstract

In the present study, we analyzed individuals of *Hypostomus soniae* (Loricariidae) collected from the Teles Pires River, southern Amazon basin, Brazil. *Hypostomus soniae* has a diploid chromosome number of 2n = 64 and a karyotype composed of 12 metacentric (m), 22 submetacentric (sm), 14 subtelocentric (st), and 16 acrocentric (a) chromosomes, with a structural difference between the chromosomes of the two sexes: the presence of a block of heterochromatin in sm pair No. 26, which appears to represent a putative initial stage of the differentiation of an XX/XY sex chromosome system. This chromosome, which had a heterochromatin block, and was designated proto-Y (pY), varied in the length of the long arm (q) in comparison with its homolog, resulting from the addition of constitutive heterochromatin. It is further

distinguished by the presence of major ribosomal cistrons in a subterminal position of the long arm (q). The Nucleolus Organizer Region (NOR) had different phenotypes among the *H. soniae* individuals in terms of the number of Ag-NORs and 18S rDNA sites. The origin, distribution and maintenance of the chromosomal polymorphism found in *H. soniae* reinforced the hypothesis of the existence of a proto-Y chromosome, demonstrating the rise of an XX/XY sex chromosome system.

Keywords
Fish cytotaxonomy, chromosome banding, rDNA FISH, chromosome polymorphism, Loricariidae

Introduction

The Teles Pires River, in the southern Amazon basin, is the home of at least 36 species of Loricariidae, and five species of *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae) (Ohara et al. 2017). *Hypostomus* is considered to be one of the taxonomically most complex genera of Neotropical fish due to its enormous diversity of morphology and body pigmentation patterns, with a total of 203 recognized species (Froese and Pauly 2019). The diversification of this genus appears to be closely related to changes in the chromosome complement, which include diploid numbers (2n) ranging from 52 in *H. emarginatus* (Artoni and Bertollo 2001) to 84 in *Hypostomus* sp. (Cereali et al. 2008). However, a phylogenetic analysis of mitochondrial DNA sequences (Montoya-Burgos 2003) indicated that *H. emarginatus* does not belong to the principal *Hypostomus* clade, which would mean that the lowest diploid number in the genus is 2n = 64, found in *H. cochliodon* (Bueno et al. 2013; Rubert et al. 2016), *H. faveolus* (Bueno et al. 2013), and *Hypostomus* sp. (Artoni et al. 1998; Fenerich et al. 2004; Milhomem et al. 2010).

A number of cytogenetic studies have examined various aspects of the differentiation of the *Hypostomus* karyotype, including complex karyotype evolution (Martinez et al. 2011; Alves et al. 2012; Pansonato-Alves et al. 2013; Bueno et al. 2014), heterochromatin polymorphism (Traldi et al. 2012; Baumgärtner et al. 2014), inter-individual chromosome polymorphism (Artoni and Bertollo 1999; Ferreira et al. 2019), and morphologically differentiated sex chromosomes (Artoni et al. 1998; Oliveira et al. 2015; Kamei et al. 2017). A range of sex chromosome systems found in 705 fish species are available in the Tree of Sex Consortium (2014) database. Differentiated sex chromosome systems are not very common in the loricariid catfishes, although simple (Alves et al. 2006; de Oliveira et al. 2007; Prizon et al. 2017) and multiple systems (Centofante et al. 2006; de Oliveira et al. 2008; Blanco et al. 2014) have been described in this family. In the genus *Hypostomus*, only a simple sexual chromosomal system has been described, with a XX/XY system being found in *H. ancistroides* and *H. macrops*, identified as *Plecostomus ancistroides* and *P. macrops*, respectively (Michele et al. 1977; Rocha-Reis et al. 2018), and a ZZ/ZW system in *Hypostomus* sp. G (Artoni et al. 1998), *H.* cf. *plecostomus* (Oliveira et al. 2015) and *H. ancistroides* (Kamei et al. 2017).

Highly differentiated sex chromosomes have been analyzed in a number of different groups of animals, although the initial stages of the evolution of sex chromosome

systems have not often been described. Even so, an overview of the literature shows that our understanding of the various stages in the evolution of sex chromosome systems has increased progressively over time (Nanda et al. 1992; Bergero and Charlesworth 2009; Wright et al. 2016; Abbott et al. 2017; Kottler and Schartl 2018). The present study describes a karyotype with a putative initial stage of the differentiation of sex chromosomes in a population of *H. soniae* from the basin of the Teles Pires River, in southern Amazonia.

Material and methods

We analyzed 17 *Hypostomus soniae* individuals (5 ♂ and 12 ♀) collected from urban streams located in Alta Floresta (9°54'30.82"S, 56°03'33.86"W; 9°53'50.47"S, 56°03'39.50"W; 9°53'30.53"S, 56°04'18.75"W), in Mato Grosso, Brazil. This area is part of the Teles Pires River drainage in the southern Amazon basin. The individuals were collected according to Brazilian environmental legislation (Collecting license MMA/IBAMA/SISBIO, number 31423-1). The individuals were anesthetized and euthanized by clove-oil overdose (Griffiths 2000). Voucher specimens were deposited in the ichthyological collection of the Núcleo de Pesquisa em Limnologia, Ictiologia e Aquicultura (Nupélia) of Universidade Estadual de Maringá (**UEM**) under catalogue number NUP 14991.

Chromosome preparations were obtained from kidney cells using the technique of Bertollo et al. (1978). The NORs were detected by impregnation with silver nitrate ($AgNO_3$) (Howell and Black 1980). The constitutive heterochromatin was identified by C-banding (Sumner 1972), and stained with propidium iodide (Lui et al. 2012). Fluorescence *in situ* Hybridization (FISH) followed the protocol of Pinkel et al. (1986), using 18S rDNA probes from *Prochilodus argenteus* (Hatanaka and Galetti Jr. PM 2004), labeled with a Biotin Nick Translation kit, and 5S rDNA probes from *Leporinus elongatus* (Martins and Galetti Jr. PM 1999) labeled with a Digoxigenin Nick Translation kit. The chromosomes were classified according to Levan et al. (1964), i.e., metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a).

Results

Hypostomus soniae has the diploid chromosome number of 2n = 64, fundamental number (FN) equal to 112, and a karyotype composed of 12m + 22sm + 14st + 16a chromosomes, in both males and females (Fig. 1A). Small heterochromatin blocks were observed in some chromosomes, primarily in the terminal regions, and conspicuous heterochromatic blocks were observed in the q arms of pairs Nos. 25 and 26 (Fig. 1B). The Giemsa staining and C-banding also revealed size heteromorphism between the homologs of pair No. 26 in the males and, to a lesser extent, in the females (Fig. 1A, B).

Pair No. 25 was highlighted for comparisons with pair No. 26, to determine more precisely the size difference between the homologs of the latter (Fig. 1C, D). This allowed us to identify three variant chromosomes that may correspond to pair No. 26 in the karyotypes of the individuals from the study population (Fig. 1C, D): (i) a chro-

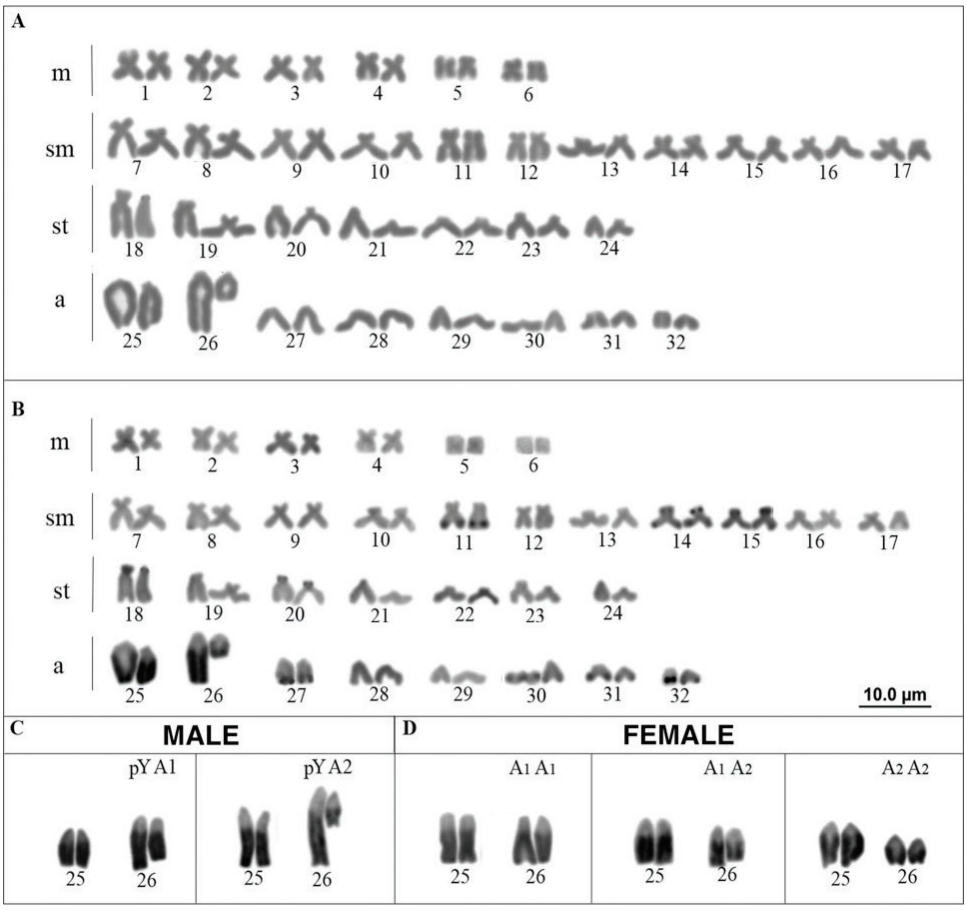


Figure 1. Karyotype of a male *Hypostomus soniae* obtained from **A** Giemsa-stained and **B** sequentially C-banded chromosomes. Variant chromosomes of pair No. 26, with pair No. 25 for comparison, in **C** males and **D** females. The dark regions in the chromosomes represent the heterochromatic blocks.

mosome larger than that of pair No. 25, which was found only in the males, and was designated pY (proto-Y); (ii) a chromosome similar in size to pair No. 25, designated A1, and (iii) a chromosome smaller than pair 25, designated A2. Considering a panmictic population, these chromosomes may form the following combinations for pair No. 26: in the males, pYA1 (found in 3 individuals) and pYA2 (2 individuals), whereas in the females, there are three possible combinations: A1A1 (3 individuals), A1A2 (6 individuals), and A2A2 (3 individuals) (Fig. 2).

The Ag-NOR-staining and FISH with the 18S rDNA probe revealed multiple nucleolus organizer regions (NORs) in a terminal portion of the short arms (p) of two pairs of sm chromosomes (Nos. 14 and 15) and in a terminal position of the q arms of three pairs of a chromosomes (Nos. 25, 26 and 31). Inter-individual variation in the 18S rDNA sites revelead six different phenotypes (Fig. 3). In all phenotypes, FISH revealed positive 18S rDNA sites in pair No. 26. The 18S rDNA sites corresponded to heterochromatin blocks in all cases.

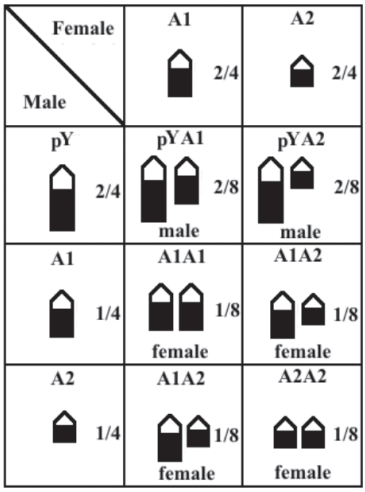


Figure 2. Combinations of the homologous pair No. 26 resulting from crossing males and females of the *Hypostomus soniae* study population. The dark regions in the chromosomes represent the heterochromatic blocks.

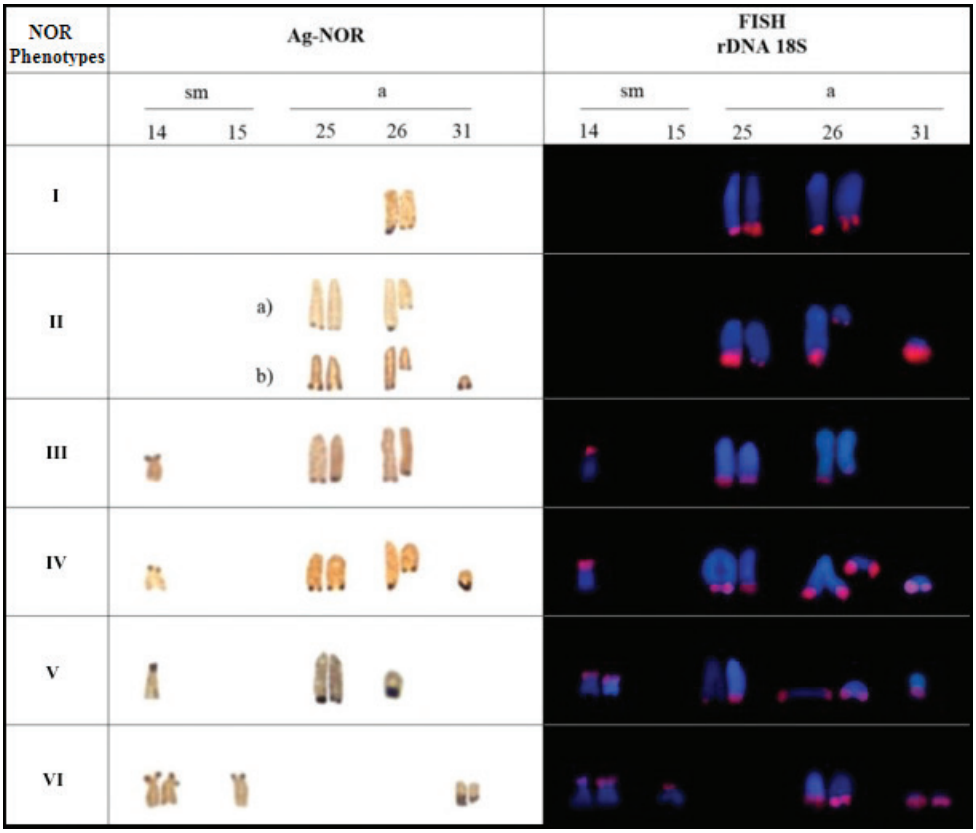


Figure 3. The Ag-NOR phenotypes observed in the karyotypes of *Hypostomus soniae*, detected by silver nitrate impregnation, FISH with 18S probes. The numbers 14, 15, 25, 26 and 31 represent the chromosomal pairs; sm = submetacentric; a = acrocentric.

