Notable homologous variation in chromosomal races of the common shrew

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Abstract

This paper is a review of the rare phenomenon of chromosome intraspecies variation manifested in monobrachial homology series in the comprehensively investigated karyotype of the common shrew Sorex araneus Linnaeus, 1758 (Eulipotyphla, Mammalia). The detailed dataset on the account of this mammalian species was drawn from the recently published monograph by Searle et al. (2019) “Shrews, Chromosomes and Speciation”. The parallels to the law of homologous series in variation by Nikolai Vavilov are discussed.

Keywords

Chromosome rearrangements (CRs), mammal karyotype, N.I. Vavilov heritage, Robertsonian fusions, Sorex araneus

Genetics started in the XXth century with rediscovery of G. Mendel’s hereditary laws, T.H. Morgan's chromosome theory of heredity and prior evolutionary generalizations of W. Bateson, the author of the term “genetics”. Advances of the first two decades in the emerging field of plant genetics have been promptly consolidated into the law of homologous series in variation by Nikolai Vavilov, who was considered himself to be a student of William Bateson. A concise first presentation of the law idea (Vavilov 1920) in Russian was soon published in English in the Journal of Genetics edited by W. Bateson and R.C. Punnett (Vavilov 1922). Since and till now, homology problems remain in focus of different scientific disciplines exploring homologous variation,
from molecular genetics to paleontology and bioinformatics (i.e., Korochkin 1985; Rozhnov 2006; Suslov et al. 2018). For decades, cytogenetic analysis was developing towards the correct assessment of chromosome homology, and today the use of methods of differential staining and, in particular, of chromosome painting makes possible the interspecies comparison on generic and higher taxonomic levels (Ferguson-Smith and Trifonov 2007). This paper aims to review features of homologous chromosomes variation in a mammalian species with one of the best investigated karyotypes, the common shrew *Sorex araneus* Linnaeus, 1758 (Searle et al. 2019). Taking into account the upcoming date of the 100th anniversary of Vavilov’s law, it could be a challenge to examine the variety of chromosomal races of *S. araneus* as the phenomenon of series of homologous variation.

The record of chromosomal variation within the common shrew was recently reviewed in essential details in the monograph “Shrews, Chromosomes and Speciation” which summarized more than the 30-year period of joined multidisciplinary studies of *S. araneus* chromosomal races in Eurasia initiated by the International *Sorex araneus* Cytogenetics Committee, ISACC (Searle et al. 2019). Chapter 5 of this book presents the list of chromosomal races discovered over the whole vast species range of *S. araneus*. As generally, geographic names of 76 chromosomal races were listed in an alphabetical order, accompanied with the diagnostic chromosomal formulas (Bulatova et al. 2019). G-band nomenclature was used for the chromosome identification (Searle et al. 1991) and chromosomal races were defined prioritizing the karyotypic and geographic separation adhering to the ISACC rules (Hausser et al. 1994).

The chromosomes of *S. araneus* are composed of 21 chromosomal arms that can be fused in a variety of combinations in different populations forming an astonishing array of chromosomal races. According to the nomenclature, 21 arms are designated by Latin letters (*a* to *s*) in correspondence with the arrangement in decreasing size from largest to smallest.

In the karyotype of *S. araneus*, chromosomes in pairs appear in either the bi-armed (metacentric) or one-armed (acrocentric) form. Among autosomes, three pairs are always bi-armed and demonstrate stable combination of chromosome arms (*af*, *bc* and *tu*). One other pair is always composed of arms *j* and *l*, but can display individual or population Robertsonian polymorphism appearing in acrocentric or/and metacentric forms (*j*, *l*/*jl*) (Ford and Hamerton 1970).

Sex chromosomes of *S. araneus* have complex origin because of the ancient event of the autosome to sex chromosome translocation: a fusion between the original “true” *X* (arm *e*) and an autosome (arm *d*). Thus, in females, XX pair is represented by bi-armed (*de*) chromosomes, and in males, by a system of triple sex chromosomes – *X(de) Y1(“true” Y)Y2(d)*.

Ten other chromosome arms (*g* to *r*, except *j*, *l*) are fused in a variety of combinations which show the remarkable intraspecies polytypic variation. Such arm reshuffling creates a variety of chromosomal races: 37 different combinations of chromosomal arms fused into metacentrics were detected in 76 described chromosomal races (see tables 5.2 and 5.3 in: Bulatova et al. 2019). So, here the set of chromosomes/arms in
Chromosomal homologous variation

Table 1. Serial presentation of chromosomal race specific metacentrics (monobrachial homologs) defined in *Sorex araneus*. Asterisks mark the fusions absent* in the list of chromosomal races, and potential** for the race/species karyotypes. A double letter designation is given in the alphabetical order following the standard nomenclature of chromosomes of *S. araneus* (Searle et al. 1991). *o, q* – NOR-bearing arms.

<table>
<thead>
<tr>
<th>Arm</th>
<th>g</th>
<th>b</th>
<th>i</th>
<th>k</th>
<th>m</th>
<th>n</th>
<th>o</th>
<th>p</th>
<th>q</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>g</td>
<td>*</td>
<td>gi</td>
<td>gk</td>
<td>gm</td>
<td>*</td>
<td>go</td>
<td>gp</td>
<td>qq</td>
<td>gr</td>
<td>gb**</td>
</tr>
<tr>
<td>b</td>
<td>*</td>
<td>bi</td>
<td>bk</td>
<td>*</td>
<td>bn</td>
<td>ho</td>
<td>bq</td>
<td>*</td>
<td>gr</td>
<td>gh**</td>
</tr>
<tr>
<td>i</td>
<td>gi</td>
<td>hi</td>
<td>ik</td>
<td>im</td>
<td>*</td>
<td>io</td>
<td>ip</td>
<td>iq</td>
<td>*</td>
<td>gh**</td>
</tr>
<tr>
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<td>gi</td>
<td>hi</td>
<td>ik</td>
<td>*</td>
<td>km</td>
<td>ko</td>
<td>kp</td>
<td>*</td>
<td>kr</td>
<td>h**</td>
</tr>
<tr>
<td>m</td>
<td>gi</td>
<td>hi</td>
<td>ik</td>
<td>*</td>
<td>km</td>
<td>ko</td>
<td>kp</td>
<td>*</td>
<td>kr</td>
<td>h**</td>
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<td>n</td>
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<td>io</td>
<td>ko</td>
<td>no</td>
<td>*</td>
<td>op</td>
<td>*</td>
<td>qr</td>
<td>nor</td>
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<td>p</td>
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<td>kp</td>
<td>mp</td>
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<td>*</td>
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<td>nor</td>
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<td>Total of 9</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>9</td>
<td>8</td>
<td>9</td>
<td>7</td>
</tr>
</tbody>
</table>

Karyotypes of the *S. araneus* is represented in symbols of the standard nomenclature in Latin letters with variable chromosomes being marked with an asterisk:

\[ af, bc, de, (XX)/d, Y2, *g*, *b*, *i*, *j/l, *k*, *m*, *n*, *o*, *p*, *q*, *r*, *s, (Y1), tu. \]

To analyse the peculiarities of the variable group of chromosome arms, the list of the synoptic table 5.3 from Bulatova et al. (2019) was restructured to follow each chromosome variation in Table 1 here. The acrocentric state and fusion variants were labelled with one and two letters, correspondingly, revealing thus all defined series.

In our analysis, each chromosome series begins with an acrocentric state (for instance, *g*) and accumulates varying fusion combinations with other elements of the variable group (in this case – *gi, gk, gm, go, gp, gq, gr*). That is, from nine possible combinations, two variants of the arm *g* fusions are absent from this series (*gh, gn*) – but probably could still be found in nature.

All nine possible fusion variants were realized in two cases, for the arms *o* and *q* (Table 1). Along with aforementioned *g* group (lacking *gb* and *gn*), incomplete series are shown for other arms, namely *b* (*-bh, bm, hp, hr*), *i* (*-in, ir*), *k* (*-kn*), *m* (*-hm*), *n* (*-gn, in, kn*), and *p* (*-hp*), and, correspondingly, for their fusion partners (arms *g, b, i, k, m, n, p, r*). It is worth noting that some fusions, for instance *gb* and *hm* absent in the *h* (as well as *g* and *m*) series, were found outside the current list of chromosomal races. These are *hm*, present in an F1 interracial hybrid karyotype due to proposed whole arm reciprocal translocation (WART) (Pavlova et al. 2008), and *gb*, identified in the karyotype of a sibling species, *S. satunini* Ognev, 1922 (Borisov and Orlov 2012). Besides, it seems remarkable that the chromosomes *o* and *q*, most “active” in fusions, are carriers of nucleolus organizing region (NOR), located distally at an acrocentric end (Searle et al. 1991).

Fusions predominate among evolutionary changes of karyotypes in the genus *Sorex* Linnaeus, 1758. Cascades of fusions have happened in the past karyotype evo-
olution of this genus according to refined cytogenetic studies (Biltueva et al. 2011) based on chromosome differential staining (homology of G-banding or R-banding patterns) combined with chromosome painting (chromosome homology revealed via fluorescent in situ hybridization). Based on the study of 12 congeneric taxa, the authors proposed an ancestral karyotype of Palearctic *Sorex*, AKPS, consisting of 28 chromosomes (2n = 56). Some of these ancestral elements are homologous to whole *S. araneus* chromosomes, while others to parts of the modern chromosomal arms, indicating that multiple evolutionary fusion events led to the formation of *S. araneus* karyotype. Nine autosomes were completely conserved (*h, i, m, n, o, p, q, r* and *tu*), while fusion combinations were defined in nine other chromosomes (*a, b* consisting of three parts each: *a1, a2, a3* and *b1, b2, b3*; and those consisting of two parts each – *c, d, f, g, j, k, l*) (Table 2). Subsequent fusions between initially formed acrocentrics shaped two characteristic metacentrics of the *S. araneus* karyotype – *jl* and *bc* (Biltueva et al. 2011). The fusion *jl* is species specific apomorphy, while *bc* is a common fusion for the clade consisting of three sibling species, *S. araneus*, *S. satunini*, *S. antinorii* Bonaparte, 1840 (reviewed by Bulatova et al. 2019). The variants of combination of 10 acrocentrics into 1 to 5 permissible race specific metacentrics are multiplied in series of further fusion combinations of every monobrachial homolog (for instance, *gm* with *hi*, *hk*, *hn* – *hi* with *ko*, *kp*, *kg*, *kr* – *ko* with *nr*, *np*, *nq*; and so on). In such a way, at least 58 distinct karyotypic variants were formed which are attributed to 76 known chromosomal races (note that, sometimes, geographically distant races do not show karyotypic differences).

In this review, numerous series of fusion variations in race specific chromosomes of *Sorex araneus* were considered (Tables 1, 2). It is noteworthy that methodical progress ensures the estimation of chromosome homology within and out of the species limits equally. The data on fully complete and incomplete series are available and allow thinking on and filling in the corresponding gaps. That makes the work in this respect to some extent predictable. It is concluded that homologous series recognition could be in particular demonstrative in cases of interspecies and intraspecies karyotypic variation of the Robertsonian type where it can concur with, for instance, routinely proposed understanding of hybrid origin of metacentric homologs (Bakloushinskaya et al. 2019).

The law of homologous series in chromosome variation may be considered a new notion of comparative cytogenetic studies not only of commemorative (Bulatova 2019) but also of fundamental genetic interests.

### Table 2.
The chromosomal arms of the common shrew in alphabetical order (top line), without *s* (*Y1*). Homology to the elements of the *Sorex* ancestral karyotype, AKPS, is indicated in the rows below. Number of ancestral homologous elements that formed present-day arms through past fusions was identified by G-banding and chromosome painting by Biltueva et al. (2011).

<table>
<thead>
<tr>
<th>a1</th>
<th>b1</th>
<th>c1</th>
<th>d1</th>
<th>e1</th>
<th>f1</th>
<th>g1</th>
<th>h1</th>
<th>i1</th>
<th>j1</th>
<th>k1</th>
<th>l1</th>
<th>m</th>
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<td>c2</td>
<td>d2</td>
<td>e2</td>
<td>f2</td>
<td>g2</td>
<td>h2</td>
<td>i2</td>
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<tr>
<td>a3</td>
<td>b3</td>
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References


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Cytogenetic characterization, rDNA mapping and quantification of the nuclear DNA content in *Seriolella violacea* Guichenot, 1848 (Perciformes, Centrolophidae)

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Abstract

*Seriolella violacea* Guichenot, 1848 is an important component of the fish fauna of the Chilean coast and is of great economic interest. Cytogenetic information for the family Centrolophidae is lacking and the genomic size of five of the twenty-eight species described for this family are is barely known. This study aimed to describe for the first time the karyotype structure via classical and molecular cytogenetics analysis with the goal of identifying the constitutive heterochromatin distribution, chromosome organization of rDNA sequences and quantification of nuclear DNA content. The karyotype of *S. violacea* is composed of 48 chromosomes, with the presence of conspicuous blocks of heterochromatin on chromosomal pairs one and two. FISH assay with a 5S rDNA probe, revealed the presence of fluorescent markings on the heterochromatic block of pair one. The 18S rDNA sites are located exclusively on pair two, characterizing this pair as the carrier of the NOR. Finally, the genomic size of *S. violacea* was estimated at 0.59 pg of DNA as C-value. This work represents the first effort to document the karyotype structure and physical organization of the rDNA sequences in the *Seriolella* genome, contributing with new information to improve our understanding of chromosomal evolution and genomic organization in marine perciforms.
Keywords
chromosomal status, CMA3 staining, genome size, Repetitive DNAs

Introduction

In recent years fish cytogenetics has accumulated data that establish evolutionary trends, phylogenetic relationships among different families, species and populations (Arai 2011). This information is of great importance for the management and conservation of natural stocks (Carvalho-Costa et al. 2008). Currently the karyotypes of only 2% of all global marine fish are known (Galetti et al. 2000; Vega et al. 2002; Arai 2011). These studies have been focusing on just a few families of reef and pelagic fish, such as Gerreidae (Calado et al. 2013), Scombridae (Soares et al. 2013), Gobiidae (Lima-Filho et al. 2012), Labridae (Molina et al. 2012; Paim et al. 2014), Haemulidae (Nirchio et al. 2007; Neto et al. 2011) Carangidae (Chai et al. 2009) and Rachycentridae (Jacobina et al. 2011) preferably distributed in the Atlantic Ocean. According to Jara-Seguel et al. (2011) the marine fish fauna of Chile has been little studied, with known cytogenetic data for only some species of the Atherinidae, Galaxiidae, Kyphosidae, Mugilidae, Ophidae and Paralichthyidae families being available.

*Seriolella violacea* (Guichenot, 1848) is an important component of the fish fauna of the Chilean coast and has great economic value (Ojeda et al. 2000). This species has an epipelagic gregarious behavior, forming schools near the coast; adults are found in areas of the continental shelf, as well as within protected bays, along the entire northern coast of Chile. Due to their rapid growth, adaptability and potential market, they currently represent an important candidate for the start of cultivation programs (Angel and Ojeda 2001; Navarrete et al. 2014).

No cytogenetic information is available for the family Centrolophidae, and the chromosomal constitution of the 28 species described in this family is unknown (Arai 2011). In addition the genomic size of five species (Hardie and Hebert 2004) is barely known. Due to this lack of biological information and the high potential for aquaculture that these species represent, it is essential to carry out a cytogenetic characterization; the karyotype and genome size are two primary genetic characteristics of the species, which are of great importance, when studying taxonomy, phylogenetic relationships, evolution and molecular biology.

Considering the absence of cytogenetic information on the Centrolophidae and the biological and economic importance of these pelagic fish, this study aims to describe for the first time the karyotype structure using classical and molecular cytogenetics analysis and quantification of nuclear DNA content in *Seriolella violacea*.

Material and methods

Six individuals, four males and two females, of *S. violacea* were obtained from the Laboratorio Central de Cultivos Marinos belonging to the Universidad Católica del Norte,
Coquimbo-Chile. Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, following the protocol established by Foresti et al. (1993). Approximately 20 metaphase spreads from different individuals were analyzed to confirm the diploid number and karyotype structure of *S. violacea*. The C-banding was carried out according to Sumner (1972); and the use of GC-specific fluorochrome Chromomycin A3 (CMA3) following Schweizer (1976). The chromosomes were classified according to Levan et al. (1964).

The 18S rDNA and the 5S rDNA probes were obtained by PCR (Polymerase Chain Reaction) from genomic DNA of *Seriolella violacea* using primers NS1F (5′-GTAGTCATATGCTTGTCTC-3′), and NS8R (5′-TCCGCAGGTTCACTACCTACGCCAGA-3′) (Cioffi et al. 2009) and 5SA (5′-TACGCCCCATCTCGTGCCGATC-3′) and 5SB (5′-GCTGGTTAGGCCGGTGAGC-3′) (Pendás et al. 1994), respectively, and subsequently labeled with biotin-16-dUTP and digoxigenin-11-dUTP.

FISH was performed under high stringency conditions using the method described by Pinkel et al. (1986). Slides were incubated with RNase (50 μg/ml) for 1 h at 37 °C. Then the chromosomal DNA was denatured in 70% formamide/2× SSC for 5 min at 70 °C. For each slide, 30 μl of hybridization solution was denatured for 10 min at 95 °C, dropped on the slides and hybridized overnight at 37 °C in a 2× SSC moist chamber. Probe detection was carried out with Avidin-FITC (Sigma) or anti-digoxigenin-rhodamine (Roche). Chromosomes were counterstained with DAPI (4′,6-diamidino-2-phenylindole, Vector Laboratories).

Measurements of nuclear DNA content (C-value) were done by microdensitometry in erythrocytes obtained from adult specimens (2♀ and 2♂), analyzing 200 nuclei per sample, using the software Image Pro-Plus 4.0. (Media Cybernetics). The blood was dispersed on slides, air dried, fixed in methanol-acetic acid (3:1 v/v) at 4 °C for 24 h and stained with the Feulgen reaction (Jara-Seguel et al, 2008). Nuclear optic density (OD) is calculated by the software according to the formula $\text{OD} = \log_{10} (1/T) = - \log_{10} T$; where $T$ = intensity of transmitted light/intensity of incident light. From this estimation, the computer integrates the values of OD obtained for each one of the pixels and it calculates the integrated optical density (IOD = ΣOD). The IOD values, in arbitrary units, were converted to absolute mass of DNA by comparison with erythrocyte smears of rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792), $2C = 5.5$ pg, $2n = 58–60$) (Hartley and Horne 1985).

**Results**

The karyotype of *S. violacea* shows 24 pairs of chromosomes ($2n = 48$; FN = 48), all acrocentric (Fig. 1A). No morphologically differentiated sex chromosomes were found when metaphase plates from males and females were compared. C-positive blocks of constitutive heterochromatin (HC) were observed in pericentromeric regions of few chromosomes, highlighting the presence of two conspicuous HC blocks, one of them in the pericentromeric region of pair one, while the other was in the telomeric region of pair two (Fig. 1B). In addition, these two conspicuous blocks were positive for Chromomycin A3 staining (Fig. 2A).
Figure 1. Conventional Giemsa-stained (A) and C-banding (B) in *Seriolella violacea*. Scale bar: 10 μm.

Figure 2. Karyotypes of *Seriolella violacea* after CMA₃ staining (A) and dual color FISH with 18S rDNA (green) and 5S rDNA (red) probes (B). Scale bar: 10 μm.

Table 1. Known genomic sizes C-Value(pg) for representatives of the Centrolophidae family.

<table>
<thead>
<tr>
<th>Species</th>
<th>C-Value</th>
<th>Method</th>
<th>Cell Type</th>
<th>St. Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrolophus niger</td>
<td>0.70</td>
<td>FIA</td>
<td>RBC</td>
<td>BS, GD, OM, RP</td>
<td>Hardie and Hebert 2004</td>
</tr>
<tr>
<td>Hyperoglyphe antarctica</td>
<td>0.77</td>
<td>FIA</td>
<td>RBC</td>
<td>BS, GD, OM, RP</td>
<td>Hardie and Hebert 2004</td>
</tr>
<tr>
<td>Schedophilus huttoni</td>
<td>0.76</td>
<td>FIA</td>
<td>RBC</td>
<td>BS, GD, OM, RP</td>
<td>Hardie and Hebert 2004</td>
</tr>
<tr>
<td>Seriolella punctata</td>
<td>0.78</td>
<td>FIA</td>
<td>RBC</td>
<td>BS, GD, OM, RP</td>
<td>Hardie and Hebert 2004</td>
</tr>
<tr>
<td>Tubbia tasmanica</td>
<td>0.76</td>
<td>FIA</td>
<td>RBC</td>
<td>BS, GD, OM, RP</td>
<td>Hardie and Hebert 2004</td>
</tr>
<tr>
<td>Seriolella violacea</td>
<td>0.59</td>
<td>FIA</td>
<td>RBC</td>
<td>GD, OM</td>
<td>in this work</td>
</tr>
</tbody>
</table>


Dual FISH detected 18S and 5S rDNA probes on different chromosome pairs (Fig. 2B). Mapping the 5S rDNA probe revealed the presence of fluorescent markings on the heterochromatic block of pair one. The 18S rDNA sites are located exclusively on pair two, in a position coincident to heterocromastics/CMA₃ positive blocks, characterizing pair two as the pair carrying the NOR.