Discussion

Hypostomus soniae belongs to the "*H. cochliodon* species group" (Hollanda-Carvalho and Weber 2004) and has 2n = 64, similar to *H. cochliodon*, analyzed by Bueno et al. (2013) and Rubert et al. (2016), which is the lowest 2n found in the genus. Considering a basal 2n = 54 for the family Loricariidae (Artoni and Bertollo 2001), 2n = 64 would be the basal character for the genus *Hypostomus* (Bueno et al. 2014).

In *Hypostomus*, several cases of chromosomal polymorphism associated with the amplification of the heterochromatin, with or without ribosomal genes, have been reported (Artoni and Bertollo 1999; Traldi et al. 2012; Baumgärtner et al. 2014; Lorscheider et al. 2018), but in none of these cases was the polymorphism found in only one of the sexes. In the present paper, all the *H. soniae* individuals analyzed had the same karyotype structures, although differences were found between the sexes in pair No. 26, indicating a putative incipient process of sex chromosome differentiation. This differentiation pattern was supported by the presence of size heteromorphism in the heterochromatic block between the homologs of pair No. 26. This remarkable heterochromatin size polymorphism may indicate an early stage of the sex chromosome differentiation, where the chromosome with a large block of heterochromatin, designated here the proto-Y (pY), was observed only in the males. In the females, the corresponding homologs of pair No. 26 were also polymorphic, with one of the chromosomes having a heterochromatic block of medium size (designated A1) and the other (designated A2), a much smaller block. The detection of these variant chromosomes in both sexes reinforces the hypothesis of an initial process of heteromorphic sex chromosome formation, in which heterochromatinization plays a fundamental role.

The proto-Y chromosome in the genome of *H. soniae* is larger than the X chromosome, as observed in the Y chromosome of *H.* aff. *ancistroides* analyzed by Rocha-Reis et al. (2018). Thus, the larger size of the proto-Y chromosome may be the result of the apparent accumulation of heterochromatin, mediated by transposable elements, which may play an important role in the differentiation process, as observed in other species of fish (see Chalopin et al. 2015).

One other ancestral trait in the Loricariidae is the existence of a chromosome pair with NORs, which has been described in a number of fish species (Artoni and Bertollo 1996; Alves et al. 2005; Bueno et al. 2014; Rubert et al. 2016), including some species of the genus *Hypostomus* (Mendes-Neto et al. 2011; Rubert et al. 2011; Alves et al. 2012). Multiple NORs, as observed in *H. soniae* in the present study, are considered to be a derived characteristic, and are the most common pattern in the genus *Hypostomus* (Rubert et al. 2016; Brandão et al. 2018). In the "*H. cochliodon* group", multiple NORs were noted in *H. cochliodon* from the Paraguay River basin (Rubert et al. 2016), although Bueno et al. (2014) observed a simple NOR in *H. cochliodon* individuals from the Paraná River basin. While *H. soniae* is part of the monophyletic "*H. cochliodon* species group", the lack of data limits conclusions on which phenotype (simple or multiple NORs) is derived, because this feature has only been investigated

in two species of this group, i.e., *H. soniae* (present paper) and *H. cochliodon* (Bueno et al. 2014; Rubert et al. 2016).

We observed inter-individual numerical variation in the Ag-NOR and 18S rDNA sites among the *H. soniae* individuals. This reflects the enormous mobility of the rDNA cistrons, and suggests the existence of dispersal mechanisms for these sites. The variation observed by silver staining is assumed to be the result of shifts in the control of the expression of ribosomal cistrons. The FISH 18S revealed that chromosome pair No. 26 was present in all of the different NOR phenotypes. These findings may reflect the transposition of rDNA genes, which had been located in pair No. 26, compared to the other chromosomes that bear major ribosomal cistrons. A similar hypothesis has been used to account for the variability in the number of NORs found in previous studies (Santi-Rampazzo et al. 2008; Porto et al. 2014). The presence of heterochromatin associated with all the ribosomal cistrons, as observed here, may indicate that mobile elements are part of the structure and organization of the adjacent heterochromatin found at these sites. While we did not investigate the presence of transposable elements (TEs) in the present study, these sequences are known to be associated with the 28S/18S rDNA in fish (Mandrioli et al. 2001; Symonová et al. 2013; Gouveia et al. 2017) and, more commonly, with the heterochromatin, including *Hypostomu*s (Pansonato-Alves et al. 2013).

The proto-sex chromosomes of *H. soniae* were also characterized by the presence of 18S rDNA cistrons. The association between the 18S rDNA sites and sex chromosomes has been reported in fishes (Artoni and Bertollo 2002, Chen et al. 2008; Cioffi and Bertollo 2010), including in the genus *Hypostomus* (Rocha-Reis et al. 2018). Repetitive sequences have been recorded at high frequencies in heterochromatic sex chromosomes and Chalopin et al. (2015) linked the evolution and emergence of sex chromosomes to the dynamics of the repeats and transposable elements. Therefore, the possible association of TEs with the ribosomal genes and adjacent heterochromatic blocks in pairs Nos. 25 and 26 in the *H. soniae* karyotype may indicate a possible link with TEs in the initial steps of the differentiation of the sex chromosomes.

Conclusion

The data presented here on *H. soniae* include previously unpublished karyotypic arrangements, which represent an important contribution to future taxonomic studies of the *H. cochliodon* species group. In *Hypostomus*, the addition of heterochromatin to some chromosomes is the cause of polymorphisms resulting in different cytotypes, although this is the first cytological evidence of this mechanism emerging in sex chromosomes in this group. The apparent emergence of novel sex chromosomes in *H. soniae* makes this species an excellent potential model for the study of the differentiation and evolution of mechanisms of sexual determination, and the role of the accumulation and amplification of repetitive sequences in the origin and differentiation of sex chromosomes and its implications for the speciation process.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001- Universidade Estadual de Maringá (UEM, PR) and FAPEMAT (Fundação de Amparo a Pesquisa do Estado do Mato Grosso, Brazil), Universidade Estadual de Mato Grosso.

References

Abbott JK, Nordén AK, Hansson B (2017) Sex chromosome evolution: historical insights and future perspectives. Proceedings Biological Sciences 284: 20162806. https://doi.org/10.1098/rspb.2016.2806

Alves AL, Borba RS, Oliveira C, Nirchio M, Granado A, Foresti F (2012) Karyotypic diversity and evolutionary trends in the Neotropical catfish genus *Hypostomus* Lacépède, 1803 (Teleostei, Siluriformes, Loricariidae). Comparative Cytogenetics 6(4): 443–452. https://doi.org/10.3897/compcytogen.v6i4.4028

Alves AL, Oliveira C, Nirchio M, Granado A, Foresti F (2006) Karyotypic relationships among the tribes of Hypostominae (Siluriformes: Loricariidae) with description of XO sex chromosome system in a Neotropical fish species. Genetica 128: 1–9. https://doi.org/10.1007/s10709-005-0715-1

Alves AL, Oliveira C, Foresti F (2005) Comparative cytogenetic analysis of eleven species of subfamilies Neoplecostominae e Hypostominae (Siluriformes: Loricariidae). Genetica 128: 127–136. https://doi.org/10.1007/s10709-004-7561-4

Artoni RF, Bertollo LAC (2002) Evolutionary aspects of the ZZ/ZW sex chromosome system in the Characidae fish, genus *Triportheus*. A monophyletic state and NOR location on the W chromosome. Heredity 89: 15–19. https://doi.org/10.1038/sj.hdy.6800081

Artoni RF, Bertollo LAC (2001) Trends in the karyotype evolution of Loricariidae fish (Siluriformes). Hereditas 134: 201–210. https://doi.org/10.1111/j.1601-5223.2001.00201.x

Artoni RF, Bertollo LAC (1999) Nature and distribution of constitutive heterochromatin in fishes, genus *Hypostomus* (Loricariidae). Genetica (The Hague) Netherlands 106: 209–214. https://doi.org/10.1023/A:1003957719178

Artoni RF, Venere PC, Bertollo LAC (1998) A heteromorphic ZZ/ZW sex chromosome system in fish, genus *Hypostomus* (Loricariidae). Cytologia 63: 421–425. https://doi.org/10.1508/cytologia.63.421

Artoni RF, Bertollo LAC (1996) Cytogenetic studies on Hypostominae (Pisces, Siluriformes, Loricariidae). Considerations on karyotype evolution in the genus *Hypostomus*. Caryologia 49: 81–90. https://doi.org/10.1080/00087114.1996.10797353

Baumgärtner L, Paiz LM, Zawadzki CH, Margarido VP, Portela-Castro AL (2014) Heterochromatin polymorphism and physical mapping of 5S and 18S ribosomal DNA in four populations of *Hypostomus strigaticeps* (Regan, 1907) from the Parana River Basin, Brazil: Evolutionary and Environmental Correlation. Zebrafish 11: 479–487. https://doi.org/10.1089/zeb.2014.1028

Bertollo LAC, Takahashi CS, Moreira-filho O (1978) Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). Revista Brasileira de Genética 2: 103–120.

Blanco DR, Vicari MR, Lui RL, Artoni RF, Almeida MC, Traldi JB, Margarido VP, Moreira-Filho O (2014) Origin of the $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system of *Harttia punctata* (Siluriformes, Loricariidae) inferred from chromosome painting and FISH with ribosomal DNA markers. Genetica 142(2): 119–126. https://doi.org/10.1007/s10709-014-9759-4

Brandão KO, Rocha-Reis DA, Garcia C, Pazza R, de Almeida-Toledo LF, Kavalco KF (2018) Studies in two allopatric populations of *Hypostomus affinis* (Steindachner, 1877): the role of mapping the ribosomal genes to understand the chromosome evolution of the group. Comparative Cytogenetics 12(1): 1–12. https://doi.org/10.3897/compcytogen.v12i1.22052

Bueno V, Venere PC, Konerat JT, Zawadzki CH, Vicari MR, Margarido VP (2014) Physical mapping of the 5S and 18S rDNA in ten species of *Hypostomus* Lacépède 1803 (Siluriformes: Loricariidae): Evolutionary tendencies in the genus. The Scientific World Journal 2014: 1–8. https://doi.org/10.1155/2014/943825

Bueno V, Venere PC, Zawadzki CH, Margarido VP (2013) Karyotypic diversification in *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae): biogeographical and phylogenetic perspectives. Review in Fish Biology and Fisheries 23: 103–112. https://doi.org/10.1007/s11160-012-9280-8

Centofante L, Bertollo LAC, Moreira-Filho O (2006) Cytogenetic characterization and description of XX/XY_1Y_2 sex chromosome system in catfish *Harttia carvalhoi* (Siluriformes, Loricariidae). Cytogenetic and Genome Research 112: 320–324. https://doi.org/10.1159/000089887

Cereali SS, Pomini E, Rosa R, Zawadzki CH, Froehlich O, Giuliano-Caetano L (2008) Karyotype description of two species of *Hypostomus* (Siluriformes, Loricariidae) of the Planalto da Bodoquena, Brazil. Genetics and Molecular Research 7: 583–591. https://doi.org/10.4238/vol7-3gmr404

Chalopin D, Volff JN, Galiana D, Anderson JL, Schart M (2015) Transposable elements and early evolution of sex chromosomes in fish. Chromosome Research 23(3): 545–560. https://doi.org/10.1007/s10577-015-9490-8

Chen J, Fu Y, Xiang D, Zhao G, Long H, Liu J, Yu Q (2008) XX/XY heteromorphic sex chromosome systems in two bullhead catfish species, *Liobagrus marginatus* and *L. styani* (Amblycipitidae, Siluriformes). Cytogenetis and Genome Research 122: 169–174. https://doi.org/10.1159/000163095

Cioffi MB, Bertollo LA (2010) Initial steps in XY chromosome differentiation in *Hoplias malabaricus* and the origin of an X(1)X(2)Y sex chromosome system in this fish group. Heredity 105(6): 554–561. https://doi.org/10.1038/hdy.2010.18

de Oliveira RR, Feldberg E, Anjos MB, Zuanon J (2008) Occurrence of multiple sexual chromosomes (XX/XY_1Y_2 and $Z_1Z_1Z_2Z_2/Z_1Z_2W_1W_2$) in catfishes of the genus *Ancistrus* (Siluriformes, Loricariidae) from the Amazon Basin. Genetica 134(2): 243–249. https://doi.org/10.1007/s10709-007-9231-9

de Oliveira RR, Feldberg E, Anjos MB, Zuanon J (2007) Karyotype characterization and ZZ/ZW sex chromosomes heteromorphism in two species of the catfish genus *Ancistrus* Kner,

1854 (Siluriformes: Loricariidae) from the Amazon basin. Neotropical Ichthyology 5(3): 301–306. https://doi.org/10.1590/S1679-62252007000300010

Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208: 191–364. https://doi.org/10.1016/S0044-8486(02)00057-1

Fenerich PC, Foresti F, Oliveira C (2004) Nuclear DNA content in 20 species of Siluriformes (Teleostei: Ostariophysi) from Neotropical region. Genetics and Molecular Biology 27: 350–354. https://doi.org/10.1590/S1415-47572004000300008

Ferreira GEB, Barbosa LM, Prizon-Nakajima AC, Paiva S, Vieira MMR, Gallo RB, Borin-Carvalho LA, Rosa R, Zawadzki CH, Santos ICM, Portela-Castro ALB (2019) Constitutive heterochromatin heteromorphism in the Neotropical armored catfish *Hypostomus regani* (Ihering, 1905) (Loricariidae, Hypostominae) from the Paraguay River basin (Mato Grosso do Sul, Brazil). Comparative Cytogenetics 13(1): 27–39. https://doi.org/10.3897/CompCytogen.v13i1.30134

Froese R, Pauly D (2019) FishBase: World Wide Web electronic publication. http://www.fishbase.org [accessed 13. October 2019]

Gouveia JG, Wolf IR, Vilas-Boas LA, Heslop-Harrison JS, Schwarzacher T, Dias AL (2017) Repetitive DNA in the catfish genome: rDNA, microsatellites, and Tc1-mariner transposon sequences in *Imparfinis* species (Siluriformes, Heptapteridae). Journal of Heredity 108: 650–657. https://doi.org/10.1093/jhered/esx065

Griffiths SP (2000) The use clove oil as an anaesthetic and method for sampling intertidal rockpool fishes. Journal of Fish Biology 57: 1453–1464. https://doi.org/10.1111/j.1095-8649.2000.tb02224.x