Finally, the nuclear DNA content measured in erythrocytes of *S. violacea* was estimated to be 1.18 ± 0.04 pg (average IOD = 14345 arbitrary units), with a coefficient of variation of 4.2%. Since *S. violacea* is a diploid organism (2n = 48, n = 24), the C-value of 0.59 pg of DNA (Table 1), is equivalent to 578.2 megabase pairs (Mbp).
Discussion

There are no data related to the organization of the repetitive fraction of the genome in the family Centrolophidae. Nevertheless, studies within the marine perciform order, specifically in representatives of the families Ephippidae, Serranidae, Lutjanidae, and Haemulidae have permitted the recognition of a diploid number of 48 chromosomes (completely acrocentric); the non-syntenic state of sequences 5S rDNA and 18S rDNA; and the presence of a single NOR, establishing this pattern as a plesiomorphic characteristic for marine perciforms (Chai et al. 2009; Arai 2011; Neto et al. 2011; Costa et al. 2016; Paim et al. 2017). The repetitive fraction of the genome can be a useful tool for identifying recent genomic changes that have occurred during the evolutionary process, as well as act as potential hotspots for chromosomal rearrangements (Ozouf-Costaz et al. 2004; Valente et al. 2011; Yano et al. 2014). In this sense, *S. violacea* presents exactly the cytogenetic pattern described for marine perciforms, highlighting the association of ribosomal clusters with heterochromatin blocks rich in CG bases in specific chromosome pairs. An association between 18S and 28S rDNA sequences and heterochromatin has been found in other fish, such as salmonids (Pendás et al. 1994; Fujiwara et al. 1998), species of the genera *Epinephelus* Bloch, 1793, *Imparfinis* Eigenmann & Norris, 1900 and *Pimelodella* Eigenmann &Eigenmann, 1888 (Gouveia et al. 2013), *Orestias* Valenciennes, 1839 (Araya-Jaime et al. 2017) and sturgeon species (Fontana et al. 2003). This suggests that the repeated HC sequences play an important role and exercise diverse functions in the eukaryotic genome (Grewal and Jia 2007). It has even been postulated that heterochromatin is involved in maintaining the structure of the nucleolus and the integrity of ribosomal DNA repeats (McStay and Grummt 2008). Visualization of a single carrier pair sequence for 18S rDNA is one of the most common features observed in the fish genome, unlike what was observed for the gene 5S ribosomal which may present variations in the chromosomal distribution, apparently through its association with transposable elements, suggesting independent evolutionary pathways for both types of rDNA (Pendás et al. 1994; Martins and Galetti 2001; Cabral-de-Mello et al. 2011; Scacchetti et al. 2012; Sene et al. 2014; Santos et al. 2017; Usso et al. 2019). Teleosts exhibit low levels of compartmentalization in their genomes, which would suggest that the configuration in *S. violacea*, observed for the two types of ribosomal DNA, would represent a relatively simple to organization state (Medrano et al. 1988).

Finally, 0.59 pg of DNA (C-value) measured in erythrocytes of *S. violacea* represents a significantly (20%) lower nuclear DNA content than that of the five species of the Centrolophipadae family analized (Table 1), which on average reach 0.75 pg DNA. Thus, this value represents the smallest genome size known to the family. Currently there are data of nuclear DNA content for 634 species of Perciformes, estimating an average of 0.94 pg of DNA (C-value) for this order of fish, with minimum values of 0.39 pg in *Scienops ocellatus* (Linnaeus, 1766) and maximum of 2.60 in *Lagodon rhomboides* (Linnaeus, 1766) (Hardie and Hebert 2004; Gregory 2020). The evolutionary role genome size plays is the subject of much discussion, but computational biology
has helped to model some patterns. These patterns are clearer when the nuclear DNA content is related to species life history attributes, especially with regards to effective population sizes and their gene flow rates, showing an inverse relationship between population size and the size of the genome (Vinogradov 2004; Labar and Adami 2017; Bobay and Ochman 2018).

**Conclusion**

In this work, the karyotype of a representative of the Centrolophidae family, *S. violacea*, is described for the first time. Its karyotype is made up of 48 acrocentric chromosomes (2n = 48; FN = 48), simple NOR and ribosomal cistrons (5S-18S rDNA) are not synthetic. Meanwhile, the nuclear DNA content, C-value, was found to be 0.59 pg. It is necessary to perform additional studies physically mapping repetitive DNAs in the other representatives of the genus *Seriolella* Guichenot, 1848, in order to understand the involvement of these sequences in the process of chromosomal evolution that these fish may be experiencing. It is especially necessary to analyze the chromosomal microstructure, given the chromosomal stasis that most marine perciforms present, as this will also expand knowledge of fish fauna which is facing serious conservation issues.

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**References**


Cytogenetic characterization in Seriolella violacea (Guichenot, 1848)


Archives: The law of homologous series in variation (N. I. Vavilov)*

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A century has passed since the days when the law of homologous series in variation was first manifested. This event happened in 1920 in Saratov, in the third post-revolution year, in the frameworks of the III All-Russian Conference on Plant Breeding, then mobilized in view of current needs of agricultural practice, science and education. The report of a 33-year-old professor Nikolai Vavilov, who was accompanied by his students from the Saratov University, caused a sensation. Vavilov’s generalization on the phenomenon of the homologous series in variation of cultivated plants was reported on June, 4, 1920 and enthusiastically appreciated by the qualified breeders as a great scientific achievement comparable with the Mendeleev’s periodic Law of the chemical elements. On June 21, 1920, a message of the provincial Saratov branch of the Russian Telegraph Agency shared internationally the information on “the greatest discovery of world significance” which was addressed to the State government by the decision of the meeting. Very soon after the initial Russian publication (Vavilov 1920), the paper entitled “The Law of Homologous Series in Variation” was published in the Journal of Ge-

netics, edited by W. Bateson and R.C. Punnett, the elder statesmen of genetics (Vavilov 1922). In 1925, William Bateson, Director of the John Innes Horticultural Institute, with colleagues, visited experimental fields and laboratories of Nikolai Vavilov, Director of the Bureau of Applied Botany and Plant Breeding (future N.I. Vavilov Institute of Plant Breeding) in a Petrograd – Leningrad (now Saint Petersburg) suburb (Fig. 1). The paper took 42 pages of Volume XII (1) (April, 1922, p. 47–89). The substance of this work by Vavilov was recalled in the chapters of N. Timofeeff-Ressovsky (1940) and N. Vavilov (1940) in the monograph “The New Systematics” (Huxley 1940), a synoptic book, preceding the publication on the new synthesis of theory of evolution (Huxley 1942). Since then and till now, genetic nature of homologous variation, the matter of the Vavilov’s law, has been in the focus of various disciplines, from agriculture to paleontology, being rejuvenated with the progress of molecular biology. Nowadays, molecular homology can be established universally at various levels, from unique genes to gene orders in chromosomes through genetic, cytogenetic and molecular analyses (Zakharov 1987) up to gene networks studied by bioinformatics (Suslov et al. 2008). It seems rational to meet the 100th anniversary of this significant event of young hereditary science with a digital copy saving the author’s idea for future readers and investigators. The text is here reproduced in the Archives format from printed pages of the Introduction (p. 48–53) and the concluding section (p. 86–89) of the original English version (Vavilov 1922). The title page copied on Fig. 2 presents the whole contents of this work. Details of punctuation and citation are generally saved.

N. Bulatova

Introduction [p. 48–53]

Evolution of the study of systematics of plants

The characteristic feature of the history of plant investigation, from Tournefort up to the present, has been the varied conception of systematic units. Further investigation did away with the former conception of species, as introduced by Linné. The history of systematics of plants gives a vivid illustration of attempts to arrange in a convenient and harmonious system all newly discovered morphological and physiological characteristics, the number of which grows rapidly with improved methods of discerning hereditary forms, and with the study of new specimens of the same plants, gathered in different regions. The Linnean species had to be divided into subspecies and varieties (in sensu bot.); varieties into races. Genetical studies of the last decades have proved even the divisibility of the minutest morphological and physiological units in systematics (races, Elementararten of de Vries), and established that, although outwardly similar, they can be different genotypically. The same is applicable to the animal world.

Lotsy, in his book Evolution by Means of Hybridization (1916), proposes to introduce a new terminology to distinguish fundamental units in the classification of hereditary forms. He proposes to call the old Linnean species, which, as was shown in the nineteenth century, are of collective nature – “Linneons”; races, varieties, which make up the elementary species of Jordan and de Vries he proposes to define as “Jordanons”.

N. Bulatova
The term “species”, Lotsy would retain (as it seems to us not very successfully) for the modern conception of genetics – the genotype, as a fundamental unit covering similar hereditary groups of individuals.

**Statistics of the diversity of the plant world**

Up to the present, statistics of the plant and animal world are available only for “Linneons”. According to Hooker and Engler there are known altogether about 130,000–140,000 Linnean species of higher seed plants, including Coniferae. Families most abounding in Linneons are, according to Engler*, those of Compositae (ca. 13,100), Leguminoseae (ca. 12,000), Gramineae (ca. 4,000).

Although these numbers of Linneons are quite large, they give a very superficial representation of the real diversity of the plant world. Only a closer study of Jordanons and genotypes would give a true idea of this diversity.

The systematic study of numerous varieties among Linnean species, which was initiated by Lindley (Monograph on Roses), de Candolle (Brassica), Kraus, Metzger,
and Alefeld on cultivated plants, and by Séringe, Jordan and Naegeli on wild plants, and is continued nowadays by plant breeders and by botanists (Swedish school of systematists: Wittrock, Dalstedt, Almquist and others), has revealed a total absence of monotypical Linneons. Linnean species, which, in the nineteenth century were re-

* Musee helveticus, p. 115 (Aconitum).
garded as uniform, in the twentieth century were separated by plant breeders and systematists into large numbers of Jordanons, easily distinguishable both morphologically and physiologically; e.g. many species of Gramineae, Compositae, Cruciferae, Leguminosae, Sesamum indicum, Viola tricolor, Linnea borealis, etc. Up to the present, not many Linneons of wild and cultivated plants have been studied thoroughly, but still the data available shows an immense diversity of Jordanons among Linneons.

Thus, after investigations of local Russian and Asiatic wheats at our experimental station, the existence was proved of about 3000 Jordanons of Triticum vulgare Vill., perfectly recognizable morphologically and physiologically*. This number does not include many hundreds of varieties of hybrids created artificially by plant breeders of Western Europe during the last thirty or forty years, but only the natural local varieties of wheat.

For barley we know at least 600 to 700 Jordanons, for oats more than 600. In Rye, Secale cereale, many hundreds of forms, differing in hereditary morphological and physiological characters, were collected by Mrs V. P. Antropova, from different parts of Persia, Bokhara, Asiatic and European Russia. Hundreds of easily distinguished forms are found in sorghum by American investigators. Investigations in Japan and India discovered thousands of varieties in rice. Thousands of varieties might be established in Indian corn, Zea mays. Hundreds of varieties were found in peas, Pisum sativum; vetches, Vicia sativa; lentils, Ervum Lens; beans, Phaseolus vulgaris. Hundreds of varieties are found among Soya beans, Soya hispida. Jordan and Rosen found about 200 constant varieties in wild Draba verna. Miss Sinskaja, at our experimental station, found more than 300 well recognizable varieties of Eruca sativa, a weed occurring in field of flax in Turkestan and Bokhara. Thousands of forms, perfectly distinguishable, exist among species of Cucurbita Pepo, Cucurbita maxima, Citrullus vulgaris – watermelon, Cucumis sativus, and Cucumis Melo**. Hundreds of forms are found among wild Linnea borealis (Wittrock), Picea excelsa (Wittrock), etc.

Wild and cultivated plants

The majority of cultivated and wild Linneons propagated by seeds, are represented by hundreds of well-defined Jordanons. There is no essential difference in this respect between wild and cultivated plants. Wild Linneons, like clover (Trifolium pratensis), Agropyrum cristatum, Agropyrum repens, yellow alfalfa (Medicago falcata), Alopecurus pratensis, Brassica elongata, studied in detail at Russian Experimental Stations by plant breeders (Roudzinski, Lorch, Jegalov, Bogdan), proved to be no less variable than cultivated wheats, barleys, oats, and peas. The monotypic nature of many wild Linnean species is kept only so long as they are studied by a few specimens in the herbarium. The individual study in culture of many samples of the same Linneon inevitably discovers its polymorphic nature.

* This data is given in the address by the author and his co-workers at the All-Russian Conference on Plant Breeding, 1920. Saratov. Now in the press.
** These plants were studied at our experimental station by Mr S.M. Boukasov and Mrs S.A. Kartashov.
Still greater diversity is observable in plants multiplying vegetatively or apogamically, like roses, potatoes, apples, *Hieracium* (Naegeli), and *Dahlia*.

We do not exactly know if there are really monotypic Linnean species in nature, fairly well specific and separated from other Linnean species and represented by one variety, one Jordanon only. The whole impression is that the more we study our plants and animals, the more variable they are, the more varieties we find among Linnean species. Several Linnean species of plants and animals, like roses, wheats, Indian corn, rice, squashes, *Drosophila*, seem to be extremely variable, but these have attracted more attention than others. We easily notice sharp differences in colour, size, and shape of several organs and are rather inattentive to others.

The differences of Jordanons within the limits of the same Linneon, in the shape and colour of their flowers, form and size of leaves, fruits and other organs, are very often no less marked than the differences between Linneons themselves. For instance, some varieties of *Cucurbita Pepo* are characterized by fruit the size of hen’s eggs; other varieties, growing under the same conditions, bear fruit three and four feet in diameter. Some varieties of *Sesamum indicum* have opposite leaves and fruits, others have alternate. Some varieties of wheat and rye have simple leaves, without differentiation into vaginae and plates, having no “ligula”, or “auriculae”; others have the usual complicated leaves, with “ligula”, and “auriculae”.

Plants self-fertilized, as wheat, barley, peas, soya, etc., and cross-fertilized, as rye, maize, beet, ale alike polymorphous. The seeming uniformity of several cross-fertilized wild and cultivated plants is only apparent when they are not studied carefully. The difference consists only in the homozygotic nature of many characters in cross-fertilized plants, and in the homozygotic nature of self-fertilized plants. Some recessive characters may be hidden in cross-fertilized plants by the dominance of other characters, but by artificial self-fertilization of these plants, and by inbreeding, it is possible to re-establish them. From what we know at present from the study of Indian corn (Emerson, Collins, and others), of rye, beetroot, *Drosophila*, man himself, cross-fertilized organisms are not less variable than self-fertilized.

The above mentioned numbers of Jordanons are in reality still greater, because, up to the present time, African and Asiatic varieties of even the most important cultivated plants, like wheat, oats, barley, peas, lentils, *Cruciferae*, are almost unknown.

Problems of the future

There is a real need for the study and systematizing of these Jordanons, especially in cultivated plants and domesticated animals, for the benefit of geneticists, as well as systematists and agriculturists. Only the closest study of Jordanons and genotypes will give a real re-presentation of what a Linneon is. To construct the general genetic schemes, it is necessary to know the composition of Linnean species. Before creating new varieties by crossing we ought to know what exists in nature. Even for cereals, *Leguminoseae*, and other most important plants, we have no adequate knowledge of even easily recognizable botanical varieties. Regions of ancient culture in Asia, Africa, and America still preserve numbers of varieties unknown to systematists and plant breeders.
In 1880, Alphonse de Candolle wrote in his remarkable book *La Phytographie*: “Un jour la science traitera les éléments de l’espèce comme les éléments des genres, comme ceux de la famille et tous ces groupes seront coordonnés, les uns au-dessus des autres d’une manière parfaitement uniforme” (p. 80). This day has arrived, but the task is not very simple. The closest study of some Linneons of cereals, *Leguminosae*, *Cruciferae*, *Compositae*, and *Cucurbitaceae*, persuades one of the immensity of this work. The diversity of plants and animals is too great to admit of giving a complete list of existing forms. There comes the necessity to establish some principles and schemes of classification.

The near future promises to differentiate the Linneons still more, and to multiply the number of Jordanons and species in Lotsy’s sense. Artificial hybridization threatens considerably to enlarge the external diversity of forms.

It may be expedient to define even at the present time the multi-formity in Linneons, not by the number of described and possible compositions, but by the number and list of *varietal characters* through which Jordanons differ from each other, not forgetting that separate characters can be dependent on several hereditary factors or genes, involving complicated genotypical formulae. The complete genotypical compositions of Linneons is a problem for the future.

The multitudinous chaos of innumerable forms obliges investigators to look for some way of simplification. The process of differentiation will go on inevitably, adding to the records of existing forms, and giving a true conception of Linneons. But parallel to differentiation it is natural to search for ways of integration of our knowledge of Jordanons and Linneons themselves. If some 130,000 Linneons are difficult to manage for investigation, the work with tens and hundreds of millions of Jordanons will be still more complicated.

As formerly, in the study of dead organic and inorganic worlds, so at the present, the problem before the investigator of the animal and plant world is to explore the regularities in polymorphism, and to establish its classes.

The object of this work

Below is an attempt to integrate the phenomena of polymorphism which we define as “The Law of Homologous Series of Variation”. These regularities were noted by the author during the study of innumerable varieties of cultivated and wild plants.

The ideas expounded below in some parts are not foreign to biological literature. Separate facts of regular variation were known long ago. Naudin noticed them in his classical study of *Cucurbitaceae*. Darwin, who was in general rather the adherer of fortuitous variations in all directions in his Origin and Variation, paid attention to regular variation, which, as he states, “occasionally” happens in plants and animals.

M.J. Duval-Jouve collected a great many data on the variation of wild Linnean species of *Gramineae*, *Juncaceae* and *Cyperaceae* in his paper on “Variations paralleles des types congeneres” published in 1865 in *Bull. De la Ste. Botanique de France*, Vol. XII. His conclusions in some part come near to the statements of our study. De Vries

* Darwin, Variation of Animals and Plants, Part 2; “Analogous or Parallel Variation.”
notices in his *Mutationstheorie* the existence of series of variation. Eimer* in his study of Orthogenesis approached the same subject from a different point of view. Several palaeontologists (Cope, Osborn) noticed regular variation in animals. More recently Saccardo** and Zederbauer*** gave extremely instructive instances of regular variation in fungi and Coniferae.

The detailed study of variation among many different groups, and the great number of new facts permits us to take this subject anew and bring all known facts into the form of a general law to which all organisms are submitted.

**X. General conclusions [p. 86–89]**

Parallelism in varietal polymorphism, and the existence of regularity in differentiation of greater groups as Linneons, genera, and families, is a great help in the study of varieties in self- and cross-fertilized plants and animals. Instead of searching for unknown forms, the investigator can definitely look for, and foresee, forms lacking in a system, by noticing the similarities with the nearest known Linneons and genera. In this respect a biologist places himself in the position of a chemist, who classifies substances according to their place in a system, and creates them through synthesis.

The investigation of polymorphism and the description of new forms become full of scientific meaning and interest. New forms have to fill vacancies in a system. The collections of immense numbers of butterflies and beetles in our museums and herbariums will play a more worthy role in the immediate future than ever before. For a systematist is not a man who knows all the curiosities of nature, but one who grasps the order and sense of it all.

The existing systems of Linneons and varieties ought to be fundamentally changed, and constructed according to a general plan. Instead of occasional characters, which usually determine species and varieties, it would be more rational to follow a general system. The greatest problem of systematists is to build up a general well sustained monotypical system, where similarity and homological series of variation would be considered as the fundamental basis, instead of an indefinite tangle of names impossible to remember. This may seem rather revolutionary for systematists, and it must be done very carefully, in consideration of existing orders. It would be easier to arrange in general systems of minutest systematical units, varieties and races which are as yet almost untouched by systematists. We have tried this for cultivated plants, and have found it expedient. Instead of remembering endless forms, usually named after occasional places of origin or in honour of persons, we have the possibility of studying a system and introducing into it individual additions, where it may be necessary to do


so, for single Linneons and genera. We realize well the size and difficulty of the whole problem. Without a differential work, and without studying in detail, the integral work will be groundless. To integrate it is necessary to differentiate. We know that perhaps a century will pass before botanists and zoologists will create, through collective work, an organized world system; but this way is historically necessary and inevitable.

**Analogy with chemistry**

The above-mentioned analogy of the present day position of the biologist and chemist is deeper than it might seem at first. We have spoken conventionally about characters, colours, hairiness, beardedness, etc. Chemistry says little about the exterior of its substances; it considers the chemical nature of its compounds and their formulas. Numerous chemical substances are required to a harmonious system of combinations of a few elements. The biologist is still far behind. During the last decades, however, genetics has advanced greatly and is rapidly overtaking chemistry – at least the old chemistry of complicated organic compounds. Genetics is creating a laconic language of signs for hereditary factors, determining external characters. The biologist has learned to analyze organisms, and to get a hold on methods for the synthesis of new forms.

The regularities in polymorphism of plants, established by a minute examination of variation in different genera and families which we have examined, can be compared to homologous series of organic chemistry, e.g. carbohydrogen (CH₄, C₂H₄, C₂H₂, ...). Its series of compounds differing from each other, are still characterized by many common properties in reactions, by definite cycles of compounds, by definite reactions of exchange and adhesion. Every single hydrocarbon gives a series of compounds similar to that of other hydrocarbon.

In general, genera (G₁, G₂, G₃, ...) and Linneons (L₁, L₂, L₃, ...) of plants and animals display, in just the same manner, their homologous series of varieties, corresponding to different homologous series of hydrocarbons.

\[
\begin{align*}
G₁L₁ (a+b+c...) & \quad _{\cdots} \quad G₂L₁ (a+b+c...) \\
G₁L₂ (a+b+c...) & \quad _{\cdots} \quad G₂L₂ (a+b+c...) \\
G₁L₃ (a+b+c...) & \quad _{\cdots} \quad G₂L₃ (a+b+c...) \\
L₁a₁, L₁a₂, L₁a₃, & \quad \ldots \\
L₂a₁, L₂a₂, L₂a₃, & \quad \ldots \\
L₃a₁, L₃a₂, L₃a₃, & \quad \ldots 
\end{align*}
\]

Where a₁, a₂, a₃, ... are different characters which distinguish different varieties. The series of forms are strikingly analogous to homologous series of organic chemistry.

Besides their chemical structure, different forms of organized nature are characterized by physical structure, and perhaps it would be better to trace also the analogy of homologous series of plants and animals, with systems and classes of crystallography with definite chemical structure (Crystallo-Chemistry of Fedoroff).

We leave the question, in detail, of these analogies, which is already discussed in literature (Johannsen, Lohmann, Tischler). Further investigations will establish more
precisely the law of homologous variation in plants and animals, and it may be possible to bring the same series into mathematical expression. The variation in form might be reduced to some geometrical scheme.

The problem of the origin of species cannot be separated from the problem of variation. A great many forms are undoubtedly only different combinations of the same genes, some primary types. The study of variation will give us the possibility of establishing these primary types, the fundamental series of variation of organisms.