Hatanaka T, Galetti Jr PM (2004) Mapping of the 18S and 5S ribosomal RNA genes in the fish *Prochilodus argenteus* Agassiz, 1829 (Characiformes, Prochilodontidae). Genetica 122: 239–244. https://doi.org/10.1007/s10709-004-2039-y

Hollanda-Carvalho P, Weber C (2004) Five new species of the *Hypostomus cochliodon* group (Siluriformes: Loricariidae) from middle and lower Amazon System. Revue Suisse de Zoologie 111(4): 953–978. https://doi.org/10.5962/bhl.part.80281

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. https://doi.org/10.1007/BF01953855

Kamei MCSL, Baumgärtner L, Paiva S, Zawadzki CH, Martins-Santos IC, Portela-Castro ALB (2017) Chromosomal diversity of three species of *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae), from the Paraná River Basin, Brazil: A species complex in *Hypostomus ancistroides* reinforced by a ZZ/ZW sex chromosome system. Zebrafish 14: 1–4. https://doi.org/10.1089/zeb.2017.1429

Kottler VA, Schartl M (2018) The colorful sex chromosomes of Teleost fish. Genes (Basel) 9(5): 1–233. https://doi.org/10.3390/genes9050233

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

Lorscheider CA, Oliveira JIN, Dulz TA, Nogaroto V, Martins-Santos I, Vicari MR (2018) Comparative cytogenetics among three sympatric *Hypostomus* species (Siluriformes: Loricariidae): an evolutionary analysis in a high endemic region. Brazilian Archives of Biology and Technology 6: e18180417. https://doi.org/10.1590/1678-4324-2018180417

Lui RL, Blanco DR, Moreira-Filho O, Margarido VP (2012) Propidium iodide for making heterochromatin more evident in the C-banding technique. Biotechnic and Histochemistry 87: 433–438. https://doi.org/10.3109/10520295.2012.696700

Mandrioli M, Manicardi GC, Machella N, Caputo V (2001) Molecular and cytogenetic analysis of the goby *Gobius niger* (Teleostei, Gobiidae). Genetica 110: 73–78. https://doi.org/10.1023/A:1017547005143

Martinez ERM, Zawadzki CH, Foresti F, Oliveira C (2011) Cytogenetic analysis of five *Hypostomus* species (Siluriformes, Loricariidae). Genetics and Molecular Biology 34(4): 562–568. https://doi.org/10.1590/S1415-47572011005000038

Martins C, Galetti Jr PM (1999) Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Research 5: 363–367. https://doi.org/10.1023/A:1009216030316

Mendes-Neto EO, Vicari MR, Artoni RF, Moreira-Filho O (2011) Description of karyotype in *Hypostomus regani* (Iheringi, 1905) (Teleostei, Loricariidae) from the Piumhi river in Brazil with comments on karyotype variation found in *Hypostomus*. Comparative Cytogenetics 5: 133–1425. https://doi.org/10.3897/compcytogen.v5i2.964

Michele JL, Takahashi CS, Ferrari I (1977) Karyotypic study of some species of the family Loricariidae (Pisces). Cytologia 42: 539–546. https://doi.org/10.1508/cytologia.42.539

Milhomem SSR, Castro RR, Nagamachi CY, Souza ACP, Feldberg E, Pieczarka JC (2010) Different cytotypes in fishes of the genus *Hypostomus* Lacépède, 1803, (Siluriformes: Loricariidae) from Xingu river (Amazon region, Brazil). Comparative Cytogenetics 4: 45–54. https://doi.org/10.3897/compcytogen.v4i1.31

Montoya-Burgos JI (2003) Historical biogeography of the catfish genus *Hypostomus* (Siluriformes: Loricariidae), with implications on the diversification of Neotropical ichthyofauna. Molecular Ecology 12: 1855–1867. https://doi.org/10.1046/j.1365-294X.2003.01857.x

Nanda I, Schartl M, Feichtinger W, Epplen JT, Schmid M (1992) Early stages of sex chromosome differentiation in fish as analysed by simple repetitive DNA sequences. Chromosoma 101(5–6): 301–310. https://doi.org/10.1007/BF00346009

Ohara WM, Lima FCT, Salvador GN, Andrade MC (2017) Peixes do Rio Teles Pires: Diversidade e Guia de Idenficação. Goiânia, Gráfica e Editora Amazonas, 408 pp.

Oliveira LC, Ribeiro MO, Dutra ES, Zawadzki CH, Portela-Castro ALB, Martins-Santos IC (2015) Karyotype structure of *Hypostomus* cf. *plecostomus* (Linnaeus, 1758) from Tapajós River basin, Southern Amazon: occurrence of sex chromosomes (ZZ/ZW) and their evolutionary implications. Genetics and Molecular Research 14: 6625–6634. https://doi.org/10.4238/2015.June.18.5

Pansonato-Alves JC, Serrano EA, Utsunomia R, Scacchetti PC, Oliveira C, Foresti F (2013) Mapping five repetitive DNA classes in sympatric species of *Hypostomus* (Teleostei: Siluriformes: Loricariidae): analysis of chromosomal variability. Review in Fish Biology and Fisheries 23: 477–489. https://doi.org/10.1007/s11160-013-9303-0

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences 83: 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Porto FE, Vieira MMR, Barbosa LG, Borin-Carvalho LA, Vicari MR, Portela-Castro ALB, Martins-Santos IC (2014) Chromosomal polymorphism in *Rineloricaria lanceolata*

Günther, 1868 (Loricariidae: Loricariinae) of the Paraguay basin (Mato Grosso do Sul, Brazil): evidence of fusions and their consequences in the population. Zebrafish 11(4): 318–324. https://doi.org/10.1089/zeb.2014.0996

Prizon AC, Bruschi DP, Borin-Carvalho LA, Cius A, Barbosa LM, Ruiz HB, Zawadzki CH, Fenocchio AS, Portela-Castro ALB (2017) Hidden diversity in the populations of the armored catfish *Ancistrus* Kner, 1854 (Loricariidae, Hypostominae) from the Paraná River Basin revealed by molecular and cytogenetic data. Frontiers in Genetics 8: 1–185. https://doi.org/10.3389/fgene.2017.00185

Rocha-Reis DA, Brandão KO, Almeida-Toledo LF, Pazza R, Kavalco KF (2018) The persevering cytotaxonomy: discovery of a unique XX/XY sex chromosome system in catfishes suggests the existence of a new, endemic and rare species. Cytogenetic and Genome Research 45(1): 45–55. https://doi.org/10.1159/000492959

Rubert M, Rosa R, Zawadzki CH, Mariotto S, Moreira-Filho O, Giuliano-Caetano L (2016) Chromosome mapping of 18S ribosomal RNA genes in eleven *Hypostomus* species (Siluriformes, Loricariidae): Diversity analysis of the sites. Zebrafish 13: 360–368. https://doi.org/10.1089/zeb.2016.1279

Rubert M, Rosa R, Jerep FC, Bertollo LAC, Giuliano-Caetano L (2011) Cytogenetic characterization of four species of the genus *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae) with comments on its chromosomal diversity. Comparative Cytogenetics 5: 397–410. https://doi.org/10.3897/compcytogen.v5i5.1589

Santi-Rampazzo AP, Nishiyama PB, Ferreira PEB, Martins-Santos IC (2008) Intrapopulational polymorphism of nucleolus organizer region in *Serrapinnus notomelas* (Characidae, Cheirodontinae) from the Paraná River. Journal of Fish Biology 72(5): 1236–1243. https://doi.org/10.1111/j.1095-8649.2007.01784.x

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

Symonová R, Majtánová Z, Sember A, Staaks GBO, Bohlen J, Freyhof J, Rábová M, Ráb P (2013) Genome differentiation in a species pair of coregonine fishes: an extremely rapid speciation driven by stress activated retrotransposons mediating extensive ribosomal DNA multiplications. BMC Evolutionary Biology 13: 1–42. https://doi.org/10.1186/1471-2148-13-42

The Tree of Sex Consortium (2014) Tree of Sex: A database of sexual systems. Scientific Data 1(140015): 1–8. https://doi.org/10.1038/sdata.2014.15

Traldi JB, Vicari MR, Blanco DR, Martinez JDF, Artoni RF, Moreira-Filho O (2012) First karyotype description of *Hypostomus iheringii* (Regan, 1908): a case of heterochromatic polymorphism. Comparative Cytogenetics 6: 115–125. https://doi.org/10.3897/compcytogen.v6i2.2595

Wright AE, Dean R, Zimmer F, Mank JE (2016) How to make a sex chromosome. Nature Communications 7: 1–12087. https://doi.org/10.1038/ncomms12087

CompCytogen 13(4): 423–434 (2019)
doi: 10.3897/CompCytogen.v13i4.39576
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Cytogenetic and molecular characteristics of rye genome in octoploid triticale (× *Triticosecale* Wittmack)

Elena V. Evtushenko[1*], Yulia A. Lipikhina[1], Petr I. Stepochkin[2], Alexander V. Vershinin[1,3*]

1 *Institute of Molecular and Cellular Biology SB RAS, acad. Lavrentiev ave. 8/2, Novosibirsk, 630090, Russia* **2** *Institute of Cytology and Genetics SB RAS, acad. Lavrentiev ave. 10, Novosibirsk, 630090, Russia* **3** *Novosibirsk State University, Pirogova str. 1, Novosibirsk, 630090, Russia*

Corresponding author: *Elena V. Evtushenko* (evt@mcb.nsc.ru)

Academic editor: *J. R. Daviña* | Received 30 August 2019 | Accepted 27 November 2019 | Published 16 December 2019

http://zoobank.org/CBEADA73-E45A-40BA-9754-ED39DB199721

Citation: Evtushenko EV, Lipikhina YA, Stepochkin PI, Vershinin AV (2019) Cytogenetic and molecular characteristics of rye genome in octoploid triticale (× *Triticosecale* Wittmack). Comparative Cytogenetics 13(4): 423–434. https://doi.org/10.3897/CompCytogen.v13i4.39576

Abstract

Alloploidization resulting from remote (interspecific or intergeneric) hybridization is one of the main factors in plant evolution, leading to the formation of new species. Triticale (× *Triticosecale* Wittmack, 1889) is the first artificial species created by crossing wheat (*Triticum* spp.) and rye (*Secale cereale* Linnaeus, 1753) and has a great potential as a grain and forage crop. Remote hybridization is a stress factor that causes a rapid reorganization of the parental genomes in hybrid progeny ("genomic shock") and is accompanied by abnormalities in the chromosome set of hybrids. The formation of the hybrid genome and its subsequent stabilization are directly related to the normalization of meiosis and the correct chromosome segregation. The aim of this work was to cytogenetically characterize triticale (× *Triticosecale rimpaui* Wittmack, 1899, AABBDDRR) obtained by crossing *Triticum aestivum* Linnaeus, 1753. Triple Dirk D × *Secale cereale* L. Korotkostebel'naya 69 in F_3–F_6 generations of hybrids, and to trace the process of genetic stabilization of hybrid genomes. Also, a comparative analysis of the nucleotide sequences of the centromeric histone *CENH3* genes was performed in wheat-rye allopolyploids of various ploidy as well as their parental forms. In the hybrid genomes of octoploid triticale an increased expression of the rye *CENH3* variants was detected. The octoploid triticale plants contain complete chromosome sets of the parental subgenomes

* these authors contributed equally to this work

maintaining the chromosome balance and meiotic stability. For three generations the percentage of aneuploids in the progeny of such plants has been gradually decreasing, and they maintain a complete set of the paternal rye chromosomes. However, the emergence of hexaploid and new aneuploid plants in F_5 and F_6 generations indicates that stabilization of the hybrid genome is not complete yet. This conclusion was confirmed by the analysis of morphological features in hybrid plants: the progeny of one plant having the whole chromosome sets of parental subgenomes showed significant morphological variations in awn length and spike density. Thus, we expect that the results of our karyotyping of octoploid triticales obtained by crossing hexaploid wheat to diploid rye supplemented by comparative analysis of CENH3 sequences will be applicable to targeted breeding of stable octo- and hexaploid hybrids.

Keywords

Aneuploidy, centromeric histone H3 (CENH3), fluorescence *in situ* hybridization (FISH), remote hybridization, triticale

Introduction

Triticale, derived from crossing wheat (*Triticum* spp.) and rye (*Secale cereale* Linnaeus, 1758) was the first synthetic allopolyploid cereal. It incorporates favorable alleles from both progenitor species (wheat and rye), enabling adaptation to environments that are less favorable for wheat yet providing better biomass yield and forage quality (Ayalew et al. 2018). Triticale is a promising model for studying the rapid changes in the hybrid genomes associated with diverse rearrangements in the genomes of parental forms, which occur when parents' genomes are combined in complex allopollyploid genome (Ma and Gustafson 2008).

The formation of a hybrid genome and its subsequent stabilization are directly related to the normalization of the meiosis process and the correct chromosome segregation (Giacomin et al. 2015). Incompatibility of centromeres of different species appears to be the main reason for the elimination of chromosomes of one of the parental genomes in hybrids (Sanei et al. 2011). According to studies of the last decade, a special role among centromeric proteins is given to the centromeric modification of the histone H3, designated as CENH3 in plants (De Rop et al. 2012, Comai et al. 2017). This is due to the fact that at the molecular level, the most specialized and universal characteristic of the active centromere is the presence of CENH3 instead of the canonical histone H3 in the nucleosomes of centromeric chromatin. As it was shown in some mammalian species and in *Drosophila* Fallén, 1823, in case of its loss the kinetochore does not form and the chromosomes do not segregate correctly during cell division (Talbert et al. 2004).

The variations in the amount and distribution of heterochromatin have facilitated the identification of rye chromosomes in different triticales (Seal and Bennett 1981). The DNA repetitive clone pSc200 has proved to be extremely useful to characterize rye heterochromatin. This highly repeated sequence has been reported to predominantly occupy the subtelomeric regions of all rye chromosome arms. Its monomers of 379–380 bp are organized as long tandem arrays up to hundreds kilobases, and they

account for 2.5% of the *S. cereale* genome (Vershinin et al. 1995). Another valuable property contributing to its widespread use in the FISH analysis of triticale karyotypes (Fu et al. 2010, Fu et al. 2013, Fradkin et al. 2013) is the lack of pSc200 hybridization signals on wheat chromosomes.