The idea of the homologous series in variation in its essence is only a development of the general idea of Goethe’s “Metamorphosis of plants”, the idea of the unity in variety of C. Dresser*.

In conclusion, we take the liberty of expressing our strong conviction that the most rational and expedient method of studying the diversity of plants and animals open to breeders of both, even for practical purposes, is through the establishment of parallelism and homologous series of variation.

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Chromosomal and reproductive features of some Oriental and Australasian scale insects (Homoptera, Coccinea)

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Abstract
Fourteen species of scale insects from the families Margarodidae s.l., Pseudococcidae, Eriococcidae, and Coccidae were investigated for the first time in respect to karyotypes, genetic systems, modes of reproduction and general anatomy of the female reproductive system. One of the studied species, Steatococcus samaraius Morrison, 1927, showed hermaphroditic reproduction of the female-like specimens, the other species demonstrated bisexual reproduction with a peculiar “Lecanoid” heterochromatinization of the paternal set of chromosomes in male embryos or thelytocious parthenogenesis. Antonina parazonata Williams, 2004 and Saccharolecanium krugeri (Zehntner, 1897) are recorded here for the first time from Thailand, Antonina vietnamensis Williams, 2004 and Geococcus satellitum Williams, 2004 – for the first time from Laos.

Keywords
scale insects, giant scales, mealybugs, soft scales, felt scales, chromosome number, karyotype, genetic system, reproductive system

Introduction
Here, 14 previously unstudied species from 13 genera of the families Margarodidae s.l., Pseudococcidae, Eriococcidae, and Coccidae are considered in respect of their karyotypes, genetic systems, modes of reproduction, and general anatomy of the female reproductive system. Unusual aberrant genetic systems of scale insects have been reviewed several times previously (e.g., Hughes-Schrader 1948, Nur 1980, Gavrilov 2007, Gavrilov-Zimin et al. 2015) and will not be discussed here. General evolutionary aspects of scale insect reproductive biology and ontogenesis were analyzed in a special monograph (Gavrilov-Zimin 2018a), that can also be used by readers for the clarifying of the terminology and the higher-level taxa system, explored below.

General anatomic types of the female reproductive system in the scale insects were previously reviewed by De Marzo et al. (1990) basing on a few, mainly European species. However, subsequent studies (for example, Gavrilov and Trapeznikova 2007, Gavrilov-Zimin 2012, 2018a), including the present work, support the view of the mentioned authors (l.c.) that the main types of the reproductive system are characteristic of the higher taxa of scale insects (families, subfamilies, tribes).

Material and methods

Material was collected by the author in different years in Thailand, Laos, Malaysia and Indonesia (Sulawesi, Bali, New Guinea). The detailed collecting data are provided below for each species. All numbers with the letter “K” mean unique collecting numbers for both acetoethanol material and Canada balsam slides. All material is deposited at the Zoological Institute, Russian Academy of Sciences (ZIN RAS), St. Petersburg, Russia.

Both the method for the preparation of permanent morphological slides mounted with Canada balsam and the method of squashing the embryonic cells in lactoacetic orcein for chromosome studies were reported, for example, by Danzig and Gavrilov-Zimin (2014).

All figures and photos, excluding the colour ones, were prepared by the author. The colour photos were prepared by the author with a kind help of D.A. Gapon.

Results and discussion

Family Margarodidae s.l.

Steatococcus samarains Morrison, 1927
Figs 1a, 2

Figure 1. Embryonic cells and chromosomes of the studied species (Margarodidae, Pseudococcidae, Eriococcidae). a Steatococcus samaraius (2n = 4) b Antonina parazonata (2n = 30) c A. vietnamensis (2n = 20) d Mollicoccus guadalcanalanus (2n = 10) e Acanthococcus prope onukii (2n = 16) f, g Gossypariella siamensis (2n = 16). g Shows interphase cells of male embryos with a Lecanoid heterochromatinization of the paternal set of chromosomes (arrowed in each cell). Scale bars: 10 μm.

New data. 2n = 4; hermaphroditism: the studied female-like ultimolarvae contain sperm bundles in the ovo-testicles. Early stages of embryogenesis (before anatrepsis) occur inside of ovary; then the eggs are laid in the marsupium, where the embryogen-
Figure 2. General anatomy of the female (or female-like hermaphrodite in *Steatococcus samaraius*) reproductive system in the studied species (Margarodidae, Pseudococcidae, Eriococcidae).

Figure 2. General anatomy of the female (or female-like hermaphrodite in *Steatococcus samaraius*) reproductive system in the studied species (Margarodidae, Pseudococcidae, Eriococcidae).

 Comments. Hermaphroditism is an exceptionally rare phenomenon in Insecta (see, for example, Royer 1975). Up to now hermaphroditic species are known for sure only in the scale insect tribe Iceryini (Margarodidae: Monophlebinae) (Hughes-Schrader 1948, Gavrilov 2007, Gavrilov-Zimin 2018a). Previously, the presence of ovo-testicles in female-like diploid insects has been shown for *Icerya bimaculata* De Lotto, 1959 (Hughes-Shrader 1963), *I. multicicatrices* (Kondo & Unruh, 2009)
Chromosomal and reproductive peculiarities of some scale insects

(Gavrilov-Zimin 2018a: 27, 190) and I. purchasi Maskell, 1879 (Schrader & Hughes-Schrader 1926, Royer 1975). Closely related genus Steatococcus Ferris, 1921 (18 species), which differs from Icerya Signoret, 1876 (45 species) by the presence of peculiar marsupium, was previously almost unstudied in respect of cytogenetics and reproductive biology, excluding the only American species, S. tuberculatus Morrison, 1941. This species was investigated by Hughes-Schrader & Ris (1941) who found that it had 2n = 4 and reproduced bisexually with the appearance of haploid males via facultative parthenogenesis. Here, another species of the genus, S. samaraius, a widely distributed Oriental and Australasian pest, was studied and the same chromosome number, 2n = 4, was discovered. However, males were totally absent in any populations of S. samaraius, inspected by me in the different countries of the Oriental region and seem to have never been reported in the literature. The preparation of the mature females and larvae expectedly revealed a hermaphroditic condition of the reproductive system of S. samaraius. Such a combination of hermaphroditism and haplo-diploidy in closely related species of one genus and even in different populations of the species (as is the case of Icerya purchasi; Schrader & Hughes-Schrader 1926) is a peculiar feature of the tribe Iceryini (Hughes-Shrader 1963, Gavrilov-Zimin 2018a). Some authors (Unruh and Gullan 2007) do not consider Steatococcus as a separate genus and place its species either in the genus Icerya or in another related genus Crypticerya Cockerell, 1895. However, such approach leads to the total overlapping of the generic diagnostic characters and to the practical impossibility of assigning newly described species to a certain genus (see Gavrilov-Zimin 2018a: 174, 184, Gavrilov-Zimin and Stekolshikov 2018).

Family Pseudococcidae

Antonina parazonata Williams, 2004
Figs 1b, 2


New data. 2n = 30; bisexual reproduction with a Lecanoid heterochromatinization of paternal chromosomes in male embryos; complete ovoviviparity. Female reproductive system is similar to that of other studied mealybugs, i.e. with numerous ovarioles located on the paired oviducts, accessory glands attached to the proximal part of the common oviduct, and a spermatheca located at the same place as accessory glands (Fig. 2)

Comments. Special study of cytogenetics and reproductive biology of the genus Antonina Signoret, 1875 and other “legless mealybugs” was done recently (Gavrilov-Zimin 2016). Nine species from 3 genera of legless mealybugs were considered in that paper based on original and literature data and a significant variation of chromosome number was shown: 2n = 10, 12, 16, 20, 22+ Bs, 24, 24 + Bs, and 30. Antonina parazonata, studied here showed 2n = 30 as a species from the related monotypic genus Komodesia Gavrilov, 2016, namely, Komodesia circuliplurima Gavrilov, 2016. For the genus Antonina, such a high chromosome number was revealed for the first time.
A. parazonata was previously known from the type localities in India only. It is the first record of this species for Thailand.

Antonina vietnamensis Williams, 2004
Figs 1c, 2


New data. 2n=20; bisexual reproduction with a Lecanoid heterochromatinization in male embryos; complete ovoviviparity. Female reproductive system is the same type as in A. parazonata (Fig. 2).

Comments. Antonina vietnamensis has the same chromosome number as a closely related Oriental species of the genus, A. diversiglandulosa Gavrilov, 2016.

A. vietnamensis was previously known from the type localities in Vietnam only. It is the first record of the species for Laos.

Geococcus satellitum Williams, 2004
Fig. 2


New data. All studied embryos from 3 available females were unsuitable for chromosomal studies due to numerous yolk inclusions. Eggs are laid in loose ovisac at the stage of anatremesis suggesting incomplete ovoviviparity. Female reproductive system is characterized by an extremely small number of ovarioles and the absence of a spermatheca (Fig. 2).

Comments. Up to now, the genus Geococcus Green, 1902 (14 species) has not been studied in terms of cytogenetics and reproductive biology. This is the case with most other related genera of tribe Rhizoecini (or group of the genus Rhizoecus Künckel d’Herculais, 1878). Diploid chromosome numbers, 8, 10, and 12, are known only for 5 species of Rhizoecus (Danzig & Gavrilov-Zimin, 2015: 428–429); all these species are characterized by a Lecanoid genetic system and bisexual reproduction.

G. satellitum was previously known from the type localities in China and Thailand only. This is the first record of this species for Laos.

Mollicoccus guadalcanalanus Williams, 1960
Figs 1d, 2

Material. K 917, Indonesia, New Guinea, Manokwari, forest near the airport, on leaves of undetermined dicotyledonous herb, 8.XI. 2011, I.A. Gavrilov-Zimin.
New data. 2n = 10; bisexual reproduction with a Lecanoid heterochromatinization in male embryos; eggs are laid in loose ovisac at stage of anatrepsis suggesting incomplete ovoviviparity. Female reproductive system is similar in general details to that of Geococcus satellitum (Fig. 2).

Comments. These are the first cytogenetic and reproductive data for monotypic Australasian genus Mollicoccus Williams, 1960. The diploid number 10 is considered a modal chromosome number for the family Pseudococcidae as a whole (Nur 1980, Gavrilov 2007, Gavrilov-Zimin et al. 2015).

Family Eriococcidae

Acanthococcus prope onukii (Kuwana, 1902)
Figs 1e, 2

Material. K 1513, Thailand, Chiang Mai, slope of Doi Suthep Mt. near the University, on leaves of bamboo, 8.XI.2019, I.A. Gavrilov-Zimin.

New data. 2n = 16; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Eggs are laid in dense wax ovisac at the stage of anatrepsis, i.e. incomplete ovoviviparity is characteristic of the species. Female reproductive system consists of a spermatheca attached at the junction of the oviducts and accessory glands attached at the base of a common oviduct (Fig. 2).

Comments. Only two species of the large genus Acanthococcus Signoret, 1875 have been previously studied cytogenetically, i.e. European A. agropyri (Borhcsenius, 1949) and A. insignis (Newstead, 1891), both with 2n = 16 (Gavrilov 2004, 2007). [Nota bene! The studied specimens differ from a common Acanthococcus onukii (=Anophococcus onukii) in the conical setae with blunt apices. The generic name Anophococcus Balachowsky, 1954 is considered here as a synonym of Acanthococcus (synonymized by Danzig 1980: 205)].

Gossypariella siamensis (Takahashi, 1942)
Figs 1f–g, 2, 5a

Material. K 1521, Thailand, Chiang Mai, city street near the University, on branches and twigs of an undetermined dicotyledonous tree, probably Ficus sp., 9.XI.2019, I.A. Gavrilov-Zimin.

New data. 2n = 16; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Complete ovoviviparity. Female reproductive system is similar with that in the previous species, but accessory glands are located in the middle part of the common oviduct (Fig. 2).

Comments. The genus Gossypariella Borchsenius, 1960 includes 4 species distributed in the Oriental region. G. siamensis is the first species of the genus studied cytogenetically.
Family Coccidae

*Coccus viridis* (Green, 1889)
Figs 3a, 4

**Material.** K 939, Indonesia, Bali, mountain forest above Lake Buyan, about 1200 m altitude, on leaves of an undetermined tree, 13.XI. 2011, I.A. Gavrilov-Zimin.

**New data.** 2n = 18; there is no heterochromatinization (and thus no Lecanoid system) in all 50 studied embryos from 3 females, no sperm in spermathecae and no males in the studied population; so, the thelytocous reproduction is characteristic of this species. Complete ovoviviparity. Female reproductive system is of the usual for the soft scales type (Fig. 4).

**Comments.** The type species of the genus, *Coccus hesperidum* Linnaeus, 1758, shows 2n =14 and different variants of parthenogenesis (Thomsen 1927, 1929, Nur 1979), whereas two other studied species, *C. longulus* (Douglas, 1887) and *Coccus* sp., were reported by Moharana (1990) as having 2n=18, but without any comments on genetic system and reproductive peculiarities. All other (more than 110) species of the genus *Coccus* Linnaeus, 1758, are still unstudied cytogenetically.

*Discochiton expansum* (Green, 1896)
Figs 3b, 4

**Material.** K 1121, Thailand, Malay Peninsula, Khao Lak, forest above the city, on leaves of an undetermined bush, 8.XI. 2013, I.A. Gavrilov-Zimin.

**New data.** 2n = 18; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Complete ovoviviparity. Female reproductive system has the usual structure, but accessory glands are poorly visible (Fig. 4).

**Comments.** The recently erected genus *Discochiton* Hodgson & Williams, 2018 comprises 21 species, and *D. expansum* is the first species of the genus studied cytogenetically.

*Drepanococcus chiton* (Green, 1909)
Fig. 4

**Material.** K 864, Indonesia, New Guinea, vicinity of Jayapura, Entrop, on stem of a dicotyledonous herb, 30.X. 2011, I.A. Gavrilov-Zimin.

**New data.** There were no embryonic cells suitable for chromosomal analysis in the available material. The reproduction is bisexual with a Lecanoid heterochromatinization in male embryos. All studied females contained embryos at early stages of embryogenesis (up to anatrepsis). Female reproductive system has the usual structure (Fig. 4).

**Comments.** The only other species of the genus, *D. cajani* (Maskell, 1891), was previously studied cytogenetically by Moharana (1990), who reported 2n = 18 with no other comments on the species.
**Figure 3.** Embryonic cells and chromosomes of the studied species (Coccidae). a *Coccus viridis* (2n = 18) b *Discochiton expansum* (2n = 18) c *Luzulaspis australis* (2n = 18) d *Megalocryptes buteae* (2n = 18) e *Megapulvinaria maxima* (2n = 20) f *Saccharolecanium krugeri* (2n = 18). Scale bars: 10 μm.

**Luzulaspis australis** (Maskell, 1894)
Figs 3c, 4

**Material.** K 861, Indonesia, New Guinea, vicinity of Jayapura, Entrop, under leaf sheathes of a Poaceae grass, 30.X. 2011, I.A. Gavrilov-Zimin.

**New data.** 2n = 18; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. The eggs are laid in a long wax ovisac at the stage of late anatrepsis; i.e. incomplete ovoviviparity is characteristic of the species. Female reproductive system has the usual structure (Fig. 4).

**Comments.** The genus *Luzulaspis* Cockerell, 1902 comprises about 25 species, but only one of them, European *L. dactylis* Green, 1928, has been thus far studied cytogenetically (Gavrilov 2004). This species was found to have 2n = 18 and a bisexual reproduction with a Lecanoid heterochromatinization as well as presently studied Australasian *L. australis*.
Figure 4. General anatomy of the female reproductive system in the studied species (Coccidae).

*Megalocryptes buteae* Takahashi, 1942  
Figs 3d, 4, 5c

**Material.** K 1536, Thailand, Pai, on twigs of an undetermined dicotyledonous tree, 13.XI.2019, I.A. Gavrilov-Zimin.

**New data.** $2n = 18$; there is no heterochromatinization in all 72 studied embryos from 3 females, no sperm in spermathecae and no males in the population suggesting thus thelytokous reproduction. Female reproductive system is distinguished by unusually long and thin lateral oviducts (Fig. 4). Cleavage divisions in the egg start just prior to oviposition.

**Comments.** These are the first cytogenetic and reproductive data for the small Oriental genus *Megalocryptes* Takahashi, 1942 which comprises two species only.
Figure 5. Females of some species on twigs of host plants. a Gosypariella siamensis b Megapulvinaria maxima c Megalocryptes buteae (with a colony of Kerria sp. at the background) d Saccharolecanium krugerii.
Megapulvinaria maxima (Green, 1904)
Figs 3e, 4, 5b


New data. 2n = 20; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Incomplete ovoviviparity: embryogenesis (until the late anatrepsis) partially occurs inside of the mother’s body. Female reproductive system has the usual structure (Fig. 4).

Comments. Different European members of the tribe Pulvinariini have been previously studied cytogenetically (Gavrilov 2007, Gavrilov and Trapeznikova 2008). Four Oriental species from the genera Chloropulvinaria Borchsenius, 1952, Pseudopulvinaria Atkinson, 1889 and Pulvinaria Targioni Tozzetti, 1866 were studied by Moharana (1990), who reported chromosome numbers with no comments or details. M. maxima is the first species of the genus Megapulvinaria Yang, 1982 studied in terms of chromosome number; the karyotype 2n = 20 is found for the first time in the tribe Pulvinariini in general.

Saccharolecanium krugeri (Zehntner, 1897)
Figs 3f, 4, 5d

Material. K 1368, Thailand, vicinity of Chiang Rai, forest above the Mae Fah Luang University, under the leaf sheathes of Saccharum sp., 8.VI.2017, I.A. Gavrilov-Zimin.

New data. 2n = 18; bisexual reproduction with a Lecanoid heterochromatinization in male embryos. Complete ovoviviparity. Female reproductive system has the usual structure (Fig. 4).

Comments. These are the first cytogenetic and reproductive data for the small Oriental genus Saccharolecanium Williams, 1980, which comprises two species only. S. krugeri is noted here for the first time for the territory of Thailand.

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References


The highly rearranged karyotype of the hangingfly
*Bittacus sinicus* (Mecoptera, Bittacidae):
the lowest chromosome number in the order

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Abstract

Cytogenetic features of the hangingfly *Bittacus sinicus* Issiki, 1931 were investigated for the first time using C-banding and DAPI (4′,6-diamidino-2-phenylindole) staining. The karyotype analyses show that the male *B. sinicus* possesses the lowest chromosome number (*2n* = 15) ever observed in Mecoptera, and an almost symmetric karyotype with *MCA* (Mean Centromeric Asymmetry) of 12.55 and *CVCL* (Coefficient of Variation of Chromosome Length) of 19.78. The chromosomes are either metacentric or submetacentric with their sizes decreasing gradually. Both the C-banding and DAPI+ patterns detect intermediate heterochromatin on the pachytene bivalents of *B. sinicus*, definitely different from the heterochromatic segment at one bivalent terminal of other bittacids studied previously. The male meiosis of *B. sinicus* is chiasmate with two chiasmata in metacentric bivalents and one in the submetacentric bivalent. The sex determination mechanism is X0(♂), which is likely plesiomorphic in Bittacidae. Two alternative scenarios of karyotype origin and evolution in *Bittacus* Latreille, 1805 are discussed.

Keywords

C-banding technique, chromosome rearrangement, cytogenetics, DAPI, evolution, Holometabola, meiosis

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Introduction

Bittacidae is the second largest family of Mecoptera, and currently consists of over 200 species in 18 genera in the world (Zhang et al. 2020). The adults of Bittacidae comprise an exclusive group that possesses three pairs of elongated raptorial legs with a single claw at pretarsus and adopts a predacious feeding strategy (Bornemissa 1966; Byers and Thornhill 1983; Penny 2006; Tan and Hua 2008; Ma et al. 2014). They are commonly known as hangingflies because between flights they are unable to stand on a surface but hang themselves from the edges of leaves or twigs using the prehensile foretarsi (Thornhill 1977; Tan and Hua 2008). *Bittacus* Latreille, 1805 is the largest and most widespread genus of Bittacidae, and comprises more than 2/3 species of the family recorded from all zoogeographical regions (Penny and Byers 1979). Owing to considerable morphological variations (Lambkin 1988; Chen et al. 2013) and complicated distribution patterns (Penny 1975; Li and Ren 2009), the evolutionary relationship within this genus remains largely unknown to date.

Chromosomes of eukaryotic organisms may carry crucial information related to the species diversification and evolution (Gokhman and Kuznetsova 2006; Noor et al. 2007; Faria and Navarro 2010). The variations of chromosome number reflect the result of complicated chromosomal rearrangements and may help reveal the evolutionary relationships of sibling species (White 1974; Lukhtanov et al. 2005; Kandul et al. 2007; Faria and Navarro 2010). The chromosomal morphology may provide substantial information related to structural rearrangements, which may contribute to the increased level of divergence among taxa (Rieseberg and Burke 2001; Navarro and Barton 2003; Butlin 2005). Such studies have been well documented in many insect groups, including aquatic bugs (Stoianova et al. 2020), psyllids (Nokkala et al. 2019), bush crickets (Kociński et al. 2018), beetles (Dutrillaux and Dutrillaux 2019), butterflies (Dincă et al. 2011), warrior wasps (Menezes et al. 2019), and ants (Pereira et al. 2018). In Bittacidae, however, the cytogenetic information is poorly documented, with only six species reported to date (Matthey 1950; Atchley and Jackson 1970; Miao and Hua 2017, 2019).

According to the limited cytogenetic data available, the chromosome number varies extensively in Bittacidae (Matthey 1950; Atchley and Jackson 1970; Miao and Hua 2017, 2019). It is $2n = 25$ in *B. italicus* (Müller, 1766), $2n = 27$ in *B. flavidus* Huang et Hua, 2005, $2n = 29$ in *B. pilicornis* Westwood, 1846, $2n = 31$ in *B. stigmaterus* Say, 1823, $2n = 35$ in *B. planus* Cheng, 1949, and $2n = 41$ in *Terrobittacus implicatus* (Huang et Hua in Cai et al., 2006). Each species examined has a distinctive karyotype, which represents an important diagnostic feature in Bittacidae and provides useful information on the evolutionary relationship of Mecoptera (Miao and Hua 2017, 2019).

In this paper, we present for the first time information on the karyotype and male meiosis of the hangingfly *Bittacus sinicus* Issiki, 1931, attempting to enrich our knowledge of the chromosome evolution of *Bittacus* and to contribute to the cytogenetic data for a better understanding of the evolutionary history of Bittacidae.
Materials and methods

Adult collecting

Adults of *B. sinicus* (Fig. 1A) were collected from Shimian County (29°03'00"N, 102°21'00"E, elev. 1800–1890 m), Sichuan Province in China from July to August in 2016 and Paomashan (30°02'36"N, 101°57'33"E, elev. 2600 m), Sichuan Province in China in late July 2018, respectively.

Insect rearing

Live adults were reared in screen-wired cages (40 × 60 × 60 cm) containing twigs and leaves of plants and moist absorbent cotton (Miao and Hua 2019). Eggs, larvae and pupae were incubated and reared in plastic containers with humid humus. Live flies and frozen pupae of *Musca domestica* Linnaeus, 1758 (Diptera, Muscidae) were provided as food for the adults and larvae, respectively. Temperature was kept at 16 ± 2 °C for larvae, 21 ± 2 °C for pupae, and 23 ± 2 °C for adults. Relative humidity was maintained at 75 % ± 10 % (Miao and Hua 2017).

Cytogenetic analyses

Chromosome spreads were prepared using the testes of larvae and pupae following Imai et al. (1988). The mitotic metaphase and early stages of meiosis were obtained from males of the third and fourth (last) instar larvae, and the male meiosis I/II mainly from young pupae. Totally 66 larvae (46 from Shimian County and 20 from Paomashan) and 12 pupae (nine from Shimian County and three from Paomashan) of *B. sinicus* were used for chromosome preparations.

C-banding was obtained using the same technique as in Miao and Hua (2019). The fluorochrome DAPI (4’,6-diamidino-2-phenylindole) staining was performed to characterize the DAPI+ heterochromatin (the shiny blue regions rich in AT bases) on chromosomes, following Rebagliati et al. (2003).

Photographs were taken with a Nikon DS-Fil digital camera mounted on a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). The fluorescence signals were observed with a UV filter (330–385 nm).

Statistical analyses

Five spermatogonial cells with well-spread chromosomes at mitotic metaphase were used to statistically analyze the chromosomes of *B. sinicus* following the procedures of Miao and Hua (2017). The captured images were quantified using the NIS-Element
D 3.22 software (Nikon, Tokyo, Japan). The chromosomal morphology was determined based on the arm ratio where chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or telocentric (t) (Levan et al. 1964). The following features of chromosomes were measured: absolute chromosome length ($AL$), long arm length ($L$), short arm length ($S$), arm ratio ($r = L/S$), centromeric index ($i = S \times 100/AL$), and relative chromosome length ($RL$) of each chromosome ($RL = AL \times 100/\sum AL$). The evaluated data are presented as mean ± SD.

The karyotype asymmetry is represented by two components, the heterogeneous degree of chromosome lengths (interchromosomal asymmetry) and the prevalence of telo-/subtelocentric chromosomes (intrachromosomal asymmetry) (Astuti et al. 2017). Two separate parameters were assessed, i.e. Coefficient of Variation of Chromosome Length ($CV_{CL}$) (Paszko 2006) and Mean Centromeric Asymmetry ($MCA$) (Peruzzi and Eroğlu 2013).

**Results**

**Karyology**

The males of *B. sinicus* possess $2n = 15$ (Fundamental Number $FN = 30$), with the karyotype formula of $13\ m + 2\ sm$ (Fig. 1B, C).

The $AL$ ranges from 7.47 ± 0.26 to 3.72 ± 0.05 μm, and the $RL$ from 8.43 ± 0.29 to 4.20 ± 0.05. Autosomal bivalents decrease gradually in size, and the sex chromosome (X) is the smallest of the set. The total length of all chromosomes is 88.65 μm (Table 1).

**Table 1.** Morphometric analyses of the chromosomes of *Bittacus sinicus* based on five spermatogonial cells from a male larva.

<table>
<thead>
<tr>
<th>Pair No.</th>
<th>$AL \pm SD$ (μm)</th>
<th>$RL \pm SD$</th>
<th>$L \pm SD$ (μm)</th>
<th>$S \pm SD$ (μm)</th>
<th>$(L - S)/(L + S)$</th>
<th>$i$</th>
<th>$r$</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.98 ± 0.06</td>
<td>4.49 ± 0.07</td>
<td>2.62 ± 0.05</td>
<td>1.36 ± 0.18</td>
<td>0.32</td>
<td>34.11</td>
<td>1.93</td>
<td>sm</td>
</tr>
<tr>
<td></td>
<td>4.29 ± 0.02</td>
<td>4.83 ± 0.03</td>
<td>2.75 ± 0.03</td>
<td>1.53 ± 0.02</td>
<td>0.29</td>
<td>35.74</td>
<td>1.80</td>
<td>sm</td>
</tr>
<tr>
<td>2</td>
<td>4.97 ± 0.24</td>
<td>5.61 ± 0.27</td>
<td>2.67 ± 0.10</td>
<td>2.30 ± 0.10</td>
<td>0.07</td>
<td>46.27</td>
<td>1.16</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>5.38 ± 0.04</td>
<td>6.07 ± 0.05</td>
<td>3.18 ± 0.22</td>
<td>2.20 ± 0.15</td>
<td>0.18</td>
<td>40.84</td>
<td>1.45</td>
<td>m</td>
</tr>
<tr>
<td>3</td>
<td>6.00 ± 0.17</td>
<td>6.77 ± 0.19</td>
<td>3.45 ± 0.05</td>
<td>2.55 ± 0.12</td>
<td>0.15</td>
<td>42.55</td>
<td>1.35</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>6.12 ± 0.08</td>
<td>6.90 ± 0.09</td>
<td>3.35 ± 0.03</td>
<td>2.76 ± 0.06</td>
<td>0.10</td>
<td>45.19</td>
<td>1.21</td>
<td>m</td>
</tr>
<tr>
<td>4</td>
<td>6.45 ± 0.08</td>
<td>7.27 ± 0.09</td>
<td>3.48 ± 0.05</td>
<td>2.97 ± 0.12</td>
<td>0.08</td>
<td>46.00</td>
<td>1.17</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>6.50 ± 0.21</td>
<td>7.33 ± 0.24</td>
<td>3.68 ± 0.22</td>
<td>2.83 ± 0.13</td>
<td>0.13</td>
<td>43.45</td>
<td>1.30</td>
<td>m</td>
</tr>
<tr>
<td>5</td>
<td>6.59 ± 0.15</td>
<td>7.44 ± 0.17</td>
<td>3.49 ± 0.13</td>
<td>3.10 ± 0.29</td>
<td>0.06</td>
<td>47.08</td>
<td>1.12</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>6.60 ± 0.15</td>
<td>7.44 ± 0.17</td>
<td>3.49 ± 0.11</td>
<td>3.11 ± 0.20</td>
<td>0.06</td>
<td>47.16</td>
<td>1.12</td>
<td>m</td>
</tr>
<tr>
<td>6</td>
<td>6.92 ± 0.64</td>
<td>7.80 ± 0.72</td>
<td>3.93 ± 0.09</td>
<td>2.99 ± 0.12</td>
<td>0.14</td>
<td>43.18</td>
<td>1.32</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>6.62 ± 0.61</td>
<td>7.46 ± 0.69</td>
<td>3.56 ± 0.26</td>
<td>3.05 ± 0.17</td>
<td>0.08</td>
<td>46.14</td>
<td>1.17</td>
<td>m</td>
</tr>
<tr>
<td>7</td>
<td>7.04 ± 0.11</td>
<td>7.94 ± 0.12</td>
<td>3.92 ± 0.09</td>
<td>3.12 ± 0.01</td>
<td>0.11</td>
<td>44.31</td>
<td>1.26</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>7.47 ± 0.26</td>
<td>8.43 ± 0.29</td>
<td>3.97 ± 0.26</td>
<td>3.50 ± 0.25</td>
<td>0.06</td>
<td>46.90</td>
<td>1.13</td>
<td>m</td>
</tr>
<tr>
<td>8 (X)</td>
<td>3.72 ± 0.05</td>
<td>4.20 ± 0.05</td>
<td>1.98 ± 0.13</td>
<td>1.75 ± 0.09</td>
<td>0.06</td>
<td>46.94</td>
<td>1.13</td>
<td>m</td>
</tr>
</tbody>
</table>

Notes: $AL$, absolute chromosome length (actual length of chromosomes); $RL$, relative chromosome length ($RL = AL/\sum AL$); $L$, long arm length; $S$, short arm length; $i$, centromeric index ($i = S \times 100/AL$); $r$, arm ratio ($r = L/S$); m, metacentric; sm, submetacentric.
The $M_{CI}$ is calculated as 12.55 and the $CV_{CI}$ is 19.78. The relatively low degrees of both intrachromosomal and interchromosomal asymmetries indicate that the karyotype of $B. sinicus$ is almost symmetric.

**Banding patterns**

Conspicuous heterochromatin was observed on the meiotic bivalents of $B. sinicus$ after C-banding and DAPI staining (Fig. 2). Both treatments reveal that the autosomal bivalents exhibit intermediate heterochromatin. The sex chromosome is heteropycnotic and totally heterochromatic at the early pachytene (Fig. 2A, C), but becomes isopycnic with two heterochromatic dots later (Fig. 2B, D).

**Chiasmate male meiosis**

The synaptic attraction between the homologues terminates from the pachytene to diplo- tene. The early diplotene appears to be the diffuse stage, which can be interpreted as uncondensed bivalents connected by chiasmata (Fig. 3A). During this stage, the intermediate region of the bivalents is heavily stained and arranged dispersedly, while the remaining bivalents are weakly stained and are often overlooked consequently. The chromosomes move apart in repulsion and are held together only at exchange points, which appear as visible chiasmata in the diplotene stage (Fig. 3B). Metacentric bivalents exhibit two terminal chiasmata and look like large rings, whereas the submetacentric one usually
contains only one terminal chiasma at the long-arm side as a long rod-shape. Chiasmata can be clearly visible after some condensation of the chromosomes at diakinesis (Fig. 3C). In *B. sinicus* the mean chiasma count per cell was 13.2 (50 cells, ranging from 13 to 14).

Bivalents assemble at the equatorial plate in metaphase I (Fig. 3D) and become oriented with their centromeres poleward (Fig. 3E). In *B. sinicus* the rod-shaped bivalent is bound by one chiasma at one arm end (asterisk in Fig. 3F), whereas the ring-shaped bivalents have both arms bound by chiasmata. The autosomal bivalents separate into dyads, whereas the X univalent moves undividedly to one pole (Fig. 3G–I), indicating that *B. sinicus* has the initial-/prereductional meiosis. Each dyad consists of two divergent chromatids associated only in the regions proximal to the centromere (Fig. 3G,

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**Figure 2.** Pachytene bivalents of *Bittacus sinicus*, stained with C-banding (**A, B**) and DAPI (**C, D**) **A, C** early pachytene, showing the intermediate heterochromatin on bivalents and the heteropycnotic sex chromosome (arrowhead) **B, D** late pachytene, showing the sex chromosome with a dot-shaped heterochromatic block (arrowheads). Arrows point to the intermediate heterochromatin. Scale bars: 10 μm.
Karyotype of the hangingfly Bittacus sinicus

H). Both submetacentric and metacentric dyads are four armed with a double V-shape in anaphase I. The dyads reach the opposite poles and fuse into an indistinguishable mass of chromatin in telophase I (Fig. 3I).

Meiosis II takes place immediately after the first meiotic division. The movement of the X univalent toward only one pole at anaphase I leads to the formation of two classes of nuclei (Fig. 4A, B). The sister chromatids of each dyad are widely splayed, but are held together at the centromere in prometaphase II (Fig. 4C). The centromeric

Figure 3. Meiosis I of Bittacus sinicus A diffuse diplotene with the condensed sex chromosome and decondensed bivalents B diplotene, showing the bivalents are held together only at exchange points (arrows) C diakinesis, showing the evident chiasmata (arrows) D bivalents assembling at the equatorial plate in metaphase I (polar view) E, F metaphase I in side view, showing the ring-shaped bivalents with two chiasmata and rod-shaped bivalent with one terminal chiasma (asterisk) G anaphase disjunction, showing the divided bivalents and the undivided sex chromosome H anaphase I, showing the chromosome number of B. sinicus is 2n = 15 I telophase I. Arrowheads show the sex chromosome. Scale bars: 10 μm.
cohesion between the two sister chromatids is removed in anaphase II, and the sister chromatids are pulled apart by microtubules attached to the kinetochore (Fig. 4D).

**Sex chromosome system**

The diploid somatic chromosome number \((2n)\) is reduced to the haploid gametic chromosome number \((n)\) during the first meiosis. Both the autosomes and the sex chromosome exhibit pre-reductional type of meiosis. The haploid chromosome numbers are different between the two daughter nuclei with \(n = 7 + X\) (Fig. 4A) and \(n = 7\) (Fig. 4B), indicating an X0 sex system of the male *B. sinicus*. 

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*Figure 4.* Meiosis II of *Bittacus sinicus* **A**, **B** the secondary spermatocytes: **A** with \(n = 8\) **B** with \(n = 7\) **C** prometaphase II, showing the striking repulsion between the sister chromatids of each dyad chromosome **D** anaphase II, showing the separation of sister chromatids. Arrowheads show the sex chromosome. Scale bars: 5 μm.
Discussion

The present study is the first attempt to investigate the karyotype and male meiosis of *B. sinicus*. As in other bittacids studied previously, *B. sinicus* has the chiasmate meiosis and the X0(♂) sex determination mechanism, which are likely the plesiomorphies in Bittacidae (Matthey 1950; Archley and Jackson 1970; Miao and Hua 2017, 2019).

*Bittacus sinicus* has the lowest chromosome number $2n = 15$ ever observed in Mecoptera. Previously, $2n = 17$ chromosomes recorded for *Nannochorista dipteroides* Tillyard, 1917 (Nannochoristidae) was considered the lowest number reported for this order (Bush 1966). Despite limited chromosome data available, the chromosome number exhibits considerable variations among the families of Mecoptera, from $2n = 15$ to 41 in Bittacidae, $2n = 19$ to 31 in Boreidae (Cooper 1951, 1974), $2n = 17$ to 27 in Nannochoristidae (Bush 1966), and $2n = 35$ to 47 in Panorpidae (Naville and Beaumont 1934; Ullerich 1961; Archley and Jackson 1970; Xu et al. 2013; Miao et al. 2017, 2019).

In Bittacidae, each species examined has a distinctive karyotype, and the two genera (*Bittacus* and *Terrobittacus* Tan et Hua, 2009) investigated are distinguishable cytogenetically. *Bittacus* has relatively low chromosome numbers and symmetric karyotypes, while *Terrobittacus* has a higher chromosome number and less symmetric karyotype (Miao and Hua 2017), suggesting that the chromosomal changes may have participated in the lineage differentiation of Bittacidae.

Interestingly, the sex chromosome is the smallest element in the karyotype of *B. sinicus*, but is larger than the majority of autosomes in other bittacids studied (Miao and Hua 2017, 2019). Therefore, we speculate that autosome-autosome fusions may contribute to the karyotype formation in *B. sinicus*. Similar rearrangements are also suggested for some recently differentiated species of the scorpionflies Panorpidae (Miao et al. 2019). A notable example is *Neopanorpa lipingensis* Cai et Hua, 2009, which has a distinct chromosome number of $2n = 33$, not $2n = 41$ found in most members of *Neopanorpa* van der Weele, 1909, indicating that fusion events occurred at least eight times among the autosomes.

The C-banding pattern of *B. sinicus* is represented by intermediate blocks on pachytene bivalents and is definitely different from the heterochromatic segment at one bivalent terminal in other bittacids (Archley and Jackson 1970; Miao and Hua 2017, 2019), implying that inversions may participate in the changes of chromosome morphology.

Conspicuous bands are detectable on pachytene bivalents using the DAPI staining. In general, the terminal DAPI⁺ (AT-rich) heterochromatin at one side of a bivalent is the most frequent pattern, which has been observed in the majority of Panorpidae and Bittacidae investigated (Miao and Hua 2017, 2019; Miao et al. 2019). In *B. sinicus*, however, the DAPI⁺ bands are present in the intermediate regions of all bivalents (Fig. 2C, D). Bivalents with intermediate DAPI⁺ heterochromatin were also found in the species of *Neopanorpa* and were considered as important evidence for the evolutionary reduction of chromosome number in Panorpidae (Miao et al. 2019).

Two alternative hypotheses (fission and fusion) can explain the karyotype formation in the genus *Bittacus*. The fission hypothesis assumes that the cytogenetic features of *B. sinicus* are primitive with a low chromosome number, relatively large autosomes and reduced heterochromatin. The karyotype changes of *Bittacus* (Miao and Hua
are similar to those of ants and wasps, in which the centric fissions tend to increase the chromosome number and accumulate chromatin (mainly heterochromatin) (Imai et al. 1986, 1994, 2001).

Alternatively, the fusion hypothesis may also explain the karyotype variations found in *Bittacus*. The karyotype of *B. sinicus* is considered the derived condition and is shaped by Robertsonian translocations of acrocentric chromosomes and/or reciprocal translocations between meta-/submetacentric and acrocentric ones, which are generated by pericentric inversions. During the translocation events, small centromeric chromosomes (in addition to the final fused chromosomes) may be produced and lost within a few cell cycles. Such scenarios may explain the elimination of centromeres and heterochromatin toward the *B. sinicus* karyotype, and has been suggested for many monocentric organisms, such as the plant *Arabidopsis thaliana* (Linnaeus, 1758) (Lysak et al. 2006), the flatworm *Aspidogaster limacoides* Diesing, 1834 (Bombarová et al. 2015), the pangolin *Manis javanica* (Desmarest, 1822) (Nie et al. 2009), the mouse *Akodon* Meyen, 1833 (Ventura et al. 2009), the grasshopper *Ronderosia* Cigliano, 1997 (Orthoptera, Acrididae) (Castillo et al. 2019), the beetle *Dichotomius* Hope, 1838 (Coleoptera, Scarabaeidae) (Cabral-de-Mello et al. 2011), and the ants Myrmicinae (Cardoso et al. 2014). Based on the phylogeny of the Chinese Bittacidae (YM, unpublished data), we speculate that the cytogenetic features observed in *B. sinicus* may be derived conditions, including the low number of chromosomes, relatively large sizes of autosomes and the intermediate distribution of heterochromatin.

Chromosome rearrangements are proposed as an important driving force of diversification since they lead to speciation via formation of reproductive incompatibility or recombination suppression (Navarro and Barton 2003; Ayala and Coluzzi 2005; Butlin 2005; Kandul et al. 2007; Brown and O’Neill 2010; Kirkpatrick 2010; Mills and Cook 2014). According to the models of chromosomal speciation, there is an increasing level of divergence near rearrangement breakpoints, which tend to accumulate alleles involved in the reproductive isolation (Coghlan et al. 2005; Faria and Navarro 2010). In *Bittacus*, the cytogenetic data available indicate that the chromosomal evolution involves progressive changes in chromosome number and karyotype structure. However, it remains unclear whether these chromosomal rearrangements are an integral component and driving force of the speciation process or they are established later, after speciation is completed. Further investigations of additional species, combined with molecular phylogeny and fluorescent in situ hybridization (telomere and 18S rDNA probes), are needed to shed more light on this issue.

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References


Comparative FISH-mapping of TTAGG telomeric sequences to the chromosomes of leafcutter ants (Formicidae, Myrmicinae): is the insect canonical sequence conserved?

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Abstract

Telomeric sequences are conserved across species. The most common sequence reported among insects is (TTAGG)ₙ, but its universal occurrence is not a consensus because other canonical motifs have been reported. In the present study, we used fluorescence in situ hybridization (FISH) using telomeric probes with (TTAGG)₆ repeats to describe the telomere composition of leafcutter ants. We performed the molecular cytogenetic characterization of six Acromyrmex Mayr, 1865 and one Atta Fabricius, 1804 species (Acromyrmex ambiguus (Emery, 1888), Ac. crassispinus (Forel, 1909), Ac. lundii (Guérin-Mèneville, 1838), Ac. nigrosetosus (Forel, 1908), Ac. rugosus (Smith, 1858), Ac. subterraneus subterraneus (Forel, 1893), and Atta sexdens (Linnaeus, 1758)) and described it using a karyomorphometric approach on their chromosomes. The diploid chromosome number 2n = 38 was found in all Acromyrmex species, and the karyotypic formulas were as follows: Ac. ambiguus 2K = 14M + 12SM + 8ST + 4A, Ac. crassispinus 2K = 12M + 20SM + 4ST + 2A, Ac. lundii 2K = 10M + 14SM + 10ST + 4A, Ac. nigrosetosus 2K = 12M + 14SM + 10ST + 2A, and Ac. subterraneus subterraneus 2K = 14M + 18SM + 4ST + 2A. The exact karyotypic formula was not established for Ac. rugosus. FISH analyses revealed the telomeric regions in all the chromosomes of the species studied in the present work were marked by the (TTAGG)₆ sequence. These results reinforce the premise that Formicidae presents high homology between their genera for the presence of the canonical sequence (TTAGG)ₙ.
Keywords
evolution, FISH, insects, leafcutter ants, telomere

Introduction

Cytogenetic studies have been performed on more than 750 ant species, most of which describe only the chromosome number and morphology (Lorite and Palomeque 2010; Cardoso et al. 2018a). However, the cytogenetic information available so far represents less than 5% of the known ant species. Formicidae is very diverse with respect to both karyotype and species. The subfamily Myrmicinae comprises more than 400 species with established karyotypes and haploid chromosome counts varying from $n = 2$ to $n = 35$ (Cardoso et al. 2018a). Myrmicinae includes the leafcutter ants in the genera *Atta* Fabricius, 1804 to *Acromyrmex* Mayr, 1865 that occur exclusively in the Neotropical region and are extremely important herbivores in the habitats that they occupy. They cut thousands of fresh plant pieces that are transported to nests and this habit is essential for cycling soil nutrients, mainly carbon (Farji-Brener and Ghermandi 2008). In some cases, *Atta* and *Acromyrmex* are considered agricultural pests due to the economic damages caused by their habit of cutting green leaves; therefore, most studies usually focus on their ecology, geographic distribution, and population control (Loeck et al. 2003). However, both genera need a systematic revision and a complete picture of their unclear phylogenetic relationships.