The aim of this work was to cytogenetically characterize triticale, obtained by crossing *Triticum aestivum* L. line Triple Dirk D × *S. cereale* L., cultivar Korotkostebel'naya 69, by FISH analysis of their rye chromosomes. The parental forms have a number of specific characteristics. The wheat near-isogenic line Triple Dirk D has only one dominant spring allele *Vrn-A1* (Pugsley 1971). The rye cultivar Korotkostebel'naya 69 has the dominant dwarfing gene *Ddw1* (Kobyliansky and Solodukhina 2014). Thus, the triticale lines created using these parental lines are spring short-stemmed plants, which make them a convenient object for studying the triticale karyotypes along consecutive generations. The research was conducted in $F_{3:6}$ generations of hybrids to trace their possible genetic stabilization. Also, we performed a comparative analysis of the nucleotide sequences of the N-terminal tail of the *CENH3* genes in wheat–rye allopolyploids of various ploidy as well as their parental forms.

Material and methods

Plant material

Octoploid triticales (genome constitution AABBDDRR) were created by crossing the near isogenic line of common wheat (*Triticum aestivum* L.) Triple Dirk D (genome AABBDD) with diploid rye (*Secale cereale* L.) cultivar Korotkostebel'naya 69 (genome RR) (Stepochkin and Emtseva 2017). Germinating F_1 seeds were treated with 0.05% colchicine to double the number of chromosomes and obtain allopolyploids. F_2 generation derived from self-pollination F_1 plants. F_2 plants having no less than five grains were selected for further isolated propagation. Generations F_3 through F_6 were obtained by self-pollination of plants grown from these seeds. As seeds from each plant were planted separately starting from F_1, their progeny was designated as lines. Octoploid triticales derived from the Triple Dirk D × Korotkostebel'naya 69 cross were denoted as TDKF3, TDKF4, TDKF5, and TDKF6. All plants were grown under greenhouse conditions at 22 °C/18 °C (day/night) with a 16-h day length.

Cytological techniques and fluorescence in situ hybridization

For chromosome counts in triticale somatic cells, root-tips of seedlings were treated with saturated α-bromonaphthalene solution and visualized through Feulgen staining (Singh 2003). Ten metaphase spreads per each hybrid plant were examined with Axio Star Plus microscope (Carl Zeiss GmbH, Germany). Mitotic chromosome spreads for FISH were prepared as in (Aliyeva et al. 2015). FISH analysis was carried out to iden-

tify the chromosome constitution of triticale lines, using pSc200 and pTa71 as probes. Probe pSc200 from rye repetitive sequences was used to determine the rye chromosomes (Vershinin et al. 1995). The rye tandem repetitive sequence pSc200 was labeled with biotin-16-dUTP (Roche Diagnostics, Basel, Switzerland) via PCR (Vershinin et al. 1995). The probe pTa71, containing 45S rDNA (Gerlach and Bedbrook 1979), was labeled with digoxigenin-11-dUTP and nick translational mix (Roche Diagnostics, Basel, Switzerland) according to manufacturer's recommendations. FISH procedure was performed as described by the Jenkins and Hasterok (2007). Fluorescent signals were visualized using a Zeiss Axio Scope.A1 microscope equipped with filter sets nos. 49, 10 and 20 (Carl Zeiss GmbH, Germany) for detecting DAPI, FITC and Rhodamine signals, 5–10 metaphases per slide were used for the analysis. Images were captured with a Zeiss AxioCam MRm CCD camera and ZEN lite processing package (Carl Zeiss GmbH, Germany). The brightness and contrast of all images were optimized using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

RNA extraction and sequence analysis

Total RNA was isolated from leaves of individual young seedlings with TRI Reagent RT (MRC Inc., United States) and treated with RQ-RNase-Free DNase (Promega Corporation, Madison, WI, USA) according to manufacturers' recommendations. RNA was reverse-transcribed to cDNA with a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermoscientific). Amplification primers specific to the N-terminal tail of the α*CENH3* gene from rye, wheat (Genbank accession nos. MG384772.1, JF969285.1) and triticale cDNA had been designed in (Evtushenko et al. 2017). The amplification products were cloned by using an InsTAclone PCR Cloning Kit (Thermoscientific). Both strands of 15–20 clones from each parental variety and TDK lines were sequenced using BigDye Terminator Cycle Sequencing chemistry (v. 3.1) on an ABI3100 Genetic Analyzer (Applied Biosystems, CA, USA). Coding sequences of *CENH3* were aligned with online Clustal Omega (Sievers et al. 2011) at http://www.ebi.ac.uk/Tools/msa/clustalo.

Results

Cytological and morphological features of octoploid triticale

Karyotype analysis of 30 hybrid plants in the F_3 generation showed that the number of chromosomes in their somatic cells varies from 53 to 56 (Table 1), with only one plant containing a set of 56 chromosomes. It is interesting that all aneuploid plants, regardless of the number of eliminated chromosomes, contained a complete set of the rye subgenome chromosomes, which was verified by the FISH method using the pSc200 probe (Fig. 1). The pSc200 probe contains a highly repetitive DNA sequence and gives a chromosome-specific FISH pattern allowing the identification of all rye chromo-

Table 1. The chromosome numbers in the karyotypes of somatic cells of triticale lines.

Triticale lines	Generation	Percentages of plants with		Number of chromosomes		
		56 chromosomes	42 chromosomes	Mean	Min-max	Rye
TDK 94	F3	0	0	54.3	53–55	14
TDK 94	F4	16.7	0	54.5	53–56	14
TDK 96	F3	100	0	56.0	56	14
TDK 96	F4	46.2	0	54.5	49–56	14
TDK 96.1	F5	62.5	0	55.3	55–56	14
TDK 96.2	F5	80	0	55.7	55–56	14
TDK 96.3	F5	0	0	44.4	43–47	14
TDK 96.3	F6	0	37.5	42.1	41–45	14

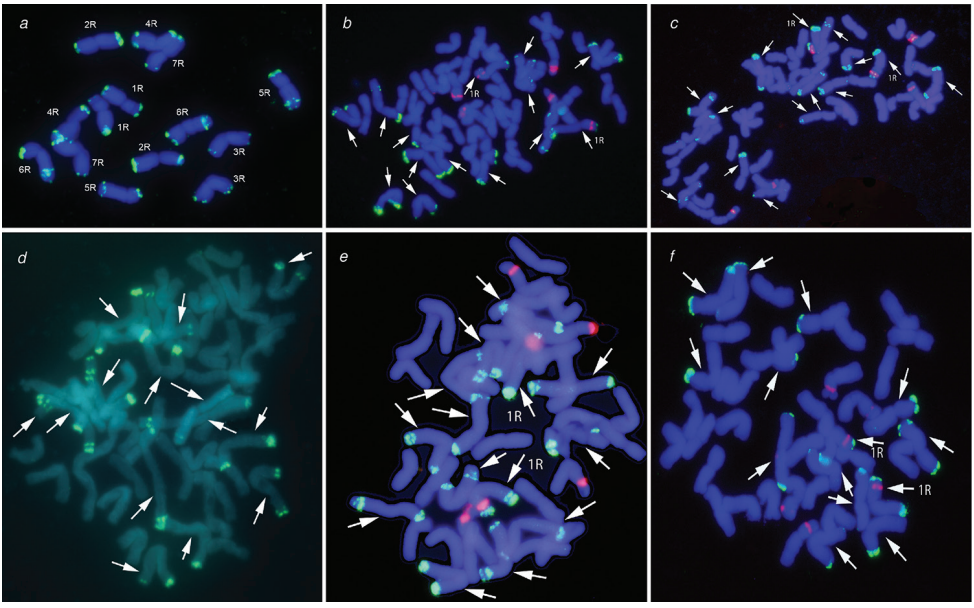


Figure 1. Identification of rye chromosomes by FISH using the pSc200 (green) and pTa71 (red) probes on metaphase chromosomes of the paternal parent and allopolyploid triticale hybrids **a** rye Korotkostebel'naya 69 **b** the line TDK96F3 (56 chromosomes) **c** the line TDK94F3 (55 chromosomes) **d** TDK96.3.F4 (49 chromosomes) **e** TDK96.3.F6 (42 chromosomes) **f** TDK96.3.F6 (41 chromosome). The arrows indicate rye chromosomes.

somes (Vershinin et al. 1995). Additionally, the pTa71 probe, which is localized on chromosomes 1R, 1B, 6B, and 5D (Cuadrado et al. 1997), was used to identify the 1R chromosome in allopolyploids. FISH signals of pSc200 probe are localized at both arms of all 14 chromosomes of the parental rye Korotkostebel'naya 69 (Fig. 1a). Plants with a set of 56 and 55 chromosomes were isolated in F_3 and were designated TDK 96 and TDK 94, respectively.(Fig. 1b, c) The progeny of these plants was analyzed in generations F_4 (TDK 94, TDK 96), F_5 and F_6 (TDK 96). The progeny of the aneuploid plant TDK94F3 (designated as TDK94F4 in Table 1) contained 16.7% of plants with 56 chromosomes. The karyotypes of the remaining plants TDK94F4 were aneuploids

with chromosome number varying from 53 to 55, but, as in TDK94F3, they were also represented by complete sets of rye chromosomes (Table 1).

The hybrid plant TDK96F3 containing a set of 56 chromosomes was also reproduced by self-pollination and its progeny (designated as TDK96F4 in Table 1) contained almost three times as many plants with 56 chromosomes (46.2%) as TDK94F4. In F_4, in most plants of the TDK96 line, the chromosome number varied slightly between 55 and 56 chromosomes, except for two plants that contained 49 chromosomes (Fig. 1d). For analysis of TDK 96 in F_5, we took two plants with the chromosome number 2n=56 (lines TDK 96.1 and TDK 96.2) and a plant with 49 chromosomes (TDK 96.3) (Table 1). In the lines TDK 96.1 and TDK 96.2 in F_5, up to 80% of plants maintained a complete set of 56 chromosomes. The progeny of the TDK 96.3 line lost individual chromosomes in the generations F_5 and F_6, reaching the minimum number of chromosomes (2n = 41, Fig. 1f) in F_6, while 37.5% of plants in F_6 had the chromosome set 2n = 42, representing hexaploid triticale (Fig. 1e). All plants of the TDK96 group, including aneuploid ones, contained complete sets of rye chromosomes; examples of some of them are shown in the Fig. 1b, d–f. However, despite maintaining the complete chromosome sets of the paternal subgenome, the emergence of aneuploid plants and hexaploid triticale in F_5 and F_6 generations indicates that stabilization of the hybrid genome in these generations is not complete yet.

This conclusion is confirmed by the analysis of some morphological features in hybrid plants. Figure 2 shows photographs of spikes of parental forms, wheat Triple Dirk D and rye Korotkostebel'naya 69 (Fig. 2a), as well as some hybrid plants with sets of 56 chromosomes (Fig. 2b). The main features that differ between parental forms are the awned spikes and higher spike density characteristic of rye. Some of the hybrid plants (TDK94F4 and TDK96.1.F5) obviously manifest features of rye, while plant TDK96.2.F5 and other plant TDK96.1.F5 manifest features of wheat. Moreover, both TDK96.1.F5 plants are the offspring of one TDK96F4 plant. A variety of morphological features of hybrid plants may be caused by numerous rearrangements leading to the exchange of relatively small regions of the genome between the original parental forms, continuing in each generation.

Analysis of the structure of centromeric histone CENH3 in octoploid triticale

Intergeneric hybridization in plants is often accompanied by elimination of some chromosomes or whole genomes. One of the putative ways to the emergence of aneuploid plants is disturbances in the functioning of centromeres of one of the parents owing to the differences in the molecular structure of centromeric histones (Sanei et al. 2011). In this regard, we analyzed the molecular structure of the coding sequences of the α*CENH3* N-terminal tail (NTT) in octoploid triticale hybrid plants in F_3–F_5 and in the parental forms. Sequencing of randomly selected clones (15–20 clones for each plant studied) of the *CENH3* NTT showed that the nucleotide sequences of wheat and rye are 99% identical, differences were observed only in a few positions. For wheat

Figure 2. Spike morphology of parental and representative triticale plants with 56 chromosomes **a** parental forms: wheat Triple Dirk D (**1**) rye Korotkostebel'naya 69 (**2**) **b** octoploid triticale hybrids: **1** – TDK 94F4 **2** – TDK 96.1.F5, **3** – TDK 96.1.F5, **4** – TDK 96.2.F5.

Table 2. The positions of species-specific non-synonymous SNPs across NTT domain of *CENH3* of wheat, rye and octoploid triticale.

Plants	The percentages of substitutions at positions across NTT domain							
	28	**32**	**73**	**82**	**84**	**99**	**122**	**145**
T. aestivum Triple Dirk D (*AABBDD*), 2n=42		11.1		55.6	55.6	27.8		
S. cereale Korotkostebel'naya 69 (*RR*), 2n=14			8.7	7.4	7.4	30.4		21.7
Octoploid triticale F_3								
Plant 1, (2n=56), TDK 96	5.6		16.7	5.6	5.6	11.1	5.6	5.6
Plant 2 (2n=52), TDK 92.4	16.7		–			16.7	33.3	
Plant 3 (2n=54), TDK 94.2	30	10	–	10				
Octoploid triticale F_4 (derived from F_3, plant 1)								
Plant 1 (2n=56), TDK 96.1	5		35	10	10	10	15	5
Plant 2 (2n=56), TDK 96.2	10		30	10	10		10	
Plant 3 (2n=49), TDK 96.3		6.7	33.3	6.7	6.7	6.7		6.7
Octoploid triticale F_5 (derived from F_4, plant 1)								
Plant 1 (2n=56), TDK 96.1.1		7.1	50				7.1	
Octoploid triticale F_5 (derived from F_4, plant 3)								
Plant 2 (2n=43), TDK 96.3.1			57.1	7.1			14.3	7.1

CENH3, these are positions 82 and 84, and for rye – positions 73 and 145. Based on the frequency of specific substitutions in hybrid plants (Table 2), one can assume that predominantly rye CENH3 variants are synthesized in these hybrids. As mentioned above FISH analysis was performed using the pTa71 probe, which confirm the presence of chromosome 1R (Fig. 1b–f) in all hybrid plants, regardless of the chromosome number. Earlier, we have shown that genes encoding the main variants of the rye centromere histone H3 are localized on chromosome 1R (Lipikhina et al. 2017). The rye-specific substitution at the position 73 of the α*CENH3* coding sequence does

not occur in the parent wheat variety Triple Dirk D. In triticale plants with different chromosome numbers (56, 49, 43) in generations F_3-F_5, the number of α*CENH3* copies carrying this substitution increases from 16 to 57%. The hybrid plants TDK94.2 (54 chromosomes in the karyotype) and TDK92.4 (52 chromosomes in the karyotype) without substitution at the position 73 in the nucleotide sequence of α*CENH3* were sterile. It can be assumed that this substitution affects the formation of the CENH3 structure and is possibly associated with the maintenance of a viable hybrid genome.