The genus *Atta* includes 17 species (Bolton 2020), of which five have an established karyotype. All species present the diploid chromosome number, $2n = 22$, and the karyotype formula, $2K = 18M + 4A$, except for *Atta robusta* Borgmeier, 1939, which has the formula $2K = 18M + 2SM + 2ST$ (reviewed in Cardoso et al. 2018a). The genus *Acromyrmex* has 34 species and 29 subspecies that are currently recognized (Bolton 2020), it has the diploid chromosome number $2n = 38$ and its karyotype formula is variable (Barros et al. 2016; reviewed in Cardoso et al. 2018a). The exceptions in the genus are *Acromyrmex ameliae* de Souza, Soares & Della Lucia, 2007, that has $2n = 36$ (reviewed in Cardoso et al. 2018a) and *Acromyrmex striatus* (Roger, 1863) which presents $2n = 22$ (Cristiano et al. 2013). The only species whose karyotype has been characterized by morphometric analyses so far is *Ac. striatus* (Cristiano et al. 2013). Such chromosomal features are essential for understanding chromosomal variants and the possible genetic barriers among phylogenetic groups (Cardoso et al. 2018b). *Ac. striatus* is a key species within the evolutionary history of leafcutter ants because molecular analyses and its karyotype establishment resulted in reclassification of *Acromyrmex* as paraphyletic. Although *Ac. striatus* shares the characteristics of both *Acromyrmex* and *Atta*, it presents peculiarities such as its karyotype formula $2K = 20M + 2SM$, indicating that *Ac. striatus* should be better classified as a genus distinct from its sibling leafcutter ants (Cristiano et al. 2013).

Karyo-evolutionary pathways can be accurately established from molecular analyses by means of fluorescence in situ hybridization (FISH), a chromosomal mapping...
technique that allows identification of specific genomic regions through hybridization of fluorescent probes to the genetic material (Speicher and Carter 2005). Probe origin may range from single or repetitive sequences to large genomic sequences and probes from telomeric repeating regions are commonly applied in cytogenetic studies (Micolino et al. 2019a, b, 2020; Travenzoli et al. 2019). Telomeres are located at terminal portions of chromosomes, which are enriched with repetitive bases of adenine (A), guanine (G), and thymine (T) and the number of repeated base pairs can be extremely conserved among some taxonomic groups (Blackburn 1991; Zakian 1995). Four different telomeric sequences have been identified in Insecta, but the pentanucleotide region (TTAGG)$_n$ is present in most insects (Okazaki et al. 1993; Sahara et al. 1999). Thus, it is presumed that this motif is derived from a common ancestor and is therefore homologous among the class orders (Vítková et al. 2005). However, many Hymenoptera families do not present the sequence in their chromosomes (Menezes et al. 2017), whereas some families have several species that show telomeric regions marked by the presence of (TTAGG)$_n$ or the vertebrate canonical repetition (TTAGGG)$_n$, as in the case of Apidae (Sahara et al. 1999), Formicidae (Okazaki et al. 1993; Meyne et al. 1995; Lorite et al. 2002; Wurm et al. 2011) and Tenthrerinidae, which has two species presenting the insect canonical sequence (Gokhman and Kuznetsova 2018).

The pentanucleotide sequence has apparently evolved from the canonical sequence (TTAGGG)$_n$ and has changed during insect diversification. This is supported by families that show the presence of (TTAGGG)$_n$ and also by genera which present a different telomeric sequence such as (TCAGG)$_n$, which is observed in some Coleoptera families (Kuznetsova et al. 2019). The differences in telomeric sequences within the class Insecta can be explained by biological mechanisms that preserve the telomere integrity. Telomerase is the enzyme responsible for maintaining repetitive sequences on telomeres; however, many alternative telomerase-independent mechanisms also act in telomere conservation. In this manner, the (TTAGG)$_n$ sequence has been lost and recovered several times during the evolution of insects (Kuznetsova et al. 2019).

Other than chromosome number, not much cytogenetic information is available regarding leafcutter ants, and FISH analyses involving telomeric probes are available only for Ac. striatus (Pereira et al. 2018). Further, the distribution of canonical repeats and telomerase systems is still an open question among insects (Kuznetsova et al. 2019). Thus, in the present study, we analyzed the homology between the telomeric regions of leafcutter ant species Ac. ambiguus (Emery, 1888), Ac. crassispinus (Forel, 1909), Ac. lundii (Guérin-Méneville, 1838), Ac. nigrosetosus (Forel, 1908), Ac. rugosus (Smith, 1858), Ac. subterraneus subterraneus (Forel, 1893), and Atta sexdens (Linnaeus, 1758) by FISH chromosome mapping using the (TTAGG)$_n$ probe. We aimed to accumulate evolutionary evidence for the presence of an insect canonical telomere motif on the chromosomes of leafcutter ants. We further performed a detailed karyomorphometric analysis to establish karyotypes and classify chromosome, and described two new chromosome counts.
Material and methods

Chromosome preparation and fluorescence in situ hybridization (FISH)

The ant colonies were collected from different Brazilian states in 2018. *Acromyrmex ambiguus* was collected from Ilha Comprida – SP (24°44’28”S, 47°32’24”W); the species *Ac. crassispinus* (Ouro Preto – 20°17’15”S, 43°30’29”W), *Ac. rugosus* (Marliéria – 19°43’21”S, 42°43’26”W), *Ac. nigrosetosus* (Ouro Preto – 20°17’15”S, 43°30’29”W), *Ac. subterraneus subterraneus* (Viçosa – 20°48’35.5”S, 42°51’31.07”W), and *At. sexdens* (Marliéria – 19°43’21”S, 42°43’26”W) were collected in Minas Gerais – MG; *Ac. lun-dii* was collected in Dom Pedrito – RS (30°58’5”S, 54°40’23”W). The nests were kept at the Laboratório de Genética Evolutiva e de Populações of the Universidade Federal de Ouro Preto. The brain ganglia of post-defective larvae were extracted in hypotonic solution of colchicine (0.005%), as described by Imai et al. (1988) with modifications described by Cardoso et al. (2012), to obtain the metaphasic chromosomes.

FISH experiments were performed as described by Micolino et al. (2019a). The (TTAGG)$_6$ motif was directly labeled with Cy3 at the 5’ terminal (Sigma, St. Louis, MO, USA). Briefly, slides were submitted to RNA degradation for 1 h in a humid chamber at 37 °C, were washed in 2× SSC, and treated with 0.005% pepsin for 10 min. After washing in 1× PBS, the slides were fixed with 10% formaldehyde for 10 min. Another wash in 1× PBS was performed and then, the slides were dehydrated in an alcohol series. Chromosomal denaturation was promoted by adding 70% formamide at 75 °C for 5 min. Another alcohol dehydration series was performed before adding 2 μL of the (TTAGG)$_6$ probe and 18 μL of HybMix to each slide in the dark. The slides were incubated overnight in a humid chamber at 37 °C. Finally, the slides were washed in 2× SSC solution, 1× SSC, 4× SSC Tween (during 5 min each solution), and then rapidly in 1× PBS. Dehydration was performed in an alcohol series and DAPI was added as a counterstain. To select 10 metaphases with chromosomal integrity and evident probe marking, the slides were visualized on a Zeiss Axio Imager Z2 fluorescence microscope coupled with an image capture system and the resulting images were further edited using Adobe Photoshop CC Software.

Karyomorphometry

The slides were stained with a 4% Giemsa solution and visualized on a Zeiss Axio Imager Z2 microscope with image capture. For each species, we selected 10 metaphases with chromosomal integrity, evident centromeres and no overlapping. Karyomorphometry and chromosomal classification were performed as described by Cristiano et al. (2017). The chromosomes were measured using Image-Pro Plus (Media Cybernetics, Rockville, MD) and some chromosome characteristics were evaluated. For each chromosome, we measured the total length (TL) end-to-end, short arm (S), and long arm (L) sizes calculated by the distance between the arm end and centromeric region. The
karyotype length (KL) was calculated by summing the total length of all chromosomes. The relative size (RL) of each chromosome was calculated in relation to the total size of all chromosomes \( (TL \times 100 / \sum TL) \). The ratio \( (r) \) between the length of the long arm and short arm \( (r = L / S) \) was calculated to classify the chromosomes as described by Levan et al. (1964) with modifications reported by Crozier (1970).

**Results**

The typical chromosome number of *Acromyrmex* \((2n = 38)\) was found in all species of the genus analyzed in the present work. The karyotype of *Ac. lundii* and *Ac. nigrosetosus* were described for the first time and, that of *Ac. ambiguus* was described for the first time from a Brazilian population. The two largest chromosomal pairs were the first subtelocentric and the first metacentric. The karyotype formula was variable (see below) and in *Ac. crassispinus*, *Ac. lundii*, *Ac. nigrosetosus*, and *Ac. subterraneus subterraneus*, most chromosomes presented an \( r \) ratio between 1.67 and 3.00; therefore, these were classified as submetacentric. The chromosomal classification of *Ac. ambiguus* was different from that of other species, as it mainly presents metacentric chromosomes. *Ac. ambiguus* has the karyotype formula \( 2K = 14M + 12SM + 8ST + 4A \) (Figure 1, Table 1). *Ac. crassispinus* presented \( 2K = 12M + 20SM + 4ST + 2A \) (Figure 2, Table 2) and its chromosomes are larger when compared to other species. *Ac. lundii* has the karyotype formula \( 2K = 10M + 14SM + 10ST + 4A \) (Figure 3, Table 3). *Ac. nigrosetosus* presented \( 2K = 12M + 14SM + 10ST + 2A \) and its chromosomes seem smaller than those of the other species (Figure 4, Table 4). *Ac. subterraneus subterraneus* has \( 2K = 14M + \)

**Figure 1.** Conventional staining of mitotic cells of *Acromyrmex ambiguus* A the metaphase and B diploid karyotype with \( 2n = 38 \). Scale bar: 5 μm.
## Table 1. Karyomorphometric analyses of the chromosomes of *Acromyrmex ambiguus*.

<table>
<thead>
<tr>
<th>Chromosomes</th>
<th>TL</th>
<th>L</th>
<th>S</th>
<th>RL</th>
<th>r</th>
<th>Classification</th>
</tr>
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<tr>
<td>1</td>
<td>5.13 ± 1.90</td>
<td>2.79 ± 1.03</td>
<td>2.34 ± 0.89</td>
<td>4.41 ± 0.39</td>
<td>1.20 ± 0.14</td>
<td>Metacentric</td>
</tr>
<tr>
<td>1</td>
<td>4.85 ± 1.87</td>
<td>2.60 ± 1.01</td>
<td>2.26 ± 0.89</td>
<td>4.17 ± 0.46</td>
<td>1.15 ± 0.15</td>
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</tr>
<tr>
<td>2</td>
<td>3.35 ± 1.11</td>
<td>1.96 ± 0.68</td>
<td>1.39 ± 0.43</td>
<td>2.91 ± 0.14</td>
<td>1.40 ± 0.14</td>
<td>Metacentric</td>
</tr>
<tr>
<td>2</td>
<td>3.18 ± 0.98</td>
<td>1.87 ± 0.58</td>
<td>1.31 ± 0.41</td>
<td>2.78 ± 0.05</td>
<td>1.43 ± 0.14</td>
<td>Metacentric</td>
</tr>
<tr>
<td>3</td>
<td>3.11 ± 0.94</td>
<td>1.83 ± 0.59</td>
<td>1.29 ± 0.36</td>
<td>2.72 ± 0.07</td>
<td>1.41 ± 0.10</td>
<td>Metacentric</td>
</tr>
<tr>
<td>3</td>
<td>3.08 ± 0.93</td>
<td>1.80 ± 0.58</td>
<td>1.28 ± 0.36</td>
<td>2.69 ± 0.07</td>
<td>1.40 ± 0.10</td>
<td>Metacentric</td>
</tr>
<tr>
<td>4</td>
<td>3.01 ± 0.92</td>
<td>1.76 ± 0.57</td>
<td>1.25 ± 0.36</td>
<td>2.63 ± 0.06</td>
<td>1.40 ± 0.13</td>
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<tr>
<td>4</td>
<td>2.92 ± 0.89</td>
<td>1.77 ± 0.54</td>
<td>1.15 ± 0.35</td>
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</tr>
<tr>
<td>5</td>
<td>2.86 ± 0.84</td>
<td>1.71 ± 0.52</td>
<td>1.15 ± 0.33</td>
<td>2.50 ± 0.08</td>
<td>1.48 ± 0.13</td>
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</tr>
<tr>
<td>5</td>
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<td>1.62 ± 0.47</td>
<td>1.14 ± 0.32</td>
<td>2.43 ± 0.15</td>
<td>1.43 ± 0.18</td>
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</tr>
<tr>
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<td>1.61 ± 0.44</td>
<td>1.04 ± 0.25</td>
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</tr>
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</tr>
<tr>
<td>18</td>
<td>1.95 ± 0.47</td>
<td>1.73 ± 0.41</td>
<td>0.22 ± 0.07</td>
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<td>8.27 ± 0.99</td>
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</tr>
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</tr>
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</table>

TL: total length; L: long arm length; S: short arm length; RL: relative length; r: arm ratio, KL: karyotype length.

Figure 2. Conventional staining of mitotic cells of *Acromyrmex crassipinus* A the metaphase and B diploid karyotype with 2n = 38. Scale bar: 5 μm.
Figure 3. Conventional staining of mitotic cells of *Acromyrmex lundii* A the metaphase and B diploid karyotype with 2n = 38. Scale bar: 5 μm.
Conventional staining of mitotic cells of *Acromyrmex nigrosetosus* A the metaphase and B diploid karyotype with 2n = 38. Scale bar: 5 μm.
Comparative FISH-mapping of TTAGG of the leafcutter ants phase and diploid karyotype with 2n = 38. Scale bar: 5 μm.

TL: total length; L: long arm length; S: short arm length; RL: relative length; r: arm ratio, KL: karyotype length.

Table 4. Karyomorphometric analyses of the chromosomes of *Acromyrmex nigrosetosus*.

<table>
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<th>Chromosomes</th>
<th>TL</th>
<th>L</th>
<th>S</th>
<th>RL</th>
<th>r</th>
<th>Classification</th>
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<td>4.34 ± 0.34</td>
<td>1.22 ± 0.11</td>
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</tr>
<tr>
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<td>2.24 ± 0.57</td>
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<td>4.12 ± 0.18</td>
<td>1.16 ± 0.08</td>
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</tr>
<tr>
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<td>1.75 ± 0.32</td>
<td>1.18 ± 0.29</td>
<td>2.90 ± 0.18</td>
<td>1.51 ± 0.12</td>
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</tr>
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<td>1.68 ± 0.34</td>
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<td>2.77 ± 0.12</td>
<td>1.51 ± 0.10</td>
<td>Metacentric</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>1.48 ± 0.13</td>
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</tr>
<tr>
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<td>2.44 ± 0.55</td>
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<td>1.54 ± 0.11</td>
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<tr>
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<td>2.21 ± 0.19</td>
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<td>1.52 ± 0.14</td>
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Figure 5. Conventional staining of mitotic cells of *Acromyrmex subterraneus subterraneus* A the metaphase and B diploid karyotype with 2n = 38. Scale bar: 5 μm.
18SM + 4ST + 2A (Figure 5, Table 5). For Ac. rugosus and At. sexdens only the chromosome number was established, but no detailed karyomorphometry was performed.

FISH analyses revealed that all chromosomes of all Acromyrmex species and Atta sexdens are positively marked at both arms in the telomeric regions with the presence of the canonical insect sequence (TTAGG)⁶ and no signals for interstitial telomeric sites were detected (Figures 6A–F, 7). The intensity and size of the probe marking varied between the chromosomes and metaphases of each species.

**Discussion**

The insect canonical repeat (TTAGG)⁶ has been observed in 30 species of ants using different methods (Okazaki et al. 1993; Meyne et al. 1995; Lorite et al. 2002; Wurm...
et al. 2011; Pereira et al. 2018), but FISH studies were mostly performed with Myrmecia species (Meyne et al. 1995). The only analysis involving a leafcutter ant has been performed on Ac. striatus, which also presents (TTAGG)$_6$ labeling in the telomeres of both arms of all 22 chromosomes and does not show markings in other chromosomal regions (Pereira et al. 2018). The present study adds information about one species of Atta (At. sexdens) and six Acromyrmex species (Ac. ambiguus, Ac. crassispinus, Ac. lundii, Ac. nigrosetosus, Ac. rugosus, Ac. subterraneus subterraneus). We also describe the chromosome number and structure of Ac. lundii and Ac. nigrosetosus for the first time. The karyotype description for Ac. ambiguus from Brazil revealed the same diploid chromo-

Figure 6. FISH mapping of mitotic metaphase chromosomes using a (TTAGG)$_6$ telomeric probe; DAPI stain in blue and Cy3 in red A Acromyrmex ambiguus B Acromyrmex crassispinus C Acromyrmex lundii D Acromyrmex nigrosetosus E Acromyrmex rugosus and F Acromyrmex subterraneus subterraneus. Scale bar: 5 μm.
some number as in previous data available from Uruguay (Goñi 1983), but distinct regarding the karyotype formula, overrepresented by subtelocentric and acrocentric chromosomes in the latter. These differences may be due the visual determination of chromosome morphology instead chromosome measurements applied here. The new chromosome counts reported in this study again corroborate the stable chromosomal number in *Acromyrmex* and the detailed karyomorphometry of the chromosomes suggests dynamism of chromosome morphology due to distinct karyotypic formulas.

Our FISH results add to the cytogenetic knowledge of new karyotypes and molecular cytogenetic analyses in leafcutter ants, and demonstrate that the pattern found in *Ac. striatus* seems to occur in *Atta* species and *Acromyrmex* species. Importantly, *Ac. striatus* is the sister clade of *Atta* and the remaining *Acromyrmex* species (Cristiano et al. 2013). The occurrence of telomeric regions marked positively by (TTAGG)ₙ reinforces the premise that Formicidae presents high homology for the presence of the insect canonical sequence. This motif has been proposed to be a plesiomorphic chromosomal feature in Hymenoptera (Gokhman and Kuznetsova 2018). In fact, the canonical motif (TTAGG)ₙ was observed in several branches of the clade of fungus-farming ants, from anciently diverged lineages such as *Mycetophylax* to recent lineages such as *Mycetomoellerius* (Micolino et al. 2019a, b, 2020). Besides, the alternative TCAGG motif present in insects seems to be restricted to some groups, but not to Formicidae (Kuznetsova et al. 2019), and we did not find any evidences for this in previously attempted experiments in our laboratory on the phylogenetic basis of fungus-farming ants (unpublished data).

Sahara et al. (1999) propose that (TTAGG)ₙ is a sequence with high homology in Insecta because it is inherited from a common primitive ancestor of the class and the
fact that some families do not show the presence of canonical repetition is explained by the group evolutionary process, where \((TTAGG)_n\) has been lost and recovered several times. This theory is supported by Frydrychová et al. (2004) who studied 22 insect species from 20 different orders selected among the main phylogenetic group lineages and found that 15 species presented the \((TTAGG)_n\) on their telomeres, whereas only seven species did not have the sequence in their chromosomes. The authors compared their results with the available literature and concluded that 16 insect orders have the primitive telomeric region conserved and eight do not present it. In contrast, Menezes et al. (2017) evaluated the presence of the canonical repeats \((TTAGG)_n\) and \((TTAGGG)_n\) in 25 representative species of eight Hymenoptera families, and surprisingly none of them showed any signs of these repetitive sequences in their telomeres or in any chromosomal regions. Therefore, the hypothesis regarding multiple losses of the sequence inherited from a primitive ancestor appears unlikely to these authors, as the number of Insecta families without the \((TTAGG)_n\) sequence is higher than the number of those bearing it. Thus, the authors propose that the most probable evolutionary scenario is that the canonical repetition has been lost in the Apocrita ancestor or even in the Hymenoptera ancestor, whereas Apidae and Formicidae have recovered the region independently. On the contrary, the phylogenetic position and the presence of \((TTAGG)_n\) as the telomeric repeat in Tentredo omissa (Fürster, 1844) and Taxonus agrorum (Fallén, 1808) (Tenthredinidae: Symphyta) were suggested to be indicative of the ancestrality of this motif in Hymenoptera (Gokhman and Kuznetsova 2018).

Ants have high variability in their karyotypes; there are species with the haploid number of chromosomes \(n = 1\) (Croslan and Crozier 1986; Taylor 1991) and species with \(n = 60\) (Mariano et al. 2008). This variation exists with respect to the chromosome number as well as the morphology and classification. Robertsonian fissions result in two acrocentric chromosomes due to the breaking of a bi-armed chromosome, whereas Robertsonian fusions involve exactly the opposite process, where two acrocentric chromosomes unite to form a single bi-armed chromosome (Lorite and Palomeque 2010). These are possibly the two most important rearrangements for karyotype evolution in ants and support the minimum-interaction theory proposed by Imai et al. (1988, 1994, 2001). This theory defines that fission processes are more significant and common than fusion processes because higher chromosome numbers reduce the possibility of interaction between non-homologous chromosomes within the nucleus, minimizing the mutation rates. Thus, it is proposed that the chromosomal number of ant species usually tends to increase. In this sense, it is also proposed that the ancestral karyotype of ants would be composed of a small number of metacentric chromosomes whereas recently divergent lineages would have more chromosomes due to several chromosomal fission processes (Imai et al. 1977). Thus, it is plausible to state that in Acromyrmex, karyotypes with 38 chromosomes arose following several Robertsonian fissions, whereas the chromosome number of the iconic Ac. striatus is a plesiomorphic feature maintained in Atta spp. (Cristiano et al. 2013).

Establishment of the karyotype (the chromosome number and determination of their morphology) is very important for the knowledge of chromosomal variations and possible genetic barriers between phylogenetic groups (Cristiano et al. 2017; Cardoso
et al. 2018b). It is necessary to go further in describing the chromosome number and morphology, as more detailed karyomorphometric analyses may reveal additional and substantial variations not observed previously, mainly when accompanied with genome size estimates (Cardoso et al. 2018b). Tsutsui et al. (2008) state that closely related species, belonging to the same genus, may have very similar genome sizes, corroborating the pattern revealed by our karyomorphometric analyses in the *Acromyrmex* species studied here.

**Acknowledgement**

We are grateful to the many people that made this work possible. We thank all of our colleagues at the Lab and Research Group of Genetics and Evolution of Ants (GEF-UFO) for their help with data or their assistance in the field of chromosome preparation. The authors thank Júlio Chaul and Rodrigo Feitosa of the Universidade Federal do Paraná that confirmed the identification of the ant species. We would like to thank Editage (www.editage.com) for English language editing. We also thank the editor and two reviewers for their helpful comments on the manuscript.

**References**


First cytogenetic information for five Nilotic elephantfishes and a problem of ancestral karyotype of the family Mormyridae (Osteoglossiformes)

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Abstract
The elephantfish family Mormyridae is the most diverse lineage of the primitive teleostean clade Osteoglossomorpha distributed in inland waters of all continents except Antarctica and Europe. The family Mormyridae is endemic to Africa and includes 22 genera and almost 230 species. The evolutionary radiation of mormyrids most probably should be attributed to their capability of both generating and receiving weak electric signals. Up-to-date cytogenetic studies have revealed substantial karyotype differentiation among the nine investigated elephantfish species and genera (a single species studied per each genus). In the present study, karyotypes of five species representing five mormyrid genera (four unexplored ones) collected from the White Nile system in southwestern Ethiopia are described for the first time. The results show substantial variety of the diploid chromosome and fundamental numbers: \(2n = 48\) and \(FN = 54\) in \(Brevimyrus nigro\) (Günther, 1866), \(2n = 50\) and \(FN = 72\) in \(Cyphomyrus petherici\) (Boulenger, 1898), \(2n = 50\) and \(FN = 78\) in \(Hippopotamyrus pictus\) (Marcusen, 1864), \(2n = 50\) and \(FN = 76\) in \(Marcusenius cyprinoides\) (Linnaeus, 1758), \(2n = 52\) and \(FN = 52\) in \(Mormyrops anguilloides\) (Linnaeus, 1758). Karyotype structure in the latter species seems to be close to the ancestral condition for the family. This hypothesis is discussed in the light of available data on karyotype diversity and phylogeny of mormyrids.

Keywords
Africa, chromosomes, karyotype evolution, \(Brevimyrus\), \(Cyphomyrus\), \(Hippopotamyrus\), \(Marcusenius\), \(Mormyrops\)

**Introduction**

The elephantfish family Mormyridae belongs to one of the most primitive groups of teleostean fishes, the cohort Osteoglossomorpha (Nelson et al. 2016). The family is endemic to the African continent and includes 22 genera and almost 230 species (Froese and Pauly 2019; Eschmeyer et al. 2020). In genus and species diversity it exceeds all other extant osteoglossomorph lineages. The evolutionary radiation of mormyrids most probably should be attributed to their ability of both generating and receiving weak electric signals that provides dual functions of ‘electrolocation’ and communication (Hopkins 2009, Carlson and Arnegard 2011).

First cytogenetic data on the osteoglossomorphs and particularly mormyrids were published by Hinegardner and Rosen (1972) and Uyeno (1973) almost half a century ago. Thereafter, the karyotype structure and cellular DNA content of osteoglossomorphs were progressively studied (reviewed by Arai 2011; Canitz et al. 2016; Barby et al. 2018; Cioffi et al. 2019). The recent works on mormyrids (Krysanov and Golubtsov 2014; Ozouf-Costaz et al. 2015; Canitz et al. 2016) raised to nine the number of mormyrid genera studied. The number of species studied is also nine because one species only has been karyotyped for all genera. The diploid chromosome numbers in most mormyrids are similar (2n = 48 or 50 excepting Pollimyrus Taverne, 1971 with 2n = 40). Nevertheless, the varying bi-armed chromosome numbers and ‘amazing’ diversity in NOR positions and C-banding patterns provide evidence for the substantial divergence in the karyotype structure with the dominating role of pericentric inversions (Ozouf-Costaz et al. 2015).

There is a coherent hypothesis about phylogenetic position of the family Mormyridae among other Osteoglossomorpha (Lavoué and Sullivan 2004; Inoue et al. 2009; Nelson et al. 2016). The phylogenetic structure of mormyrids themselves is not well-elaborated, but three basal groups in their radiation (the genera Petrocephalus Marcusen, 1854; Myomurus Boulenger, 1898; Mormyraps Müller, 1843) are reliably defined (Alves-Gomes and Hopkins 1997; Sullivan et al. 2000; Lavoué et al. 2003). This makes it possible to hypothesize about the mormyrid karyotype evolution. Based on available data Canitz et al. (2016) suggested for Mormyridae the ancestral chromosome number 2n = 48–50, that is well-coordinated with the hypothetical ancestral karyotype for the teleostean fishes and early vertebrates in general (Ohno et al. 1969; Jaillon et al. 2004; Kohn et al. 2006; Nakatani et al. 2007).

Meanwhile, only a small fraction of the total mormyrid diversity (less than 5% of species) has been yet studied cytogenetically. New findings may correct the existing views on their karyotype evolution. In the present study, new data for five mormyrid species from northern East Africa are presented using cytogenetic analysis (chromosome number and morphology). Relevance of these data to understanding of karyotype evolution within the family Mormyridae is considered.

**Material and methods**

The fifteen individuals studied represent five species of different genera – *Brevimyrus niger* (Günther, 1866), *Cyphomyrus petherici* (Boulenger, 1898), *Hippopotamyrus pic-
First cytogenetic information for five Nilotic elephantfishes

First cytogenetic information for five Nilotic elephantfishes (Marcusenius cyprinoides (Linnaeus, 1758) and Mormyrops anguilloides (Linnaeus, 1758) – of the elephantfish family Mormyridae (Table 1). Fish were collected in southwestern Ethiopia under the umbrella of the Joint Ethiopian-Russian Biological Expedition (JERBE) at three sites in November of 2017: the Baro River downstream of the City of Itang (8°10'47"N, 34°15'2"E), the Tida River half way between the cities of Gambela and Itang (8°16'15"N, 34°25'52"E) and the Alvero River downstream of the Abobo Dam (7°52'23"N, 34°29'48"E). All three rivers belong to the Sobat River drainage discharging into the White Nile in South Sudan. Fish were caught with cast or gill nets, delivered in 80-l plastic containers into the field laboratory, where they were kept in permanently aerated water for several hours before treatment.

Before preparation fish were treated intraperitoneally with 0.1% colchicine for 3–4 hours. Then fish were euthanized with an overdose of tricaine methanesulfonate (MS-222), identified based on morphological key characters (Golubtsov et al. 1995, Levin and Golubtsov 2018), measured to an accuracy of 1 mm, dissected for gonad examination and tissue sampling, and preserved in 10% formaldehyde. Vouchers are deposited at the Institute of Ecology and Evolution (Moscow) under provisional labels of JERBE.

Chromosome preparations were obtained from anterior kidney according to Kligerman and Bloom (1977). Briefly, the anterior kidney tissue was incubated with 0.075M KCl hypotonic solution for 20–30 min at room temperature and fixed with 3:1 methanol : acetic acid. To prepare slides a fixed tissue was incubated with 50% glacial acetic acid, suspended, and dropped onto a hot slides. Air-dried chromosome spreads were stained conventionally with 4% Giemsa solution in phosphate buffer at pH 6.8 for 8 min.

Chromosome spreads were analysed under “Axioplan 2 Imaging” microscope (Carl Zeiss, Germany) equipped with “CV-M4+CL” camera (JAI, Japan) and “Ikaros” software (MetaSystems, Germany). Karyotypes were established according to the centromere position following the nomenclature of Levan et al. (1964). Chromosomes were classified as metacentric (a), submetacentric (sm) and acrocentric (a), including subtelocentric and telocentric chromosomes, and grouped according to their morphology in order of decreasing size. To determine the fundamental number (FN), metacentrics and submetacentrics were considered bi-armed and acrocentrics as uni-armed. The number of complete metaphase plates studied for each specimen is presented in Table 1.

Table 1. Species, fish standard length (SL), numbers of individuals (N) and metaphases (N mt) studied, and collection site.

<table>
<thead>
<tr>
<th>Species</th>
<th>SL, mm</th>
<th>N</th>
<th>N mt</th>
<th>Collection site</th>
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<tr>
<td>Brevimyrus niger</td>
<td>81–87</td>
<td>3 (1♀, 2♂)</td>
<td>32</td>
<td>Tida River</td>
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<tr>
<td>Cyphomyrus petherici</td>
<td>69–153</td>
<td>5 (3♀, 2♂)</td>
<td>54</td>
<td>Alvero River</td>
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<tr>
<td>Hippopotamyrus pictus</td>
<td>197</td>
<td>1 (♀)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Marcusenius cyprinoides</td>
<td>196–217</td>
<td>3 (2♀, 1♂)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Mormyrops anguilloides</td>
<td>409–498</td>
<td>2 (1♀, 1♂)</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>413</td>
<td>1 (♀)</td>
<td>17</td>
<td>Baro River</td>
</tr>
</tbody>
</table>
Figure 1. Karyotypes of five elephantfishes of the family Mormyridae. Scale bar: 10 μm.
Results and discussion

*Brevimyrus niger* has a karyotype with 2n = 48 (Fig. 1) consisting of 4 metacentrics (m), 2 submetacentrics (sm) and 42 acrocentrics (a). Three taxa share the same diploid numbers of chromosomes 2n = 50 but differ in karyotypic formula: *Cyphomyrus petherici* has 18m, 4sm and 28a, *Hippopotamyrus pictus* has 24m, 4sm and 22a, and *Marcusenius cyprinoides* has 22m, 4sm and 24a. Finally, *Mormyrops anguilloides* has karyotype with 2n = 52 consisting exclusively of acrocentrics gradually decreasing in size. In the other species studied by us one or two pairs of metacentrics or submetacentrics noticeably exceed in size most acrocentrics that admits an origin of the larger chromosomes via the centric fusions.

No distinguishable sex chromosomes were observed in complements of the four species in which individuals of both sexes were studied (*B. niger*, *C. petherici*, *M. cyprinoides*, and *M. anguilloides*), while the only male of *H. pictus* was karyotyped (Table 1). This is in agreement with the lack of reports on sex chromosomes in other mormyrids, but presence of heteromorphic sex chromosomes was supposed in the Asian arowana *Scleropages formosus* (Müller & Schlegel, 1840) from the family Osteoglossidae distantly related to Mormyridae (Bian et al. 2016; but see Cioffi et al. 2019).

Data for all mormyrid taxa studied cytogenetically in the present study and earlier are presented in Table 2. Taxa within the subfamily Mormyrinae are listed in alphabetical order. Recognition of the subfamily Petrocephalinae, as a sister group to all other mormyrids, is well-grounded by morphological (including structure of electrocytes) and molecular phylogenetic data (Taverne 1972; Alves-Gomes and Hopkins 1997; Sullivan et al. 2000; Lavoué et al. 2003). For the two earlier studied taxa names are changed in accordance with recent taxonomic arrangements (Eschmeyer et al. 2020): *Brienomyrus brachyistius* (Gill, 1862) was reported as “Marcusenius brachistius Gill” by Uyeno (1973) and *Campylomormyrus rhynchophorus* (Boulenger, 1898) as *C. compressirostris* (Pellegrin, 1924) by Canitz et al. (2016). *Brienomyrus sp.*7 of Ozouf-Costaz et al. (2015) is listed as *Paramormyrops* sp.7 following to Ráb et al. (2016).

*Brevimyrus niger* shares the karyotype with 2n = 48 with three other mormyrid taxa, but differs from two of them – *Campylomormyrus rhynchophorus* with FN = 78 and *Gnathonemus petersii* (Günther 1862) with FN = 64 or 68 – by a smaller number of biarmed elements (FN = 54). For third taxon, *Brienomyrus brachyistius*, the unbalanced karyotype with FN = 53 was described in a single specimen (Uyeno 1973). Apart from the unpaired metacentric chromosome of the unclear nature, its karyotype looks similar to that of *Brevimyrus niger*. Both species have two pairs of large biarmed chromosomes, while a pair of uni-armed chromosomes in *Brienomyrus brachyistius* might be substituted by a pair of submetacentrics in *Brevimyrus niger* lineage.

The karyotype with 2n = 50 was found to be dominating in both presently and previously studied mormyrids (three and five taxa, respectively). *Cyphomyrus petherici* (FN = 72), *Hippopotamyrus pictus* (FN = 78) and *Marcusenius cyprinoides* (FN = 76) have more biarmed elements in their compliment than any other mormyrid studied except *Campylomormyrus rhynchophorus* (FN = 78). Congeneric *Marcusenius cyprinoides* and *M. moorii* (Günther, 1867) sharing the same chromosome number differ substantially in
their karyotype structure. Up to recently *Cyphomyrus petherici* was considered as belonging to the genus *Pollimyrus* (Taverne 1971; Moritz et al. 2019). Substantial cytogenetic dissimilarity between the single studied species of the latter genus (2n = 40, FN = 42) and *C. petherici* corroborates the change of its generic position (Levin and Golubtsov 2018).

*Mormyrops anguilloides* has a karyotype unique for the mormyrids studied and composed of 52 uni-armed chromosomes. There are two mormyrids — *Petrocephalus micropthalmus* Pellegrin, 1909 and *Stomatorhinus walkerii* (Günther, 1867) — with 2n = 50 and FN = 52. Karyotypes of these three taxa dominated by the uni-armed elements seem to be close to each other and to a hypothetical ancestral karyotype of the family Mormyridae. Mutual transformation of these karyotypes could occur in a few evolutionary steps (Fig. 2). It is important that two of the three genera under consideration (*Petrocephalus* and *Mormyrops*) appear to be well-defined basal groups in the family phylogeny (Sullivan et al. 2000; Lavoué et al. 2003). Phylogenetic position of the third genera (*Stomatorhinus*) is unclear. Though it appears in the rather basal position (next to *Petrocephalus*) in the small cladogram by Ozouf-Costaz et al. (2015) based of the mitochondrial cytochrome b sequences, in the more extensive mormyrid phylogenies this genus is nested deeper in the phylogenetic trees but in varying and poorly supported positions (Lavoué et al. 2003; Sullivan et al. 2016; Levin and Golubtsov 2018). Unfortunately, cytogenetic data for one more genus with the well-defined basal position in the mormyrid phylogeny (*Myomyrus*, stemming out between *Petrocephalus* and *Mormyrops*) are absent.

Based on the simultaneous phylogenetic analysis of molecular data and chromosome number, Canitz et al. (2016) recognized karyotype with n = 24 as the most parsimonious ancestral state for the order Osteoglossiformes, while the haploid chromosome number of n = 24–25 was inferred for the most recent common ancestor of the family Mormyridae. Their analysis, however, did not include the most recent cytogenetic data for several osteoglossomorph clades (Ráb et al. 2016; Barby et al. 2018; Hatanaka et al. 2018; Jegede et al. 2018; Cioffi et al. 2019; de Oliveira et al. 2019). Moreover, the recent genomic data evidence for the ancestral Euteleostomi karyotype of 50 chromosomes with domination by acrocentric elements (Nakatani et al. 2007; Sacerdot et al. 2018; de Oliveira et al. 2019). If the ancestral karyotype of Mormyridae contained 50 uni-armed elements, three chromosomal rearrangements only might produce the observed karyotype structure in the three mormyrid genera (*Petrocephalus*, *Stomatorhinus* and *Mormyrops*) tentatively recognized by us as the least cytogenetically advanced (Fig. 2). The solitary submetacentric pairs in *Petrocephalus* and *Stomatorhinus* are suggested to be not syntenic because of some differences in chromosome morphology (Ozouf-Costaz et al. 2015). If the ancestral karyotype of Mormyridae contained 50 uni-armed elements, it is apparently not retained by any extant mormyrid or osteoglossomorph, in general. Although the karyotype with 2n = 50 is dominating among mormyrids, it contains from 1 to 14 pairs of bi-armed elements (Table 2).

Based on available data the most parsimonious scenarios of the early karyotype evolution in Mormyridae are presented in Figure 2. Three different ancestral karyotypes are considered: 2n = 50 and FN = 50 (no bi-armed elements), 2n = 50 and FN = 52 (the only pair of bi-armed elements), 2n = 52 and FN = 52 (no bi-armed elements). The
Figure 2. Most parsimonious scenarios of the early karyotype evolution within the family Mormyridae including three variants (A–C) of karyotype structure in a hypothetic ancestor (HA) and three studied lineages (the genera Petrocephalus, Stomatorhinus and Mormyrops) with least advanced karyotype structure within the family. The alternative transformations of karyotype structure are joint with a dashed line. The solitary submetacentric pairs in Petrocephalus and Stomatorhinus are suggested to be not syntenic.
karyotype structure suggested for a hypothetic ancestor could not be retained in any extant mormyrid lineage (Fig. 2A) or retained in Petrocephalus (Fig. 2B) or Mormyrops (Fig. 2C). It is impossible to judge which of the scenarios considered is more preferable. There are also plenty of less parsimonious scenarios that are not considered by us.

We believe that further cytogenetic studies of various mormyrid taxa may shape the existing views on the karyotype evolution within this diverse group of fish. Looking for the probable interspecific variation of the karyotype structure within the three phylogenetically basal groups (the genera Petrocephalus, Myomurus, Mormyrops) is of special interest.

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<th>Origin</th>
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<td><strong>Subfamily Petrocephalinae</strong></td>
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<tr>
<td>Petrocephalus microphthalmus Pellegrin, 1909</td>
<td>50</td>
<td>2sm + 48a</td>
<td>52</td>
<td>Ogooué Basin, Gabon</td>
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<td><strong>Subfamily Mormyrinae</strong></td>
<td></td>
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<td>Brevisimyrus niger (Günther, 1866)</td>
<td>48</td>
<td>4m + 2sm + 42a</td>
<td>54</td>
<td>White Nile Basin, Ethiopia</td>
<td>This study</td>
</tr>
<tr>
<td>Brienomyrus brachystius (Gill, 1862)</td>
<td>48</td>
<td>1m + 4sm + 2st + 41a</td>
<td>53</td>
<td>Unknown (fish store)</td>
<td>Uyeno 1973</td>
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<td>Campylonormyrus rynchophorus (Boulenger, 1898)</td>
<td>48</td>
<td>26m + 4sm + 18a</td>
<td>78</td>
<td>Unknown (laboratory stock)</td>
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<td>50</td>
<td>18m + 4sm + 28a</td>
<td>72</td>
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<td>This study</td>
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<td>48</td>
<td>10m + 6sm + 32a</td>
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<td></td>
<td>48</td>
<td>18m + 2sm + 28a</td>
<td>68</td>
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<td>78</td>
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<td>50</td>
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<td>62</td>
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<td>4sm + 46a</td>
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<td>58</td>
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<td>40</td>
<td>2m + 38a</td>
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<td>Kryssanov and Golubtsov 2014</td>
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<td>52</td>
<td>Ogooué Basin, Gabon</td>
<td>Ozouf-Costaz et al. 2015</td>
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First cytogenetic information for five Nilotic elephantfishes

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References


First cytogenetic information for five Nilotic elephantfishes


Chromosomes of parasitic wasps of the superfamily Chalcidoidea (Hymenoptera): An overview

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Abstract
An overview of the current knowledge of chromosome sets of the parasitoid superfamily Chalcidoidea is given. Karyotypes of approximately 240 members of this group, i.e. just above one percent of described species, are studied up to now. Techniques for obtaining and analyzing preparations of chalcid chromosomes are outlined, including the so-called “traditional” and “modern” methods of differential staining as well as fluorescence in situ hybridization (FISH). Among the Chalcidoidea, the haploid chromosome number can vary from n = 3 to n = 11, with a clear mode at n = 6 and a second local maximum at n = 10. In this group, most chromosomes are either metacentric or submetacentric, but acrocentrics and/or subtelocentrics also can predominate, especially within karyotypes of certain Chalcidoidea with higher chromosome numbers. The following main types of chromosomal mutations are characteristic of chalcid karyotypes: inversions, fusions, translocations, polyploidy, aneuploidy and B chromosome variation. Although karyotype evolution of this superfamily was mainly studied using phylogenetic reconstructions based on morphological and/or molecular characters, chromosomal synapomorphies of certain groups were also revealed. Taxonomic implications of karyotypic features of the Chalcidoidea are apparently the most important at the species level, especially among cryptic taxa.

Keywords
base-specific fluorochromes, chalcid wasps, differential staining, FISH, karyotypes, phylogeny, taxonomy
Introduction

The superfamily Chalcidoidea is a very diverse, taxonomically complicated and economically important group of insects (Quicke 1997; Gokhman 2015b) that currently includes about 23 thousand described species (Huber 2017). Nevertheless, chromosomes of approximately 240 members of this group, i.e. just above one percent, are studied up to know (Gokhman 2009 onwards). The last detailed review of the chromosome study of Chalcidoidea was published more than a decade ago (Gokhman 2009, see also Gokhman and Gumovsky 2009), with only about 170 examined species. Consequently, important results of the karyotypic study of chalcids accumulated during this time, especially those obtained with the help of certain advanced techniques, substantially changed our views on the phylogenetic and taxonomic implications of chromosomal characters of this group (Gokhman 2013; Baur et al. 2014; König et al. 2019). An updated overview of the karyotypic study of the superfamily Chalcidoidea is therefore given below.