Discussion

Various chromosomal rearrangements in allopolyploid hybrids are among the most frequently described effects of remote hybridization. Significant genomic changes at remote hybridization cause instability of the hybrid genome and chromosome number imbalance. An imbalance of the hybrid genome results in a high percentage of aneuploid plants immediately after the crossing (Kalinka and Achrem 2018). In octoploid triticale, which is cytologically highly unstable, the proportion of aneuploids reaches 83% (Lukaszewski and Gustafson 1987). In our case, almost all F_3 plants, except one, were aneuploids with different numbers of eliminated chromosomes (Table 1).

Deletions and translocations of individual chromosomal regions and chromosome arms are also among the most common chromosomal alterations and have been found in the cytogenetic analysis of wheat-rye substitution and addition lines (Alkhimova et al. 1999, Fu et al. 2013), triticale, and their progeny from crosses triticale × wheat (Appels et al. 1982, Ma and Gustafson 2006). In addition to these rearrangements, cases of formation of minichromosomes and chromosomes with multiple centromeres have been described (Fu et al. 2013). Because of the meiotic instability previous studies have shown that hexaploid lines could be spontaneously derived from primary octoploid triticales, with the retention of most of A-, B- and R-genome chromosomes and the elimination of most of the D-genome chromosomes and even chromosomes of the whole wheat D-genome (Dou et al. 2006, Li et al. 2015). These results suggest that the stability of the D-genome is more strongly affected by the R genome in the octoploid triticale, comparing to the A and B genomes of common wheat. In our experiments, spontaneous emergence of plants with 42 chromosomes was also observed. These plants were found in the progeny of F_5 aneuploid plants that lost several chromosomes. The high percentage of aneuploids and the preferred elimination of wheat D-genome chromosomes in the first generations after remote hybridization should be attributed to stable signs of triticale.

Data on the effect of "genomic shock" on the chromosomes of the rye subgenome in hybrid plants are contradictory. Early studies indicated that both rye and wheat chromosomes contributed to aneuploidy (Merker 1971), as opposed to the results reported by Müntzing (1957) and Stutz (1962), who concluded that meiotic disorders and aneuploidy of triticale mainly involved rye chromosomes. Weimarck (1974) pointed out that the proportion of eliminated rye and wheat chromosomes was about 1:3.

Since the chromosome ratio in the rye and wheat genome is also 1:3, it indicates that chromosomes are eliminated in equal proportions. In recent works an opposite trend has been reported. For example, in F_5 and F_8 generations of octoploid triticale higher elimination extent of wheat chromosomes than that of rye has been found, taking into account the genomic proportions, since the ratio was on average 1:11 (Kalinka and Achrem 2018). Up to 62% of the plants had a complete set of rye chromosomes, while only about 4% had a complete set of wheat chromosomes. Among rye chromosomes, 4R and 5R were preferably eliminated (Kalinka and Achrem 2018). In advanced lines of octoploid triticale, about 70% of plants had 2n=56, however, almost all plants lost the 2R chromosome and the short arm of the 5R chromosome. The reduction of rye chromosomes was compensated by an extra pair of 2D (or 2A) chromosomes in plants with 2n=56 (Cheng and Murata 2002). The appearance in our experiments of hybrid octoploid plants of triticale with different proportions of aneuploid plants retaining complete sets of rye chromosomes indicates a complex, difficult to predict nature of inheritance of parental subgenome chromosome balance. There is a tendency indicating that F_3 plants containing complete chromosome sets of the parent subgenomes more readily maintain the chromosome balance, and more easily adapt the rye subgenome chromosomes to heterogeneous wheat cytoplasm. In subsequent generations, the percentage of aneuploidy in the progeny of such plants gradually decreases. Thus, the total chromosome number in combination with the complete set of paternal chromosomes can serve as a diagnostic indicator of the prospects for further breeding of such plants.

Differences in the CENH3 structure between the parental forms allow us to judge the regulation of the expression level of the parental protein forms in a new genomic environment that arises in a hybrid cell in case of remote hybridization. The first study of the possible relationship between differences in the CENH3 structure in parental forms and the processes of parental genome chromosome segregation during the division of hybrid cells was carried out on hybrids obtained by crossing cultured barley *H. vulgare* L. and its closest wild relative *H. bulbosum* Linnaeus, 1756. The CENH3 molecules were not included in the centromeres of *H. bulbosum* chromosomes, which were herewith inactivated and eliminated from hybrid embryos. Perhaps this was due to significant differences in protein structure between the barley species, especially in the structure of the N-terminal tail (Sanei et al. 2011). Unlike barley species, the coding sequences of α*CENH3*s in various rye and wheat species have a very high (95–99%) identity (Evtushenko et al., 2017), which noticeably complicates the search for interspecific differences and, accordingly, determining the nature of inheritance of the differences in hybrid genomes.

Our results on *CENH3* sequences analysis in hybrid plants (Table 2) indicate that both parental genomes are involved in the formation of the structure of this molecule in octoploid triticale. In the generations F_3-F_5, the proportion of plants with polymorphism at the position 73 in hybrid plants increases (from 16 to 57%), which is typical only for *CENH3* clones obtained from the rye genome. Of great importance for the discussion of these data is an extensive analysis of the expression of rye genes in cDNA isolated from various tissues of hybrid allohexaploid triticale plants obtained by cross-

ing *T. turgidum* × *S. cereale* (Khalil et al. 2015). The classes of missing (or silent) rye genes have been identified in diploid rye and triticale. A comparison of diploid rye and hexaploid triticale revealed 112 rye cDNA contigs (~ 0.5% of the total amount), which were not determined by expression analysis in any of the triticale tissues, although their expression was relatively high in diploid rye tissues (Khalil et al. 2015). It is important to note that the rye genes not expressed in triticale had significantly less homology to the corresponding homeologs in the genomes of wheat or other *Triticum* species than 200 randomly selected rye genes. Thus, rye genes with a low similarity to their homeologs in *Triticum* genomes are more likely to be repressed or absent due to deletions in the allopolyploid genomes. This conclusion is in good agreement with our results. High identity of rye and wheat CENH3 sequences does not inhibit the expression of both parental forms in the hybrid genomes of octoploid triticale.

Acknowledgments

The work was supported by the Russian fundamental scientific research program (project 0310-2019-0003); the experiments on plant crossing were funded by the Russian Foundation for Basic Research (project № 18-54-00013), cytogenetic analysis and determination of gene expression were funded by the Russian Foundation for Basic Research (project № 17-04-00748), the experiments on identifying differences in the DNA structure of parental forms were funded by the Russian Science Foundation (project № 19-14-00051).

References

Aliyeva-Schnorr L, Ma L, Houben A (2015) A fast air-dry dropping chromosome preparation method suitable for FISH in plants. Journal of Visualized Experiments 106: e53470 53470. https://doi.org/10.3791/53470

Alkhimova AG, Heslop-Harrison JS, Shchapova AI, Vershinin AV (1999) Rye chromosome variability in wheat-rye addition and substitution lines. Chromosome Research 7: 205–212. https://doi.org/10.1023/A:1009299300018

Appels R, Gustafson JP, May CE (1982) Structural variation in the heterochromatin of rye chromosomes in triticale. Theoretical and Applied Genetics 63(3): 235–244. https://doi.org/10.1007/BF00304002

Ayalew H, Kumssa TT, Butler TJ, Ma X-F (2018) Triticale improvement for forage and cover crop uses in the Southern Great Plains of the United States. Frontiers in plant science 9: 1130. https://doi.org/10.3389/fpls.2018.01130

Cheng ZJ, Murata M (2002) Loss of chromosomes 2R and 5RS in octoploid triticale selected for agronomic traits. Genes & Genetic Systems 77: 23–29. https://doi.org/10.1266/ggs.77.23

Comai L, Maheshwari S, Marimuthu PA (2017) Plant centromeres. Current Opinion in Plant Biology 36: 158–167. https://doi.org/10.1016/j.pbi.2017.03.003

De Rop V, Padeganeh A, Maddox PS (2012) CENP-A: the key player behind centromere identity, propagation, and kinetochore assembly. Chromosoma 121(6): 527–538. https://doi.org/10.1007/s00412-012-0386-5

Dou Q, Tanaka H, Nakata N, Tsujimoto H (2006) Molecular cytogenetic analyses of hexaploid lines spontaneously appearing in octoploid Triticale. Theoretical and Applied Genetics 114(1): 41–47. https://doi.org/10.1007/s00122-006-0408-x

Evtushenko E, Elisafenko E, Gatzkaya S, Lipikhina Y, Houben A, Vershinin A (2017) Conserved molecular structure of the centromeric histone CENH3 in Secale and its phylogenetic relationships. Scientific Reports 7: 17628. https://doi.org/10.1038/s41598-017-17932-8

Fradkin M, Ferrari M, Espert S, Ferreira V, Grassi E, Greizerstein E, Poggio L (2013) Differentiation of triticale cultivars through FISH karyotyping of their rye chromosomes. Genome 56(5): 267–272. https://doi.org/10.1139/gen-2012-0117

Fu S, Tang Z, Ren Z (2010) Inter- and intra-genomic transfer of small chromosomal segments in wheat-rye allopolyploids. Journal of Plant Research 123(1): 97–103. https://doi.org/10.1007/s10265-009-0264-2

Fu S, Lv Z, Guo X, Zhang X, Han F (2013) Alteration of Terminal Heterochromatin and Chromosome Rearrangements in Derivatives of Wheat-Rye Hybrids. Journal of Genetics and Genomics 40(8): 413–420. https://doi.org/10.1016/j.jgg.2013.05.005

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Research 7(7): 1869–1885. https://doi.org/10.1093/nar/7.7.1869

Giacomin RM, Assis R, Brammer SP, Nascimento JA, Da-Silva PR (2015) Backcrossing to increase meiotic stability in triticale. Genetics and Molecular Research 14(3): 11271–11280. https://doi.org/10.4238/2015.September.22.21

Jenkins G, Hasterok R (2007) BAC 'landing' on chromosomes of *Brachypodium distachyon* for comparative genome alignment. Nature Protocols 2(1): 88–98. https://doi.org/10.1038/nprot.2006.490

Kalinka A, Achrem M (2018) Reorganization of wheat and rye genomes in octoploid triticale (× *Triticosecale*). Planta 247(4): 807–829. https://doi.org/10.1007/s00425-017-2827-0

Khalil HB, Ehdaeivand MR, Xu Y, Laroche A, Gulick PJ (2015) Identification and characterization of rye genes not expressed in allohexaploid triticale. BMC Genomics 16(1): 281. https://doi.org/10.1186/s12864-015-1480-x

Kobyliansky VD, Solodukhina OV (2014) The role of the Vavilov Institute of Plant Industry in the initiation and development of new trends in winter rye breeding in Russia. Proceedings on applied botany, genetics and breeding 176(1): 5–19. [in Russ] https://doi.org/10.30901/2227-8834-2015-1-5-19

Li H, Guo X, Wang C, Ji W (2015) Spontaneous and divergent hexaploid triticales derived from common wheat × rye by complete elimination of D-Genome chromosomes. PLOS ONE 10(3): e0120421. https://doi.org/10.1371/journal.pone.0120421

Lipikhina Y, Evtushenko E, Elisafenko E, Vershinin A (2017) Chromosomal assignment of centromere-specific histone CENH3 genes in rye (*Secale cereale* L.) and their phylogeny. Comparative Cytogenetics 11(4): 821–832. https://doi.org/10.3897/compcytogen.v11i4.19953

Lukaszewski AJ, Gustafson JP (1987) Cytogenetics of triticale. Plant Breeding Reviews 5: 41–93.

Ma X, Gustafson JP (2006) Timing and rate of genome variation in triticale following alloploidization. Genome 49(8): 950–958. https://doi.org/10.1139/g06-078

Ma X, Gustafson JP (2008) Allopolyploidization-accommodated genomic sequence changes in triticale. Annals of botany 101(6): 825–832. https://doi.org/10.1093/aob/mcm331

Merker A (1971) Cytogenetic investigations in hexaploid *Triticale*. I. Meiosis, aneuploidy and fertility. Hereditas 68(2): 281–290. https://doi.org/10.1111/j.1601-5223.1971.tb02403.x

Müntzing A (1957) Cytogenetic studies in rye-wheat (Triticale). Proceedings of International Genetics Symposium Tokyo. Cytologia (Supplement): 51–56.

Pugsley AT (1971) A genetic analysis of the spring-winter habit of growth in wheat. Australian Journal of Agricultural Research 22: 21–31. https://doi.org/10.1071/AR9710021

Sanei M, Pickering R, Kumke K, Nasuda S, Houben A (2011) Loss of centromeric histone H3 (CENH3) from centromeres precedes uniparental chromosome elimination in interspecific barley hybrids. Proceedings of the National Academy of Sciences 108(33): E498–E505. https://doi.org/10.1073/pnas.1103190108

Seal AG, Bennett MD (1981) The rye genome in winter hexaploid triticales. Canadian Journal of Genetics and Cytology 23(4): 647–653. https://doi.org/10.1139/g81-071

Sievers F, Wilm A, Dineen D, Gibson T, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson J, Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular Systems Biology 7: 539. https://doi.org/10.1038/msb.2011.75

Singh RJ (2003) Plant cytogenetics. CRC Press, Boca Raton, 488 pp.