Techniques used for the chromosome study

Perhaps it is needless to mention that tissues with relatively large numbers of cell divisions should be examined to perform a successful chromosomal analysis of any given group. In the case of Hymenoptera, this for a long time meant studying immature stages (Crozier 1975; Imai et al. 1988; Gokhman 2009). Indeed, chromosome preparations made either from cerebral ganglia or from developing gonads of hymenopteran prepupae and early pupae apparently remain the best source of high-quality metaphase plates, which are the most suitable for morphometric analysis and application of advanced techniques of chromosome staining (Gokhman and Gumovsky 2009). However, obtaining that kind of preparation from many parasitic wasps, including chalcids, is impossible because the establishment of both host and parasitoid lab stocks is usually needed to get access to immature stages of parasitic wasps as well as to ensure reliable identification of this material based on a thorough morphological study of conspecific adults. Nevertheless, this limitation can be overcome in the case of gregarious species (Gokhman 2009). Ovaries of adult females of many parasitoid Hymenoptera can also provide certain numbers of mitotic divisions with discernible morphology of chromosomes, but this mainly applies to synovigenic species, in which oogonia generally continue to divide after eclosion of the female parasitoid from the host (Jervis et al. 2001). On the other hand, ovaries of chalcid wasps often contain meiotic divisions as well, although the number of these divisions is fairly low (Gokhman 2009). In addition, hymenopteran males, which are usually haploid, lack normal meiosis, including synopsis and the reductional division (Crozier 1975), and therefore many details of this process which are observed in diplo-diploid organisms, cannot be reported for parasitoid Hymenoptera. At present, examination of meiotic chromosomes is relatively scarce in Chalcidoidea (see e.g. Gokhman et al. 2014b), but, for example, it would be of considerable interest for studying hybrids between closely related forms with different karyotypes.
Nowadays, the technique developed by Imai et al. (1988) for obtaining air-drying chromosome preparations from prepupae and early pupae of ants, is generally used for karyotyping chalcids. However, stronger hypotonic treatment is usually needed to prevent overlapping of substantially longer chromosomes in the Chalcidoidea. In particular, I normally use 30 min incubation in the 0.5% sodium citrate solution before preparing cell suspension (e.g. Gokhman et al. 2017a), as opposed to 20 min treatment with the 1% solution recommended by Imai et al. (1988). The process also includes maceration of the tissue on the microscope slide in an aqueous solution containing both ethanol and acetic acid, and a subsequent treatment of the cells attached to the slide with an analogous although water-free fixative. However, the final step of chromosome preparation according to Imai et al. (1988), i.e. application of pure acetic acid as an additional fixative, is usually omitted in the case of Chalcidoidea and other parasitoids. I do not only consider this step redundant, but also suggest that the excessive amount of acids can hydrolyze DNA, which is crucial e.g. for performing fluorescence in situ hybridization (FISH). Nevertheless, to avoid washing the cells away from the slide during the subsequent treatment, post-fixation of the material, preferably by acid-free fixatives, is recommended (Gokhman et al. 2019a).

To visualize chromosomes of Chalcidoidea, modern optic microscopes are currently used. Additional epifluorescence modules are also needed to work with fluorochromes, including base-specific chromosome staining and FISH. Moreover, the resulting images must be captured by a modern digital camera, usually controlled through a computer. This camera should produce images with relatively high resolution (at least 300 dpi) and be sensitive enough to work with fluorescence. In turn, these images can be analyzed using specialized software, e.g. KaryoType (Altinordu et al. 2016), to determine absolute/relative lengths and centromere indices of particular chromosomes. As in all other Hymenoptera, chromosomes of chalcid wasps are monocentric, i.e. each of them carries a single centromere (Gokhman 2009). These chromosomes can be subdivided into four groups according to the centromere position, i.e., metacentrics (M), submetacentrics (SM), subtelocentrics (ST) and acrocentrics (A) generally following guidelines provided by Levan et al. (1964). In case of various types of differential staining, both localization and size of particular chromosomal segments have to be identified as well.

It is also noteworthy that precise species identifications are crucial for the karyotypic study of Chalcidoidea as well as of parasitoid Hymenoptera in general (Gokhman 2009). Bearing in mind an exceptional taxonomic complexity of this superfamily and the abundance of cryptic taxa (Gokhman 2018), expert identifications of the examined populations/strains and particular specimens should be obtained in every possible case.

Karyotypes of the overwhelming majority of chalcids were studied using only routine staining. Nowadays, chromosomes of Chalcidoidea are most often stained with Giemsa solution diluted in Sorensen’s phosphate buffer (Gokhman 2009). Nevertheless, routinely stained karyotypes can be further studied using morphometric analysis which already proved its effectiveness for finding both similarities and differences between closely related forms of Chalcidoidea (Gokhman and Westendorff 2000; König et al. 2019). Use of this technique in chalcids is facilitated by the generally low chromosome numbers that are characteristic of most Chalcidoidea.
In addition, karyotypes of a few dozen members of the superfamily Chalcidoidea were examined using various methods of differential staining (Gokhman 2009). The latter techniques are often subdivided into the so-called “traditional” and “modern” ones (Gokhman 2015a). Among the former methods, various techniques of chromosome banding, i.e. C-, AgNOR- and sometimes also G-banding, are used. C- and AgNOR-banding respectively visualize constitutive heterochromatin and nucleolus organizing regions (NORs) (Sumner 1972; Howell and Black 1980). However, chromosomes of only few members of the superfamily Chalcidoidea were studied using either AgNOR- or C-banding. These species belong to the families Aphelinidae (Odierna et al. 1993; Baldanza et al. 1999; Baldanza and Giorgini 2001; Giorgini and Baldanza 2004), Eulophidae (Maffei et al. 2001; Gebiola et al. 2012), Pteromalidae (Reed 1993; Gokhman and Westendorff 2000) and Trichogrammatidae (Van Vugt et al. 2005). C-banding usually visualizes small to medium-sized pericentromeric and telomeric segments of the constitutive heterochromatin on chalcid chromosomes, but a few intercalary blocks were also revealed (Reed 1993; Baldanza et al. 1999; Gokhman and Westendorff 2000). As for AgNOR-banding, it most often detects a single NOR per haploid karyotype (Baldanza et al. 1999; Baldanza and Giorgini 2001 etc.), but two sites of this kind (and an additional NOR on a particular B chromosome) were visualized in the chromosome set of *Trichogramma kaykai* Pinto & Stouthamer, 1997 (Van Vugt et al. 2005). In the superfamily Chalcidoidea, subtelocentric/acrocentric chromosomes usually carry subterminal/terminal NORs, but these sites can be situated close to the centromeres of certain metacentrics (Baldanza et al. 1999; Giorgini and Baldanza 2004). The localization of NORs can vary among members of the same genus (Giorgini and Baldanza 2004), and this is further corroborated by FISH (see below).

G-banding is usually produced by treatment of chromosomes with certain proteolytic enzymes like trypsin (Chiarelli et al. 1972 onwards). Among chalcids, karyotypes of only three members of this group, i.e. *Encarsia berlesei* (Howard, 1906) and *E. inarum* (Walker, 1839) (Aphelinidae) as well as *Nasonia vitripennis* (Walker, 1836) (Pteromalidae) (Odierna et al. 1993; Baldanza et al. 1999; Rütten et al. 2004) were studied using G-banding. This technique identifies different chromosomes within karyotypes of the same species (Gadau et al. 2015), but apparently fails to highlight homologous elements among chromosome sets of closely related parasitoids (see e.g. Odierna et al. 1993; Baldanza et al. 1999), and therefore it cannot be used for a comparative cytogenetic study of parasitoid Hymenoptera.

The modern techniques of differential chromosome staining are mostly represented by using fluorochromes which specifically visualize AT- and GC-rich chromosome segments (Schweizer and Ambros 1994; Gokhman 2015a). Among the former dyes, 4’, 6-diamidino-2-phenylindole (DAPI) is the most widely used. However, chromosomes of parasitoid Hymenoptera predominantly contain AT-rich DNA, and therefore staining chalcid karyotypes with DAPI and similar fluorochromes normally does not reveal any banding pattern (Odierna et al. 1993; Baldanza et al. 1999 etc.), sometimes except for a single negative band per haploid karyotype (Bolsheva et al. 2012). In turn, bands of this kind, which represent NORs, are usually GC-rich, and thus can be stained with chromomycin A₃ (CMA₃) or similar fluorochromes (Gokhman et
Nevertheless, multiple CMA₃-positive and DAPI-negative terminal bands were recently discovered on every chromosome of a particular member of the family Eulophidae, *Trichosipilus diatraeae* Cherian & Margabandhu, 1942, although it seems unlikely that they all represent NORs (Gokhman et al. 2017b). In addition, there are also several fluorochromes, like propidium iodide, which stain total DNA irrespective of its base composition (Bolsheva et al. 2012).

Nevertheless, FISH remains the most powerful tool for analyzing chromosomes of parasitoid Hymenoptera including chalcids (Gokhman 2015a). This technique seems to work particularly well with different DNA repeats (Van Vugt et al. 2005, 2009). Indeed, it is most frequently used, for example, to map clusters of ribosomal DNA (= NORs) in certain members of Chalcidoidea that belong to the families Eurytomidae, Torymidae, Eulophidae, Aphelinidae and Trichogrammatidae (Van Vugt et al. 2005, 2009; Bolsheva et al. 2012; Gokhman et al. 2014a, 2017a). Among other results, these data show that the number and localization of NORs vary within certain chalcid genera, e.g. *Eurytoma* Illiger, 1807 (Gokhman et al. 2014a; see above). Van Vugt et al. (2005, 2009) also mapped the whole fraction of repetitive DNA (C₀t-50) as well as the ITS2 and EcoRI repeats on chromosomes of *Trichogramma kaykai*. Analogously, Li et al. (2017) used the same approach to physically map a number of repeats on a particular B chromosome of *Nasonia vitripennis*. In addition, FISH revealed absence of the TTAGG telomeric repeat in all studied parasitoid Hymenoptera including chalcids (Gokhman et al. 2014a). Moreover, chromosome microdissection together with whole chromosome painting, a powerful technique for identifying particular chromosomes and their segments, was first applied to the karyotype of *N. vitripennis* more than 15 years ago (Rütten et al. 2004; Gadau et al. 2015). To prepare specific probes from each chromosome of this species which haploid karyotype contains five metacentrics of similar size, the chromosomes were first G-banded. Furthermore, Gokhman et al. (2019a) who applied the same technique to the chromosome sets of two cryptic species of the *Lariophagus distinguendus* (Förster, 1841) complex (Pteromalidae), were able to identify elements involved in a certain chromosomal fusion (see below).

Methods of immunocytochemistry also can be used for studying karyotypes of parasitoid Hymenoptera. Up to now, however, this technique was applied only to two closely related species, *Entedon cioni* Thomson, 1878 and *E. cionobius* Thomson, 1878 (Eulophidae) (Bolsheva et al. 2012). Specifically, chromosomes of these parasitoids were treated with antibodies against 5-methylcytosine, which visualized patterns of DNA methylation along different chromosomes.

**Overview of known data**

**General notes**

In the superfamily Chalcidoidea, haploid chromosome numbers (n) can vary from *n* = 3 to *n* = 11 (Table 1, Fig. 1). In fact, a few papers reporting *n* values outside of this range were also published during the previous century (Silvestri 1914;
Muramoto 1993), but those results still need to be confirmed. Among chalcids, the distribution of chromosome numbers at the species level has a clear mode at \( n = 6 \), with a second local maximum at \( n = 10 \) (Fig. 1). Members of this superfamily with \( n = 5 \) are also very numerous, and the proportion of Chalcidoidea with other chromosome numbers is substantially smaller (Table 1, Fig. 1).

Just a decade ago (Gokhman 2009; Gokhman and Gumovsky 2009), chalcid families were generally subdivided into two groups according to their chromosome numbers, i.e. the so-called “low-numbered” and “high-numbered” families. Within these groups, \( n \) values ranged from 3 to 7 and 8 to 11 respectively, with just a few exceptions. Most families belonged to the first group (Fig. 2a–c), whereas higher chromosome numbers were characteristic of Mymaridae, Eurytomidae, and Encyrtidae (Table 1, Fig. 2d). In addition, Aphelinidae contained taxa with both lower and higher \( n \) values. Specifically, the subfamily Aphelininae harbored parasitoids with \( n = 4–5 \), whereas Coccophaginae often had \( n = 10–11 \) (Gokhman 2009). However, \( n = 3–10 \) was found in different species of the large genus Encarsia Förster, 1878 from the latter subfamily (Baldanza et al. 1999). Moreover, \( n = 10 \) was detected in Podagrion pachymerum (Walker, 1833) and P. gibbum Bernard, 1938 (Torymidae) (Fusu 2008a). Furthermore, the above-mentioned pattern also substantially changed during the last years. For example, parasitoids with lower chromosome numbers (\( n = 5 \) to 7) were found within both Encyrtidae and Eurytomidae (Gokhman and Mikhailenko 2008; Gokhman 2010). These

### Table 1. Chromosome numbers of different families of Chalcidoidea. Spalangiinae were earlier considered as a subfamily of Pteromalidae s.l., but they deserve the family rank (Heraty et al. 2013). Torymidae s.l. include Megastigmidae (Janšta et al. 2018), but they are treated here as a single taxon because relationships of the latter group with other chalcid families remain uncertain. Data from: Fusu 2008b, 2009, 2017; Gokhman 2009, 2010, 2015b; Gokhman and Gumovsky 2009, 2013; Bolsheva et al. 2012; Gebiola et al. 2012; Gokhman et al. 2014b, 2017a, 2019bc; Santos et al. 2015; Gokhman and Nishkomaeva 2018; Wu et al. 2019, the present paper and unpublished results of the author.

<table>
<thead>
<tr>
<th>Family</th>
<th>No. species studied</th>
<th>Chromosome numbers (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mymaridae</td>
<td>3</td>
<td>9, 11</td>
</tr>
<tr>
<td>Eulophidae</td>
<td>73</td>
<td>5, 6, 7, 8, 10</td>
</tr>
<tr>
<td>Trichogrammatida</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Aphelinidae</td>
<td>31</td>
<td>3, 4, 5, 6, 7, 8, 9, 10, 11</td>
</tr>
<tr>
<td>Agaonidae</td>
<td>8</td>
<td>5, 6</td>
</tr>
<tr>
<td>Encyrtidae</td>
<td>20</td>
<td>5, 8, 9, 10, 11</td>
</tr>
<tr>
<td>Eupelmidae</td>
<td>22</td>
<td>5, 6, 7, 8, 10</td>
</tr>
<tr>
<td>Eurytomidae</td>
<td>14</td>
<td>5, 6, 7, 8, 9, 10</td>
</tr>
<tr>
<td>Spalangiinae</td>
<td>2</td>
<td>4, 6</td>
</tr>
<tr>
<td>Leucospidae</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Chalcididae</td>
<td>5</td>
<td>3, 5, 6</td>
</tr>
<tr>
<td>Ormyridae</td>
<td>2</td>
<td>5, 6</td>
</tr>
<tr>
<td>Torymidae s.l.</td>
<td>24</td>
<td>4, 5, 6, 10</td>
</tr>
<tr>
<td>Perilampidae</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Eucharitidae</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Pteromalidae</td>
<td>19</td>
<td>4, 5, 6, 7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>237</strong></td>
<td><strong>3, 4, 5, 6, 7, 8, 9, 10, 11</strong></td>
</tr>
</tbody>
</table>
Figure 1. Distribution of main lineages of Chalcidoidea by the chromosome number at the species level (based on data from Table 1).

Figure 2. Representative karyotypes of Chalcidoidea a Trichogramma principium Sugonjaev & Sorokina, 1976 (Trichogrammatidae; n = 5) b Mesopolobus mediterraneus (Mayr, 1903) (Pteromalidae; 2n = 10) c Oomyzus gallerucae (Fonscolombe, 1832) (Eulophidae; 2n = 12) d Eurytoma cynipsea Boheman, 1836 (Eurytomidae; 2n = 20 + 4B). Scale bar: 10 μm.
lower n values could be attributed to independent chromosomal fusions which took place in these groups. Finally, n = 8 to 10 were also detected in certain Eupelmidae and Eulophidae (Fusu 2008b, 2017; Gokhman and Nishkomaeva 2018). As a result of these findings, most principal lineages of Chalcidoidea now include both “high-numbered” and “low-numbered” members (Table 1, Fig. 3).

Chromosomes of Chalcidoidea are generally longer than those found in many other parasitoid Hymenoptera, mainly due to lower chromosome numbers that are characteristic of most chalcids, with average chromosome lengths ranging from 5 to 7 μm (Gokhman 2009). In this group, chromosomes of the “low-numbered” taxa mostly have two distinct arms, i.e. they are either metacentric or submetacentric (Gokhman 2013; Fig. 2b, c). Nevertheless, acrocentric and/or subtelocentric chromosomes can predominate as well, often within karyotypes of certain “high-numbered” chalcids (Gokhman and Gumovsky 2009; Fig. 2d, but see also Fig. 2a). Transitions from the latter character state to metacentrics/submetacentrics usually accompany the process of consecutive chromosomal fusions (see e.g. Gokhman and Mikhailenko 2008).

Among Chalcidoidea, meiotic chromosomes were examined in some detail in a few dozen members of the families Eulophidae, Aphelinidae, Encyrtidae, Eupelmidae, Eurytomidae, Torymidae s.l. (including Megastigmidae) and Pteromalidae (Fusu 2009, 2017; Gokhman 2009 and references therein, Gokhman and Gumovsky 2013; Gokhman et al. 2014b). Specifically, chalcid chromosomes can form rod-like, cross-like or ring-like bivalents in diplotene, as in other members of the order Hymenoptera. Each bivalent usually carries one or two terminal/subterminal chiasmata.

Chromosomal mutations

The following types of chromosomal mutations are characteristic of chalcid karyotypes: (Gokhman 2009): inversions, fusions (both central and tandem ones), translocations, polyploidy, aneuploidy and B chromosome variation. In addition, deletions/duplications probably also occur in this superfamily. Specifically, inversions were detected in certain members of the genus *Aphelinus* Dalman, 1820 (Aphelinidae) (Gokhman et al. 2017a). In this group, haploid karyotypes of most parasitoids that belong to the *varipes* species group with n = 4, contain two metacentric and two acrocentric chromosomes. However, *A. hordei* Kurdjumov, 1913 also has a similar karyotype structure, but the centromere of the second metacentric is significantly shifted towards the chromosome end, and in a certain sister species, *A. kurdjumovi* Mercet, 1930, this centromere becomes terminal, turning the particular chromosome into an acrocentric (Gokhman et al. 2017a). An inversion could also be involved in the process of karyotype transformation within the *Lariophagus distinguendus* species complex (König et al. 2019).

At present, direct evidence for translocations, which occur among Chalcidoidea, is generally scarce. For instance, reciprocal translocations are presumed in certain members of the family Eulophidae (Gokhman 2009). These rearrangements, together with deletions and duplications, are apparently responsible for the numerous size differences between chromosomes of related chalcid species with the same n values (Giorgini
Comparative studies of the genome size complemented with chromosome morphometrics can provide additional insights regarding possible deletions/duplications in closely related forms with similar karyotypes (Gokhman et al. 2017a). Nevertheless, detection of these mutations often requires sophisticated techniques of the chromosome study (see e.g. Gokhman et al. 2019a), and therefore more rearrangements of this kind are undoubtedly going to be discovered within chalcid karyotypes in the future.

Fortunately, other types of chromosomal mutations can be identified more easily among the Chalcidoidea, because these karyotypic changes usually affect the chromosome number of related forms. For example, this parameter decreases via chromosomal fusions,
and the products of these rearrangements can be instantly detected using e.g. chromosome morphometrics or whole chromosome painting (Gokhman et al. 2019a; König et al. 2019). Specifically, more or less well-documented consecutive chromosomal fusions were found in the Eurytomidae. Although parasitoids that belong to this group, and to the genus *Eurytoma* in particular, generally have \( n = 10 \) (Fig. 2d), but \( n = 5, 6 \) and 7 were found in *E. compresa* (Fabricius, 1794), *E. serratlatae* (Fabricius, 1798) and *E. robusta* Mayr, 1878 respectively (Gokhman and Mikhailenko 2008). The number of larger metacentrics observed in these chalcids also corresponded with the above-mentioned scenario. Analogously, two studied members of the genus *Sycophila* Walker, 1871 from the same family, namely, *S. submutica* (Thomson, 1876) and *S. biguttata* (Swederus, 1795), have \( n = 8 \) and 9 respectively (Gokhman and Mikhailenko 2008; Gokhman and Gumovsky 2013). Furthermore, \( n = 10 \) is characteristic of both *Metaphycus flavus* (Howard, 1881) and *M. luteolus* (Timberlake, 1916) (Encyrtidae), but \( n = 9 \) and 5 were respectively found in *M. angustifrons* Compere, 1957 and *M. stanleyi* Compere, 1940 (Gokhman 2010). In addition, Gokhman et al. (2019a) who applied chromosome microdissection and whole chromosome painting to chromosome sets of two cryptic species of *Lariophagus distinguendus* complex with \( n = 5 \) and 6, were able to identify chromosomes involved in a particular fusion. During this process, the only acrocentric and a medium-sized metacentric in the chromosome set with \( n = 6 \) fused into the largest metacentric chromosome in the karyotype with \( n = 5 \). At present, however, it is difficult to distinguish between centric and tandem fusions in the superfamily Chalcidoidea. Nevertheless, since the haploid chromosome set containing eleven subtelocentrics or acrocentrics of similar size is considered ancestral for chalcids (Gokhman 2013), centric fusions could predominate in this group.

Polyploid individuals were found in a few groups of Chalcidoidea. For example, triploid females were found in *Nasonia vitripennis* and certain Aphelinidae (Gokhman 2009 and references therein). In the former species, diploid males and tetraploid females were also detected. However, various attempts to create a stable strain of *N. vitripennis* with tetraploid females and diploid males failed, probably due to the so-called preferential segregation of chromosomes (Crozier 1975). Nevertheless, a particular stock of *N. vitripennis* with triploid females/diploid males can be supported in the lab for many generations (Leung et al. 2019).

At present, the only reliable case of aneuploidy among chalcids is known in *Torymus bedeguaris* (Linnaeus, 1758) (Torymidae). In this species, which usually has \( 2n = 12 \), three copies of the smallest acrocentric chromosome carrying NORs were found in the only specimen with \( 2n = 13 \) (Gokhman et al. 2014a). In addition, Baldanza et al. (1999) reported \( n = 11 \) in a few male individuals of *Encarsia asterobemisiae* Viggiani & Mazzone, 1980 (Aphelinidae) normally having \( n = 10 \) and \( 2n = 20 \). However, this pattern was apparently caused by presence of a particular B chromosome (see below).