Stepochkin PI, Emtseva MV (2017) Study of the interphase period "shoots–earing" of the initial parental forms and hybrids of triticale with different *Vrn* genes. Vavilov Journal of Genetics and Breeding 21(5): 530–533. [in Russ] https://doi.org/10.18699/VJ17.22-o

Stutz HC (1972) On the origin of cultivated rye. American Journal of Botany 59(1): 59–70. https://doi.org/10.1002/j.1537-2197.1972.tb10062.x

Talbert PB, Bryson TD, Henikoff S (2004) Adaptive evolution of centromere proteins in plants and animals. Journal of Biology 3(4): 18. https://doi.org/10.1186/jbiol11

Vershinin AV, Schwarzacher T, Heslop-Harrison JS (1995) The large-scale genomic organization of repetitive DNA families at the telomeres of rye chromosomes. Plant Cell 7(11): 1823–1833. https://doi.org/10.1105/tpc.7.11.1823

Weimarck A (1974) Elimination of wheat and rye chromosomes in a strain of octoploid *Triticale* as revealed by Giemsa banding technique. Hereditas 77(2): 281–286. https://doi.org/10.1111/j.1601-5223.1974.tb00940.x

CompCytogen 13(4): 435–449 (2019)
doi: 10.3897/CompCytogen.v13i4.48368
http://compcytogen.pensoft.net

RESEARCH ARTICLE

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

Linking karyotypes with DNA barcodes: proposal for a new standard in chromosomal analysis with an example based on the study of Neotropical Nymphalidae (Lepidoptera)

Vladimir A. Lukhtanov[1,2], Yaroslavna Iashenkova[2,3]

1 *Department of Karyosystematics, Zoological Institute of the Russian Academy of Sciences, Universitetskaya emb. 1, St. Petersburg 199034, Russia* **2** *Department of Entomology, St. Petersburg State University, Universitetskaya emb. 7/9, St. Petersburg 199034, Russia* **3** *Department of Genetics and Biotechnology, St. Petersburg State University, Universitetskaya emb. 7/9, St. Petersburg 199034, Russia*

Corresponding author: *Vladimir A. Lukhtanov* (lukhtanov@mail.ru)

Academic editor: *V. G. Kuznetsova* | Received 11 November 2019 | Accepted 4 December 2019 | Published 17 December 2019

http://zoobank.org/911CBEA6-C1C8-4D68-81E2-ECB5B61A5706

Citation: Lukhtanov VA, Iashenkova Y (2019) Linking karyotypes with DNA barcodes: proposal for a new standard in chromosomal analysis with an example based on the study of Neotropical Nymphalidae (Lepidoptera). Comparative Cytogenetics 13(4): 435–449. https://doi.org/10.3897/CompCytogen.v13i4.48368

Abstract

Chromosomal data are important for taxonomists, cytogeneticists and evolutionary biologists; however, the value of these data decreases sharply if they are obtained for individuals with inaccurate species identification or unclear species identity. To avoid this problem, here we suggest linking each karyotyped sample with its DNA barcode, photograph and precise geographic data, providing an opportunity for unambiguous identification of described taxa and for delimitation of undescribed species. Using this approach, we present new data on chromosome number diversity in neotropical butterflies of the subfamily Biblidinae (genus *Vila* Kirby, 1871) and the tribe Ithomiini (genera *Oleria* Hübner, 1816, *Ithomia* Hübner, 1816, *Godyris* Boisduval, 1870, *Hypothyris* Hübner, 1821, *Napeogenes* Bates, 1862, *Pseudoscada* Godman et Salvin, 1879 and *Hyposcada* Godman et Salvin, 1879). Combining new and previously published data we show that the species complex *Oleria onega* (Hewitson, [1852]) includes three discrete chromosomal clusters (with haploid chromosome numbers n = 15, n = 22 and n = 30) and at least four DNA barcode clusters. Then we discuss how the incomplete connection between these chromosomal and molecular data (karyotypes and DNA barcodes were obtained for different sets of individuals) complicates the taxonomic interpretation of the discovered clusters.

Keywords
karyotype, DNA barcoding, *COI*, meiosis, metaphase, Lepidoptera, Nymphalidae, Biblidinae, Danainae, Ithomiini, Peru

Introduction

Chromosomal data are an important source of information for taxonomic, evolutionary and comparative phylogenetic studies (White 1973). However, the application of these data is often difficult because of unclear taxonomic identity (e.g. Petrova et al. 2015) or doubtful species identification (or even due to the lack of a species identification) of the samples that were used as vouchers for karyotype analysis [e.g. some samples and identifications in Robinson (1971) and Brown et al. (2004)]. Theoretically, one can try to find these samples, provided that they were neatly labeled and can be recognized, are stored in accessible museums and have not been lost, and then check their identification using taxonomic literature or comparison with type specimens. However, it is complicated and almost impossible in practice.

To avoid this problem, here we suggest linking each karyotyped sample with its DNA barcode. It was empirically demonstrated that the mitochondrial DNA barcode, a relatively short fragment of the mitochondrial *COI* gene (658 base pairs) (i.e., a negligible part of the genome in terms of size), could differentiate up to 95% of species in many taxa (Hebert et al. 2003, 2004; Hebert and Gregory 2005; Hajibabaei et al. 2006; Lukhtanov et al. 2009). In addition, the barcoding DNA protocol provides a standardized system for storing information on vouchers that served as the basis for DNA barcoding, including the image, the exact label and the storage location of the samples. This makes it possible, if necessary, to relatively easily find and re-examine a voucher.

Obtaining barcodes is currently a simple technical task, which can be carried out in almost any laboratory or on a commercial basis. Our personal experience, based on a molecular analysis of the fauna of Central Asia, Eastern Europe and Western Asia (Lukhtanov et al. 2009, 2016; Lukhtanov 2017; Pazhenkova and Lukhtanov 2019), shows that if there are barcode libraries (Ward et al. 2009; Dincă et al. 2011) for a given region and for a given taxonomic group, barcodes ensure almost 100% success of species identification. Even if such a library is not currently available for a group or region under study, the presence of a barcode makes it possible to reliably identify the sample in the future. Thus, linking karyotypes with DNA barcodes resolves the problem of reliable species identification.

Additionally, combination of DNA barcodes and karyotypes represents a powerful tool for detection, delimitation and description of unrecognized species (Lukhtanov et al. 2015; Vishnevskaya et al. 2016, 2018). Therefore, linking karyotypes with DNA barcodes, potentially resolves the problem of unclear species identity in chromosomal studies.

The approach based on combination of chromosomal and DNA barcode data has been already used in different studies on butterflies (Lukhtanov et al. 2014, 2015;

Lukhtanov and Dantchenko 2017), fish (Marques et al. 2013), lizards (de Matos et al. 2016), mammals (Tavares et al. 2015) and mussels (Garcia-Souto et al. 2017). However, its principles have not been explicitly formulated.

In this paper, we demonstrate the algorithm, features and capabilities of the proposed approach with the butterflies of the Neotropical fauna.

Material and methods

Samples

The samples were collected in Peru in 2013 by V.A.Lukhtanov. The information on localities where the specimens were collected is presented in the Table 1. The morphology-based species identification was carried out by comparing the specimens with butterfly images figured at Butterflies of America site (https://www.butterfliesofamerica.

Table 1. List of the samples of the genera *Oleria* Hübner, 1816, *Ithomia* Hübner, 1816, *Vila* Kirby, 1871, *Pseudoscada* Godman et Salvin, 1879, *Godyris* Boisduval, 1870, *Hypothyris* Hübner, 1821, *Napeogenes* Bates, 1862 and *Hyposcada* Godman et Salvin, 1879 collected by V.A.Lukhtanov and used in the study.

Id	BOLD Id	Genus	Species	N	Exact site	Latitude / Longitude	Altitude	Collection date
A107	NOB001-17	*Oleria*	*didymaea ramona*	n = 22	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A108	NOB002-17	*Ithomia*	*salapia*	n = 34	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A111	NOB004-17	*Vila*	*emilia*	n = 30	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A112	NOB005-17	*Vila*	*emilia*	–	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A113	NOB006-17	*Vila*	*emilia*	n = 30	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A115	NOB007-17	*Vila*	*emilia*	n = 30	60 km SSW Ikitos, Puente Itaya	04°11'47"S, 73°28'39"W	114 m	30 August 2013
A121	NOB008-17	*Oleria*	*gunilla serdolis*	n = 11	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A122	NOB009-17	*Oleria*	*gunilla serdolis*	n = 11	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A123	NOB010-17	*Oleria*	*gunilla serdolis*)	n = 11	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A125	NOB011-17	*Oleria*	*onega*	n = 15	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A127	NOB012-17	*Oleria*	*gunilla serdolis*	n = 11	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A124	NOB013-17	*Oleria*	*onega*	n = 15	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A129	n/a	*Pseudoscada*	*timna*	n = 15	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A130	NOB014-17	*Ithomia*	*salapia*	n = 34	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A131	NOB015-17	*Godyris*	*zavaleta*	n = 33,35	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A132	NOB016-17	*Ithomia*	*salapia*	n = 35	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A133	NOB017-17	*Ithomia*	*salapia*	n = 36	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A135	NOB018-17	*Ithomia*	*salapia*	n = 36	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A136	NOB019-17	*Hypothyris*	*euclea*	n = 14	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A137	NOB020-17	*Napeogenes*	*sylphis*	n = 14	Tingo Maria	09°21'02"S, 76°03'21"W	835 m	4 September 2013
A140	NOB021-17	*Hyposcada*	*kena*	n = 14	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A141	NOB022-17	*Oleria*	*onega*	n = 15	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A142	NOB023-17	*Oleria*	*onega*	n = 15	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A143	NOB024-17	*Oleria*	*onega*	n = 15	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A144	NOB025-17	*Oleria*	*onega*	n = 15	Cayumba	09°29'25"S, 75°56'46"W	1020 m	5 September 2013
A145	NOB026-17	*Godyris*	*dircenna*	n = 36	Cayumba	09°29'43"S, 75°58'01"W	786 m	6 September 2013

com/L/Nymphalidae.htm). Photographs of all specimens used in the analysis, as well as collecting data, are available on the Barcode of Life Data System (BOLD) at http://www.boldsystems.org/. The specimens are deposited in the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia.

Chromosomal analysis

Gonads were removed from the abdomen and placed into freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing the butterfly in the field. Testes were stored in the fixative for 3–36 months at +4 °C. Then the gonads were stained in 2% acetic orcein for 30–60 days at +18–20 °C. Karyotypes (Figs 1–19) were analyzed as previously described (Przybyłowicz et al. 2014; Lukhtanov and Shapoval 2017). Briefly, the stained testes were placed in a drop of 40% lactic acid on a slide, and spermatocysts were dissected from gonad membranes using entomological pins before covering everything with a coverslip. Different degrees of chromosome spreading were observed by gradually increasing the pressure on the coverslip. Haploid chromosome numbers (n) were counted at meiotic metaphase I (MI) and metaphase II (MII).

DNA barcoding

Standard *COI* barcodes (658-bp 5' segment of mitochondrial cytochrome oxidase subunit I) were studied. Legs were sampled from the karyotyped specimens, and sequence data from the DNA barcode region of *COI* were obtained at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using primers and protocols described in Hajibabaei et al. (2005), Ivanova et al. (2006) and deWaard et al. (2008).

The DNA-barcode-based species identification was carried out by using the BOLDSYSTEMS Identification Engine (http://www.boldsystems.org/index.php/IDS_OpenIdEngine).

The Bayesian majority rule consensus tree of the analyzed samples (Figs 20, 21) was constructed as previously described (Sahoo et al. 2016; Lukhtanov 2017; Lukhtanov and Dantchenko 2017) using the sequences obtained in this study as well as the published sequences uploaded from GenBank (de-Silva et al. 2010). Briefly, sequences were aligned using the BioEdit software (Hall 1999) and edited manually. The Bayesian analysis was performed using the program MrBayes 3.2 (Ronquist et al. 2012) with default settings as suggested by Mesquite (Maddison and Maddison 2015): burnin = 0.25, nst = 6 (GTR + I + G). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. The consensus of the obtained trees was visualised using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Karyotypes
Subfamily Biblidinae

***Vila emilia* (Cramer, 1779)**
Fig. 1

The meiotic karyotype was found to include 30 bivalents of similar size.

Subfamily Danainae
Tribe Ithomiini

***Oleria didymaea ramona* (Haensch, 1909)**
Fig. 2

The meiotic karyotype was found to include 22 bivalents of similar size.

***Ithomia salapia* Hewitson, [1853]**
Figs 3–6

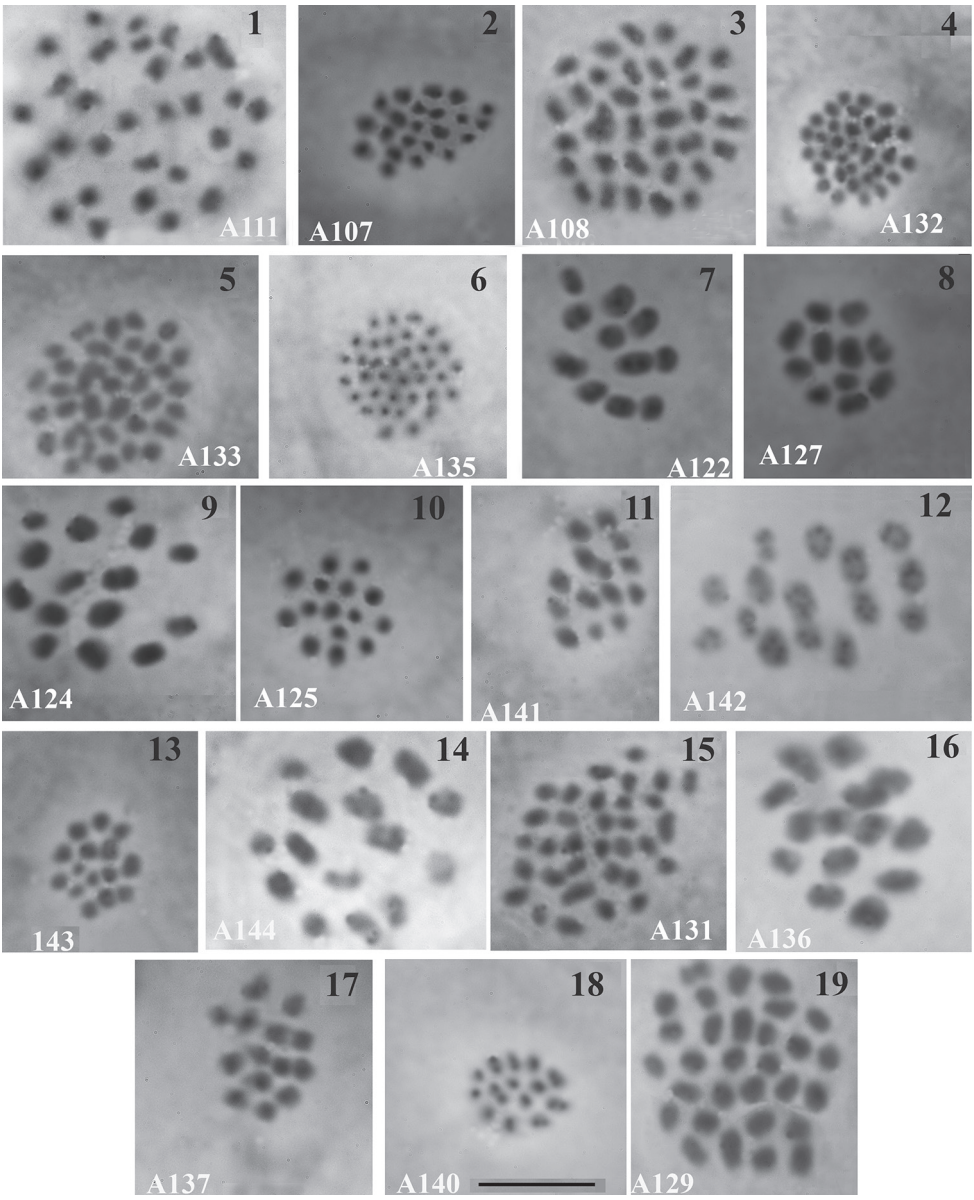
The meiotic karyotype was found to include 34 bivalents in a single studied specimen from Puente Itaya (Peru, 60 km SSW Ikitos). One bivalent was slightly larger than the rest ones. The meiotic karyotype was found to include 35–36 bivalents of similar size in the specimens from Tingo Maria.

***Oleria gunilla serdolis* (Haensch, 1909)**
Figs 7, 8

The meiotic karyotype was found to include 11 bivalents. Two bivalents were larger than the other nine ones.

***Oleria onega* (Hewitson, [1852])**
Figs 9–14

The meiotic karyotype was found to include 15 bivalents. The bivalents had different sizes and shapes.



Figures 1–19. Male metaphase I (MI) and II (MII) plates of Ithomiini and Biblidinae **1** A111, *Vila emilia*, MI, n = 30 **2** A107, *Oleria didymaea ramona*, MI, n = 22 **3** A108, *Ithomia salapia*, MI, n = 34 **4** A132, *Ithomia salapia*, MII, n = 35 **5** A133, *Ithomia salapia*, MI, n = 36 **6** A135, *Ithomia salapia*, MII, n = 36 7 **8** A122 *Oleria gunilla serdolis*, MI, n = 11 **9** A124, *Oleria onega*, MI, n = 15 **10** A125, *Oleria onega*, MII, n = 15 **11** A141, *Oleria onega*, MI, n = 15 **12** A142, *Oleria onega*, MI, n = 15 **13** A143, *Oleria onega*, MII, n = 15 **14** A144, *Oleria onega*, MI, n = 15 **15** A131, *Godyris zavaleta*, MI, n = 33 **16** A136, *Hypothyris euclea*, MI, n = 14 **17** A137, *Napeogenes sylphis*, MI, n = 14 **18** A140, *Hyposcada kena*, MII, n = 14 **19** A129, *Pseudoscada timna*, MI, n = 30. Scale bar: 10 μ in all figures.

***Godyris zavaleta* (Hewitson, [1855])**
Fig. 15

The meiotic karyotype was found to include cells with 33 and 35 chromosomal elements, presumably bivalents. 34 bivalents were counted in a single studied specimen from Tingo Maria.

***Hypothyris euclea* (Godart, 1819)**
Fig. 16

The meiotic karyotype was found to include 14 bivalents of similar size.

***Napeogenes sylphis* (Guérin-Méneville, [1844])**
Fig. 17

The meiotic karyotype was found to include 14 bivalents of similar size.

***Hyposcada kena* (Hewitson, 1872)**
Fig. 18

The meiotic karyotype was found to include 14 bivalents. The bivalents had different sizes and shapes.

***Pseudoscada timna* (Hewitson, [1855])**
Fig. 19

The meiotic karyotype was found to include 30 bivalents of similar size. The bivalents formed a gradient size row.

DNA barcodes

All studied species were found to be significantly differentiated with respect to the DNA barcode region and formed distinct clusters on the BI tree (Fig. 20). However, if additional sequences from GenBank were added, the picture became more intricate. Particularly, *Oleria onega* was found to have very complicated structure with numerous differentiated haplotypes forming three monophyletic and one paraphyletic clusters

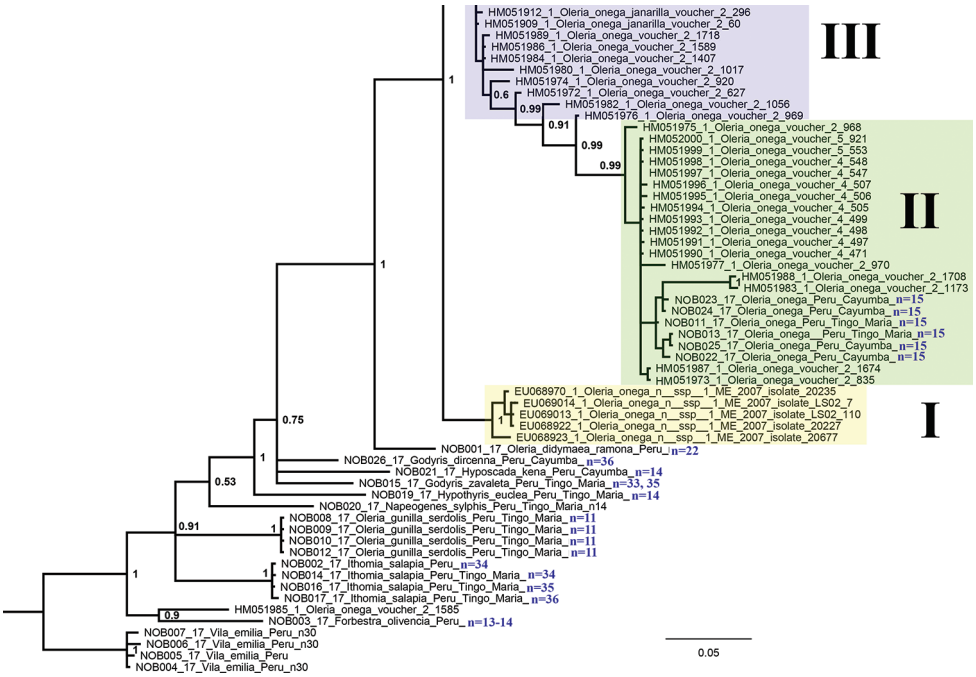


Figure 20. Fragment of the Bayesian majority rule consensus tree of the analyzed samples of Ithomiini inferred from *COI* sequences. I, II and III are the recovered clusters of the *Oleria onega* species complex (see Fig. 21 for the complete structure of the cluster III and the cluster IV). Haploid chromosome numbers (n) are shown after the tip labels. *Vila emilia* (subfamily Biblidinae) was used to root the tree. Bayesian posterior probabilities higher than 0.5 are shown next to the recovered branches.

(Figs 20, 21). The karyotyped samples of this species with the chromosome number n = 15 were found to belong to the cluster II.

Discussion

The Neotropics is one of the most species-rich regions of the world, and the nymphalids are the most speciose butterfly family (Van Nieukerken et al. 2011). Therefore, it is not surprising that the neotropical fauna of Nymphalidae is very rich in species (site (https://www.butterfliesofamerica.com/L/Nymphalidae.htm).

Chromosomal studies represent only a small part of the Neotropical nymphalid diversity (de Lesse 1967, 1970a, b; de Lesse and Brown 1971; Wesley and Emmel 1975; Suomalainen and Brown 1984; Brown et al. 1992, 2004, 2007a, b; McClure et al. 2017; Lukhtanov 2019a). However, they demonstrate an extremely high level of the interspecific karyotype variation and a potential for solving taxonomic problems within the South American nymphalid species. This potential is practically not used (but see: Suomalainen and Brown 1984; Constantino and Salazar 2010; McClure et al. 2017) in opposite to the numerous chromosomally based taxonomic studies in

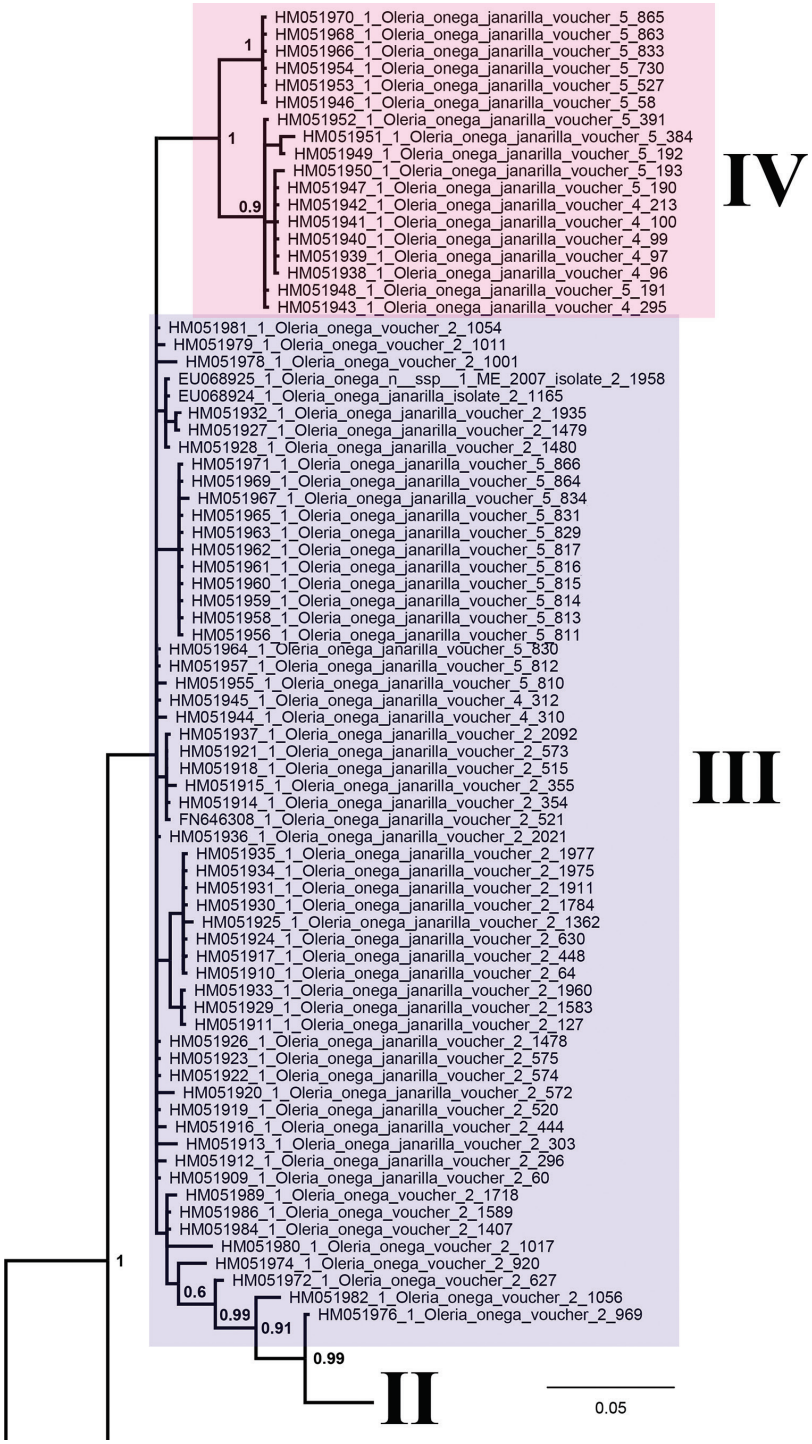


Figure 21. Fragment of the Bayesian majority rule consensus tree of the analyzed samples of Ithomiini inferred from *COI* sequences. The clusters III and IV of the *Oleria onega* species complex are shown. Bayesian posterior probabilities higher than 0.5 are shown next to the recovered branches.

palearctic butterflies (Lorković 1958; de Lesse 1960; Lukhtanov et al. 2011, 2015; Talavera et al. 2013).

In this study we suggest a plan for further analysis of the Neotropical Nymphalidae based on a parallel analysis of chromosomal and molecular markers.

Using this approach, we confirm the previously published data on the karyotypes of *Godyris dircenna* (n = 36), *Hypothyris euclea* (n = 14), *Napeogenes sylphis* (n = 14) and *Oleria gunilla* (n = 11) (Brown et al. 2004).

Haploid chromosome number n=30 is found by us in *Pseudoscada timna*, whereas n = 31 was reported for this taxon by Brown et al. (2004).

We provide the first data on karyotypes of *Vila emilia* and demonstrate a high interspecific chromosome number variation in this genus (previously n = 15 was reported for an unidentified *Vila* species from western Brazil; Brown et al. 2007a).

We show chromosome number n = 14 for *Hyposcada kena* confirming high level of interspecific variation in the genus *Hyposcada* (from n = 12 to n = 19) (Brown et al. 2004).

Different chromosome numbers were previously reported for *Godyris zavaleta* by Brown et al. (2004): n = 46 (on the page 220–221), n = 35–45 (p. 222), n = 36–46 (p. 224), n = 40 (p. 229). However, the credibility and the reason for this variation were not discussed. We provide n = 33 for this species and point out the need for further study of this taxon.

Even more interesting data were obtained regarding the species *Oleria didymaea* (Hewitson, 1876) and *O. onega*. We found n = 22 in the taxon identified by us as *Oleria didymaea ramona* (Haensch, 1909), whereas n= 15 was reported for taxon identified as *Oleria alexina didymaea* (Brown et al. 2004) raising the question of further study of the complex *Oleria didymaea – alexina.*

Based on chromosome numbers, we hypothesize that *Oleria onega* is a complex of at least three species with different chromosome numbers: n = 15 (our data), n = 22 and n = 30 (Brown et al. 2004). A similar conclusion can be made on the basis of molecular data that show the presence of at least four clusters of DNA barcodes in this complex (Figs 20, 21). The status of the detected chromosomal races and mitochondrial clusters could be theoretically resolved based on analysis of: (1) congruence of chromosomal and molecular characters in different sets of individuals, or (2) pattern imitating (vs not imitating) linkage of chromosomal and mitochondrial markers that are known to be unlinked (Lukhtanov at al 2015; Vishnevskaya et al. 2016, 2018; Lukhtanov 2019b). Unfortunately, the previously karyotyped samples (Brown et al. 2004) were not studied with respect to molecular markers, and vice versa, the vouchers for molecular studies were not karyotyped (de-Silva et al. 2010).

The incomplete connection between the chromosomal and molecular data (karyotypes and DNA barcodes were obtained for different sets of individuals) complicates the taxonomic interpretation of the discovered clusters. Nevertheless, we predict that in future linking karyotypes with DNA barcodes will result in a significant rearrangement of taxonomy of the genus *Oleria*.

Acknowledgements

We thank Fedor Konstantinov (St. Petersburg State University) for help in butterfly collecting and research. The study was supported by the projects RFBR 18-04-00263-a, RFBR 17-04-00828-a and state research project AAAA-A19-119020790106-0.

References

BOLD Systems Identification (2019) BOLD Systems Identification. http://boldsystems.org/index.php/IDS_OpenIdEngine

Brown KS, Emmel TC, Eliazar PJ, Suomalainen E (1992) Evolutionary patterns in chromosome numbers in neotropical Lepidoptera I. Chromosomes of the Heliconiini (Family Nymphalidae: Subfamily Nymphalinae). Hereditas 117: 109–125. https://doi.org/10.1111/j.1601-5223.1992.tb00165.x

Brown KS, von Schoultz B, Suomalainen E (2004) Chromosome evolution in Neotropical Danainae and Ithomiinae (Lepidoptera). Hereditas 141: 216–236. https://doi.org/10.1111/j.1601-5223.2004.01868.x

Brown KS, Freitas AVL, Wahlberg N, von Schoultz B, Saura AO, Saura A (2007a) Chromosomal evolution in the South American Nymphalidae. Hereditas 144: 137–148. https://doi.org/10.1111/j.2007.0018-0661.02015.x

Brown KS, Freitas AVL, von Schoultz B, Saura AO, Saura A (2007b) Chromosomal evolution of South American frugivorous butterflies in the Satyroid clade (Nymphalidae: Charaxinae, Morphinae and Satyrinae). Biological Journal of the Linnean Society 92: 467–481. https://doi.org/10.1111/j.1095-8312.2007.00872.x

Butterflies of America (2019) Butterflies of America https://www.butterfliesofamerica.com/L/Nymphalidae.htm [accessed 10. November 2019]

Constantino LM, Salazar JA (2010) A review of the *Philaethria dido* species complex (Lepidoptera: Nymphalidae: Heliconiinae) and description of three new sibling species from Colombia and Venezuela. Zootaxa 2720: 1–27. https://doi.org/10.11646/zootaxa.2720.1.1

de Lesse H (1960) Spéciation et variation chromosomique chez les Lépidoptères Rhopalocères. Annales des Sciences Naturelles Zoologie et Biologie Animale (12e série) 2: 1–223.

de Lesse H (1967) Les nombres de chromosomes chez les Lépidoptères Rhopalocères neéotropicaux. Annales de la Societe Entomologique de France (N.S.) 3: 67–136.

de Lesse H (1970a) Les nombres de chromosomes chez les Lépidoptères Rhopalocères en Amérique centrale et Colombie. Annales de la Societe Entomologique de France (N.S.) 6: 347–358.

de Lesse H (1970b) Formules chromosomiques de quelques Lépidoptères Rhopalocères de Guyane. Annales de la Societe Entomologique de France (N.S.) 6: 849–855.

de Lesse H, Brown Jr KS (1971) Formules chromosomiques de Lépidoptères Rhopalocères du Brésil. Bulletin de la Société entomologique de France 76: 131–137.

de Matos NB, Ferreira M, Silva F, Rodrigues MT, da Silva ES, Garcia C (2016) Taxonomy and evolution of *Tropidurus* (Iguania, Tropiduridae) based on chromosomal and DNA barcoding analysis. Journal of Herpetology 50(2): 316–326. https://doi.org/10.1670/13-221

de-Silva DL, Day JJ, Elias M, Willmott K, Whinnett A, Mallet J (2010) Molecular phylogenetics of the neotropical butterfly subtribe Oleriina (Nymphalidae: Danainae: Ithomiini). Molecular Phylogenetics and Evolution 55: 1032–1041. https://doi.org/10.1016/j.ympev.2010.01.010

deWaard JR, Ivanova NV, Hajibabaei M, Hebert PDN (2008) Assembling DNA barcodes: analytical protocols. In: Martin CC (Ed.) Environmental Genomics, Methods in Molecular Biology. Humana Press, Totowa, 410: 275–283. https://doi.org/10.1007/978-1-59745-548-0_15

Dincă V, Zakharov EV, Hebert PDN, Vila R (2011) Complete DNA barcode reference library for a country's butterfly fauna reveals high performance for temperate Europe. Proceedings of the Royal Society B Biological sciences 278(1704): 347–355. https://doi.org/10.1098/rspb.2010.1089

Garcia-Souto D, Sumner-Hempel A, Fervenza S, Perez-Garcia C, Torreiro A, Gonzalez-Romero R, Eirin-Lopez JM, Moran P, Pasantes JJ (2017) Detection of invasive and cryptic species in marine mussels (Bivalvia, Mytilidae): A chromosomal perspective. Journal for Nature Conservation 39: 58–67. https://doi.org/10.1016/j.jnc.2017.07.005

Hajibabaei M, deWaard JR, Ivanova NV, Ratnasingham S, Dooph RT, Kirk SL, Mackie PM, Hebert PDN (2005) Critical factors for assembling a high volume of DNA barcodes. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 360: 1959–1967. https://doi.org/10.1098/rstb.2005.1727

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences of the United States of America 103: 968–971. https://doi.org/10.1073/pnas.0510466103

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Hebert PDN, Gregory TR (2005) The promise of DNA barcoding for taxonomy. Systematic Biology 54: 852–859. https://doi.org/10.1080/10635150500354886

Hebert PDN, Cywinska A, Ball SL, deWaard J (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences 270: 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences of the United States of America 101(41): 14812–14817. https://doi.org/10.1073/pnas.0406166101

Ivanova NV, deWaard JR, Hebert PDN (2006) An inexpensive, automation-friendly protocol for recovering high quality DNA. Molecular Ecology Resources 6: 998–1002. https://doi.org/10.1111/j.1471-8286.2006.01428.x

Lorković Z (1958) Some peculiarities of spatially and sexually restricted gene exchange in the *Erebia tyndarus* group. Cold Spring Harbor Symposia on Quantitative Biology 23: 319–325. https://doi.org/10.1101/SQB.1958.023.01.032

Lukhtanov VA (2017) A new species of *Melitaea* from Israel, with notes on taxonomy, cytogenetics, phylogeography and interspecific hybridization in the *Melitaea persea* complex (Lepidoptera, Nymphalidae). Comparative Cytogenetics 11(2): 325–357. https://doi.org/10.3897/CompCytogen.v11i2.12370

Lukhtanov VA (2019a) Two types of highly ordered micro- and macrochromosome arrangement in metaphase plates of butterflies (Lepidoptera). Comparative Cytogenetics 13(1): 19–25. https://doi.org/10.3897/CompCytogen.v13i1.32614

Lukhtanov VA (2019b) Species delimitation and analysis of cryptic species diversity in the XXI century. Entomological Review 99(4): 463–472. https://doi.org/10.1134/S0013873819040055

Lukhtanov VA, Dantchenko AV (2017) A new butterfly species from south Russia revealed through chromosomal and molecular analysis of the *Polyommatus* (*Agrodiaetus*) *damonides* complex (Lepidoptera, Lycaenidae). Comparative Cytogenetics 11(4): 769–795. https://doi.org/10.3897/compcytogen.v11i4.20072

Lukhtanov VA, Dantchenko AV, Vishnevskaya MS, Saifitdinova AF (2015) Detecting cryptic species in sympatry and allopatry: analysis of hidden diversity in *Polyommatus* (*Agrodiaetus*) butterflies (Lepidoptera: Lycaenidae). Biological Journal of the Linnean Society 116(2): 468–485. https://doi.org/10.1111/bij.12596

Lukhtanov VA, Dincă V, Talavera G, Vila R (2011) Unprecedented within-species chromosome number cline in the Wood White butterfly *Leptidea sinapis* and its significance for karyotype evolution and speciation. BMC Evolutionary Biology 11: 1–109. https://doi.org/10.1186/1471-2148-11-109

Lukhtanov VA, Shapoval NA (2017) Chromosomal identification of cryptic species sharing their DNA barcodes: *Polyommatus* (*Agrodiaetus*) *antidolus* and *P.* (*A.*) *morgani* in Iran (Lepidoptera, Lycaenidae). Comparative Cytogenetics 11(4): 759–768. https://doi.org/10.3897/compcytogen.v11i4.20876

Lukhtanov VA, Shapoval NA, Dantchenko AV (2014) Taxonomic position of several enigmatic *Polyommatus* (*Agrodiaetus*) species (Lepidoptera, Lycaenidae) from Central and Eastern Iran: insights from molecular and chromosomal data. Comparative Cytogenetics 8(4): 313–322. https://doi.org/10.3897/CompCytogen.v8i4.8939

Lukhtanov VA, Sourakov A, Zakharov EV (2016) DNA barcodes as a tool in biodiversity research: testing pre-existing taxonomic hypotheses in Delphic Apollo butterflies (Lepidoptera, Papilionidae). Systematics and Biodiversity 14(6): 599–613. https://doi.org/10.1080/14772000.2016.1203371

Lukhtanov VA, Sourakov A, Zakharov EV, Hebert PDN (2009) DNA barcoding Central Asian butterflies: increasing geographical dimension does not significantly reduce the success of species identification. Molecular Ecology Resources 9: 1302–1310. https://doi.org/10.1111/j.1755-0998.2009.02577.x

Maddison WP, Maddison DR (2015) Mesquite: a modular system for evolutionary analysis. Version 3.04. http://www.mesquiteproject.org

Marques DF, dos Santos FA, da Silva SS, Sampaio I, Rodrigues LRR (2015) Cytogenetic and DNA barcoding reveals high divergence within the trahira, *Hoplias malabaricus* (Char-

aciformes: Erythrinidae) from the lower Amazon River. Neotropical Ichthyology 11(2): 459–466. https://doi.org/10.1590/S1679-62252013000200015

McClure M, Dutrillaux B, Dutrillaux A-M, Lukhtanov V, Elias M (2017) Heterozygosity and chain multivalents during meiosis illustrate ongoing evolution as a result of multiple holokinetic chromosome fusions in the genus *Melinaea* (Lepidoptera, Nymphalidae). Cytogenetic and Genome Research 153(4): 213–222. https://doi.org/10.1159/000487107

Pazhenkova EA, Lukhtanov VA (2019) Nuclear genes (but not mitochondrial DNA barcodes) reveal real species: Evidence from the *Brenthis* fritillary butterflies (Lepidoptera, Nymphalidae). Journal of Zoological Systematics and Evolutionary Research 57(2): 298–313. https://doi.org/10.1111/jzs.12252

Petrova NA, Cornette R, Shimura S, Gusev OH, Pemba D, Kikawada T, Zhirov SV, Okuda T (2015) Karyotypical characteristics of two allopatric African populations of anhydrobiotic *Polypedilum* Kieffer, 1912 (Diptera, Chironomidae) originating from Nigeria and Malawi. Comparative Cytogenetics 9(2): 173–188. https://doi.org/10.3897/CompCytogen.v9i2.9104

Przybyłowicz Ł, Lukhtanov V, Lachowska-Cierlik D (2014) Towards the understanding of the origin of the Polish remote population of *Polyommatus* (*Agrodiaetus*) *ripartii* (Lepidoptera: Lycaenidae) based on karyology and molecular phylogeny. Journal of Zoological Systematics and Evolutionary Research 52(1): 44–51. https://doi.org/10.1111/jzs.12040

Robinson R (1971) Lepidoptera Genetics. Pergamon Press, Oxford, 698 pp. https://doi.org/10.1016/B978-0-08-006659-2.50006-7

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

Sahoo RK, Warren AD, Wahlberg N, Brower AVZ, Lukhtanov VA, Kodandaramaiah U (2016) Ten genes and two topologies: an exploration of higher relationships in skipper butterflies (Hesperiidae). PeerJ 4: e2653. https://doi.org/10.7717/peerj.2653

Suomalainen E, Brown KS (1984) Chromosome numbers within *Philaethria* butterflies (Lepidoptera: Nymphalidae, Heliconiini). Chromosoma 90: 170–176. https://doi.org/10.1007/BF00292393

Talavera G, Lukhtanov VA, Rieppel L, Pierce NE, Vila R (2013) In the shadow of phylogenetic uncertainty: the recent diversification of *Lysandra* butterflies through chromosomal change. Molecular Phylogenetics and Evolution 69: 469–478. https://doi.org/10.1016/j.ympev.2013.08.004

Tavares JR, de Sousa TP, da Silva JM, Venere PC, Faria KD (2015) Cytogenetics and DNA barcoding of the round-eared bats, *Tonatia* (Chiroptera: Phyllostomidae): a new karyotype for Tonatia bidens. Zoologia 32(5): 371–379. https://doi.org/10.1590/S1984-46702015000500006

Van Nieukerken EJ , Kaila L, Kitching IJ, Kristensen NP, Lees DC, Minet J, Mitter C, Mutanen M, Regier JC, Simonsen TJ, Wahlberg N, Yen S-H, Zahiri R, Adamski D, Baixeras J, Bartsch D, Bengtsson B, Brown JW, Bucheli SR, Davis DR, de Prins J, de Prins W, Epstein ME, Gentili-Poole P, Gielis C, Hättenschwiler P, Hausmann A, Holloway JD, Kallies A,

Karsholt O, Kawahara AY, Koster SJC, Kozlov MV, Lafontaine JD, Lamas G, Landry J-F, Lee S, Nuss M, Park K-T, Penz C, Rota J, Schintlmeister A, Schmidt BC, Sohn J-C, Solis MA, Tarmann GM, Warren AD, Weller S, Yakovlev RV, Zolotuhin VV, Zwick A (2011) Order Lepidoptera Linnaeus, 1758. In: Zhang Z.-Q. (Ed) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. Zootaxa 3148: 212–221. https://doi.org/10.11646/zootaxa.3148.1.41

Vishnevskaya MS, Lukhtanov VA, Dantchenko AV, Saifitdinova AF (2018) Combined analysis of chromosomal and molecular markers reveals cryptic species: karyosystematics of the *Agrodiaetus* Hübner, [1822] blue butterflies. Comparative Cytogenetics 12(3): 325–326. https://doi.org/10.3897/CompCytogen.v12i3.27748

Vishnevskaya MS, Saifitdinova AF, Lukhtanov VA (2016) Karyosystematics and molecular taxonomy of the anomalous blue butterflies (Lepidoptera, Lycaenidae) from the Balkan Peninsula. Comparative Cytogenetics 10(5): 1–85. https://doi.org/10.3897/CompCytogen.v10i5.10944

Ward RD, Hanner R, Hebert PDN (2009) The campaign to DNA barcode all fishes, FISH-BOL. Journal of Fish Biology 74(2): 329–356. https://doi.org/10.1111/j.1095-8649.2008.02080.x

Wesley DJ, Emmel TC (1975) The chromosomes of neotropical butterflies from Trinidad and Tobago. Biotropica 7: 24–31. https://doi.org/10.2307/2989796

White MJD (1973) Animal cytology and evolution. Cambridge, 961 pp.