Up to now, B chromosomes were found in certain members of the superfamily Chalcidoidea. Specifically, the so-called PSR (paternal sex ratio) B chromosomes were detected in two distantly related chalcid species, i.e. *Nasonia vitripennis* and *Trichogramma kaykai* (Nur et al. 1988; Van Vugt et al. 2005). These paternally inherited chromosomes eliminate all other elements of the paternal genome from the diploid zygote, thus turning...
it into the haploid one. In addition, B chromosomes which apparently do not carry sex-ratio distorting factors, were also found in a few members of the families Aphelinidae and Eulophidae (Baldanța et al. 1999; Gebiola et al. 2012; Gokhman et al. 2014b). For example, the highest number of B chromosomes among parasitoids was detected in *Pnigalio gyamiensis* Myartseva & Kurashev, 1990 (Eulophidae) with 2n = 12 + 0–6B (Gokhman et al. 2014b). Chromosomes of this kind have also been recently found in *Eurytoma cynipsea* Boheman, 1836 with 2n = 20 + 0–4B (Fig. 2d).

**Phylogenetic implications of chromosomal characters**

Chalcid karyotype evolution was previously studied using phylogenetic reconstructions that were based on morphological and/or molecular characters (Gokhman 2009, 2013, see also Gokhman and Gumovsky 2009). Together with other papers published during the last 10–15 years (Gokhman and Mikhailenko 2008; Gokhman 2010; Santos et al. 2015; Gokhman et al. 2017a), these studies revealed a number of synapomorphies of certain higher taxa (e.g. lower chromosome numbers shared by the Eucharitidae and Perilampidae, see Fig. 3) and related species. The best known synapomorphies of the latter kind are represented either by chromosomal fusions in the Eurytomidae and Encyrtidae or by inversions in the Aphelinidae (see above). However, understanding karyotype evolution of many supraspecific taxa of parasitic wasps is far from straightforward. For instance, a detailed molecular analysis suggests n = 6 as an ancestral chromosome number for the *Lariophagus distinguendus* complex (König et al. 2019), although n = 5 is currently considered as an ancestral value for the family Pteromalidae in general (Gokhman 2009).

The problem of phylogenetic reconstruction of karyotype evolution at the level of higher taxa can be illustrated by the example of the Eulophidae, apparently the best studied group of the superfamily Chalcidoidea (Table 1). Indeed, the haploid chromosome set containing five larger metacentrics and a smaller subtelocentric/acrocentric (n = 6) was long considered ancestral for the family, since it predominates in most previously examined lineages of Eulophidae (Gokhman 2009 and references therein). In that case, the karyotype of *Trichospilus diatraeae* which contains four longer metacentric and three shorter acrocentric chromosomes (n = 7), might originate from a centric fission from the apparently ancestral chromosome set (Gokhman et al. 2017b). However, a recent study of *Ophelimus maskelli* (Ashmead, 1900), the only member of the subfamily Opheliminae with the known karyotype, revealed n = 10 (Gokhman and Nishkomaeva 2018). Since this subfamily apparently represents a less derived group of Eulophidae (see e.g. Gumovsky 2008), n = 10 is likely to be considered ancestral for the family in general, with n = 7 and 6 arose from the preceding karyotype by consecutive chromosomal fusions (Gokhman and Nishkomaeva 2018).

In addition, numerous chromosomal fusions lead to independent origins of similar karyotypes within different lineages of Chalcidoidea (Gokhman 2013). Specifically, at least some chromosome sets with n = 10 originated from the apparently ancestral
karyotype containing eleven subtelocentrics/acrocentrics through pairwise fusions. Moreover, further consecutive rearrangements of this kind also led to the multiple origins of chalcid chromosome sets with n = 6 (five larger metacentrics/submetacentrics and a smaller subtelocentric/acrocentric; Fig. 2c). In turn, numerous karyotypes with five metacentric chromosomes (n = 5; Fig. 2b) also can originate through independent fusions of the above-mentioned subtelocentrics/acrocentrics to certain metacentric chromosomes (Gokhman 2013). These parallel transitions apparently occurred in a few distantly related chalcid families, including Eulophidae, Agaonidae, and Torymidae s.l. plus Ormyridae (Fig. 3).

**Taxonomic implications of chromosomal characters**

In the superfamily Chalcidoidea, karyotypic features can have substantial taxonomic implications, and these implications are the most important at the species level (Gokhman 2015b). Specifically, in a few cases different karyotypes were reported for the same parasitoids. Although some of those reports apparently resulted from misidentifications of well-defined different species (see Gokhman 2009 and references therein), cryptic taxa were also involved in certain cases. For example, a chromosome study of the supposedly well-known synanthropic parasitoid of many stored-product pests, *Anisopteromalus calandrae* (Howard, 1881) (Pteromalidae), eventually resulted in the detection and description of a new cosmopolitan species, *A. quinarius* Gokhman & Baur, 2014, with these species respectively having n = 7 and 5 (Baur et al. 2014). Analogously, two morphologically indistinguishable cryptic species with n = 5 and 6 were found in the *Lariophagus distinguendus* complex from the same family (König et al. 2019). In addition, two newly described members of the genus *Eupelmus* Dalman, 1820 (Eupelmidae), *E. barai* Fusu, 2017 and *E. vladimiri* Fusu, 2017, were earlier misidentified as *E. vesicularis* (Retzius, 1783) and *E. impennis* Nikol’skaya, 1952, although the first, the last, and the two remaining species have n = 6, 9, and 5 respectively (Fusu 2017). Similar cases are summarized and discussed in the recent review on integrative taxonomy of parasitoid Hymenoptera (Gokhman 2018).

Variation of chromosome morphology between routinely stained karyotypes of related species with the same n values was also revealed. For instance, two reproductively isolated populations of *Encarsia sophia* (Girault & Dodd, 1915) (Aphelinidae) from Spain and Pakistan have structurally different karyotypes with n = 5 (Giorgini and Baldanza 2004). We also found that chromosome sets of two members of the genus *Trichogramma* Westwood, 1833 with n = 5, i.e. *T. pretiosum* Riley, 1879 and *T. principium* Sugonjaev & Sorokina, 1976, substantially differ in their morphometric parameters (Gokhman et al. 2017b and the present paper; Fig. 2a), contrary to some previous reports for this genus (Hung 1982). Up to now, various techniques of differential staining did not reveal karyotypic differences between closely related species with the same morphology of chromosomes, but this seems possible, given the fact that members of the same genus, for instance, can differ in the number and localization of NORs (Baldanza and Giorgini 2001; Giorgini and Baldanza 2004; Gokhman et al. 2014a).
Future directions

In the coming decades, karyotypic study is undoubtedly going to become an important tool of taxonomic and cytogenetic research on many groups of parasitic wasps, including chalcids. However, this investigation can be effective only if complemented by other modern approaches and techniques. For example, it should be used in combination with a thorough morphological analysis for detecting and identifying cryptic species of parasitoids (Gokhman 2018). This is especially true for the families with a relatively high variation in chromosomal characters, e.g. Encyrtidae, Aphelinidae, Eurytomidae, Pteromalidae etc. (Gokhman 2015a). Since the genome size is generally correlated with the total length of chromosomes, but not necessarily with the overall karyotype structure (Gokhman et al. 2017a), a combined study can highlight hidden chromosomal rearrangements among closely related forms (see e.g. Moura et al. 2020). On the other hand, cytogenetic research of the superfamily Chalcidoidea per se will also benefit from using molecular and similar approaches, which include microdissection and chromosome painting (Gokhman et al. 2019a), immunohistochemical techniques (Bolsheva et al. 2012) and other applications. In turn, some of these techniques could be used to investigate fine structure of meiotic chromosomes of hybrids between closely related chalcid species (see e.g. König et al. 2019). Finally, modern efforts for genome sequencing can also be supported by cytogenetic studies of the Chalcidoidea in a number of ways – from providing direct estimates of the number of linkage groups (which equals to the $n$ value) to the physical mapping of various DNA sequences, especially repetitive ones, using FISH (Gokhman 2009; Gokhman et al. 2017a).

Conclusion

Although a considerable amount of new data of the karyotypic study of the superfamily Chalcidoidea were collected and summarized during the last decade (see e.g. Gokhman 2015a), chromosomes of many chalcid taxa remain totally unknown. Nevertheless, conclusions based on the accumulated data already have important implications for genetics, taxonomy and phylogeny of this enormous group, as well as for its use in biological pest control (Gokhman 2015b, 2018). In turn, phylogenetic and taxonomic research provides essential information which enables better understanding of various cytogenetic phenomena occurring in the Chalcidoidea (Baur et al. 2014; Fusu 2017; Gokhman et al. 2017a; König et al. 2019), and I am sure both these trends are certainly going to continue in the observable future.

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Rapid chromosomal evolution in the bush-cricket
Gonatoxia helleri Hemp, 2016 (Orthoptera, Phaneropterinae)

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Abstract

Gonatoxia helleri Hemp, 2016 is one of the most widespread bush-crickets of the genus Gonatoxia Karsch, 1889 in East Africa. This species with seven large chromosomes (2n♂ = 7) differs from other representatives of the genus Gonatoxia drastically by its reduced chromosome number, the asymmetrical karyotype including karyomorphs rarely found in tettigoniids, as well as in irregularities in the course of meiosis. To better understand the origin of such an exceptional karyotype, chromosomes of 29 specimens from four populations/localities were studied using classical techniques, such as C-banding, silver impregnation, fluorochrome double staining and fluorescence in situ hybridization (FISH) technique with 18S rDNA and (TTAGG)n telomeric probes. FISH showed many 18S rDNA loci as well as interstitial telomeric sequences, where chromosome morphology varied in these components in terms of quantity and distribution. The 18S rDNA loci coincided with active NORs and C-banding patterns. We suggest that a combination of Robertsonian rearrangements and/or multiple common tandem fusions involving the same chromosomes contributed to the formation of this karyotype/karyomorphs. The results are the first step towards a better understanding of chromosomal reorganization and evolution within the genus Gonatoxia. Low chromosome number, together with the incidence of chromosomal polymorphism that is higher in G. helleri than previously reported in bush-crickets, implies that this species can be a valuable new model for cytogenetic and speciation studies. Our findings suggest that chromosomal translocations lead to diversification and speciation in this species and could be the driving force of adaptive radiation.

http://zoobank.org/FE323E4D-F956-459E-9B53-2DEDF78182D9


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Introduction

Chromosome number and structure, including their size and morphology, are important aspects of genome organization, because chromosomal variation may lead to species divergence. The analysis of the karyotype is also a useful feature in the systematic and evolutionary analysis because closely related species tend to have more similar karyotypes than more distinctly related ones (Sumner 2003). Changes in chromosome numbers (karyotype variability) or chromosome polymorphism within species as observed in many plant and animal groups may be involved in adaptation (e.g. Potter et al. 2017). The role of chromosomal rearrangements (translocations, inversions, changes in chromosome number) in the formation of reproductive barriers has been investigated and found to play a causal role in the isolation of species or populations in some genera of Hemiptera (Mills and Cook 2014, Chirino et al. 2017), Diptera (Coluzzi et al. 2002), Coleoptera (Kobayashi et al. 2000, Xavier et al. 2018), Lepidoptera (Vershinina and Lukhtanov 2017, Lucek 2018), and Orthoptera (e.g. Kawakami et al. 2011, Taffarel et al. 2015, Buleu et al. 2019, Silva et al. 2019).

Comparative cytogenetics, as a powerful tool to study karyotype variation, is based on accurate chromosome identification. Physical mapping involves fluorescence in situ hybridization (FISH) of specific segments of genomic DNA to their physical location on chromosomes, and it is useful in terms of gaining an insight into structural arrangements within the genome. The presence of repetitive DNA clusters in some genomic regions may represent fragile breakage sites that are associated with rearrangements during chromosome evolution (e.g. Schneider et al. 2013). Recently, series of works with FISH and conventional chromosome banding showed that the number and location of rDNA and heterochromatin sites can be useful markers for the study of tettigoniid karyotype evolution, and for the identification of genus/species-specific patterns (e.g. Grzywacz et al. 2011; Warchałowska-Śliwa et al. 2011, 2013a; Grzywacz et al. 2014a, b).

East Africa is a region of exceptional diversity of Orthoptera including Tettigoniidae bush-crickets (e.g. Hemp et al. 2013a, b, 2017). In the last few years, numerous papers have been published about East African Phaneropterinae taxa including descriptions of new genera and species combined with genetic studies, mainly on chromosome level (e.g. Hemp et al. 2010a, b, 2014, 2015a, b, c; Warchałowska-Śliwa et al. 2015; Hemp et al. 2016a, b, 2018). Gonatoxia Karsch, 1889 is a poorly known genus occurring in East Africa from which to date four species have been described. From G. maculata Karsch, 1889, little is yet known, although it is probably widely distributed throughout Tanzania, Kenya and Somalia, inhabiting deciduous dry forests and savanna woodlands in northern Tanzania; also little is known for G. immaculata Karsch, 1889, a species...
adapted to wet lowland forest in the East Usambara Mountains and along the Tanzanian coast; *G. furcata* Hemp, 2016 is probably endemic to the Udzungwa Mountains; *G. helleri* Hemp, 2016 was found syntopically at some localities with *G. immaculata* and *G. furcata*. The ecological niche of *G. helleri* seems to be broader than in the other species of the genus, as it occurs from coastal and lowland wet forests (e.g. East Usambara Mountains) up to montane elevations (e.g. Uluguru Mountains).

*Gonatoxia* is a very unusual genus within the subfamily Phaneropterinae, characterized by rarely observed high variability of chromosomes (both chromosome number and structure, $2n^{♂} = 7, 27$ or $29$) in bush-crickets (Hemp et al. 2016a). Our preliminary analysis of *Gonatoxia* showed that compared to other investigated East African Phaneropterinae, *Gonatoxia helleri* had the lowest number of chromosomes ($2n^{♂} = 7$). Our cytogenetic studies indicated that such dramatic chromosomal rearrangements probably took place during a relatively early stage of speciation in *G. helleri* which is one of the most wide-spread and intriguing species of the genus so far (Hemp et al. 2016a). In the present study, a detailed cytogenetic characterization of *G. helleri* was performed using different techniques including mapping of repetitive DNA sequences characterizing chromosomal diversity. Based on the markers obtained we try to clarify the rearrangements responsible for intra- and inter-specimen chromosomal variability. It is important to investigate the potential role of repetitive DNA in the chromosomal evolution of this species.

**Material and methods**

Cytogenetic analysis was conducted on 19 males and 10 females of *G. helleri* collected from four populations/localities in northern Tanzania: Morogoro District, Udzungwa Mountains [Ud], National Park Headquarters, Mangula Gate, lowland wet forest, 300 m (males: CH7949, CH8048, CH8087, CH8088, CH8089, CH8144, CH8145, CH8247; females: CH8072, CH8073, CH8138, CH8139, CH8146, CH8147), and Uluguru Mountains [Ul], forest above Morningside, 1800–2100 m (males: HE89, HE96, HE105, CH8246, CH8251, CH8252, CH8253; females: CH8250, CH8289) as well as East Usambara, Nilo [Ni] forest reserve, lowland wet to a submontane forest, 450–1150 m; (male CH8134, HE97, HE104; female CH8135) and Sigi Trail [Si], 450 m, East Usambara Mountains (male CH862; female CH8136).

Testes, ovaries, and somatic hepatic caeca were dissected, incubated in hypotonic solution (0.9% sodium citrate) and fixed in Carnoy’s solution [ethanol – acetic acid (3:1, v/v)], squashed in 45% acetic acid, followed by removal of coverslips using the dry ice technique and air-drying. For karyotyping and the identification of chromosome rearrangements, the preparations from all specimens were used for C-banding according to Sumner (1972). Additionally, some slides were analysed qualitatively by CMA$_3$ (chromomycin A$_3$) and DAPI (4,6-diamidino-2-phenylindole) staining (Schweizer 1976) as well as by AgNO$_3$ (silver nitrate) staining to visualize active nucleolus organizer regions (NORs) (Warchałowska-Śliwa and Maryańska-Nadachowska 1992).
The best preparations (for individuals Ud: CH7949, CH8048, CH8088; Ul: HE89, HE96, CH8252; Si: CH621; Ni: HE97) were used for fluorescence in situ hybridization (FISH). All FISH experiments with 18S rDNA and telomeric probes were carried out according to the protocol described in Grzywacz et al. (2018). Unlabelled 18S rDNA probe was generated by PCR, using the genomic DNA of bushcrickets as templates. The probe was labelled with biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany). The telomeric probe was generated by non-template PCR. The unlabelled telomeric probe was labelled with digoxigenin-11-dUTP (Roche Diagnostics GmbH). The detection of biotin-16-dUTP and digoxigenin-11-dUTP was carried out by avidin-FITC (Invitrogen, USA) and anti-digoxigenin rhodamine (Roche Diagnostics GmbH), respectively. Finally, the slides were counterstained with DAPI and mounted in the DABCO-based antifade solution. Preparations from FISH experiments were observed under a fluorescence microscope. Color images were recorded with a CCD DS-U1 camera using the NIS-Elements BR 3.0 software package. For each individual, at least 10 mitotic metaphase (oogonial/spermatogonial) and/or 20 meiotic divisions were analyzed using different markers.

Results

The study of mitotic metaphase spermatogonial, oogonial, and somatic gastric caeca cells showed 2n = 7 (6+X), FN = 10–13 in most cells of the male and 2n = 8 (6+XX), FN = 11–14 in the female. In the karyotype, the first long pair of autosomes was metacentric, whereas the second long (three main karyomorphs) and small third pairs (four main karyomorphs) were polymorphic with respect to the morphology of homologous chromosomes in specimens of the analyzed localities. The 2nd chromosome pair showed three main karyomorphs: homozygous metacentric (2A) [18 specimens: Udzungwa (Ud) 9, Uluguru (Ul) 3, Nilo (Ni) 4, Sigi (Si) 2], heterozygous – subacro/acro (2B) [9 specimens: Ud 5, Ul 4] and homozygous acrocentric (2C) [2 individuals from Ul]. The 3rd chromosome pair was greatly polymorphic and was observed in both Ud and Ul populations. It should be noted that in individuals from Si and Ni populations (few individuals analyzed), the 1st and 2nd chromosome pairs were homozygous (both bi-armed) in terms of chromosome morphology. The acrocentric sex chromosome (X) was the largest element of the set (Figs 1–3).

Constitutive heterochromatin blocks with pericentromeric thick C-bands were found in all chromosomes. Additionally, the bi-armed first pair possessed thin telomeric and two interstitial (near the centromeric region in one arm and thin near the end in the second arm) C-bands, which are a feature in distinguishing this pair from the 2nd pair, more or less similar in size. The heterochromatin in the 1st pair revealed a discrete size polymorphism in the C-patterns. Also, the 2nd (karyomorphs 2A, 2B, 2C) and 3rd chromosome pairs showed heteromorphism in terms of the size/locality of bands on respective homologous chromosomes. Pericentromeric, interstitial and terminal C-bands with differences in size were observed on the acrocentric X chromosome (Figs 1a, a’, 2a, e, 4). The secondary constriction (not always seen) of the 2nd chromosome and the
X chromosome were located near the C-band (Figs 1a, a’, 2a, e). Besides a large active NOR in 3rd chromosomes/bivalent and a smaller in the 2nd, being coincident with secondary constrictions, a site with faint silver nitrate staining was observed, indicating the occurrence of small NORs, probably “secondary NORs”, in bivalents and the X chromosome (Figs 1b, e, 2b). Positive C-blocks in the 1st chromosome pair were neutral for G+C (DAPI+) and A+T (CMA 3+) base pairs, whereas the 2nd and 3rd pair

Figure 1. Examples of C-banding (a, a’), silver nitrate staining (b, e) and fluorescence in situ hybridization (c-c”, d-d”) in individuals with karyomorph 2A from populations: Udzungwa Mts (Ud CH8089) (a), Sigi (Si CH8621) (a’), Nilo (Ni HE97) (b, c-c”, d-d”, e). C-banding karyotypes of males chromosome complement (arranged from mitotic metaphase – right side); open arrows point to interstitial C-bands in the X chromosome and chromosome pairs 1st and 2nd; black arrowheads indicate secondary constriction (a, a’). AgNO 3 staining in male spermatogonial metaphases (a) and metaphase I/ diplotene (e) revealed medium sized and large active NORs of the bivalents 2nd and 3rd (black arrows) and very small NORs seen in the X (open arrows). FISH using 18S rDNA (green – c, c’, d, d’) and telomeric DNA (red – c, c”, d, d”) probes in mitotic metaphase (c) and metaphase I (f); white arrowheads point to rDNA clusters near centromeric, interstitial and telomeric regions of the chromosomes (c’, d’) and white arrows ITS signals (c”, d”). Heterochromatin (a, a’, b, e) and hybridization areas (c, d) vary in size between homologous chromosomes, which are marked with asterisks (*). Elements (e) arisen from rearrangements were found (e). The X chromosome is indicated. Scale bars: 10 μm.
Figure 2. Examples of C-banding (a, e), silver nitrate staining (b), C-, DAPI and CMA, stained heterochromatin (f–f”) and FISH (c–c”, d–d”) in individuals with karyomorph 2B (a–d”) and 2C (e, f”) from populations: Udzungwa Mts (Ud CH7949 and CH8088) (a, b), Ud CH8088 (c–c”), Uluguru Mts (Ul HE89) (d–d”). C-banding karyotypes of males chromosome complement (arranged from mitotic metaphase – right side); open arrows point to interstitial C-bands in the X chromosome and chromosome pairs 1* and 2*; black arrowheads indicate secondary constriction (a, e). AgNO₃ staining (b) at diplotene revealed large active NOR of the 3rd bivalent (black arrows) and very small NORs seen in the X and bivalents (open arrows). FISH using 18S rDNA (green – c, c’, d, d”) and telomeric DNA (red – c, c”, d, d”) probes in mitotic metaphase (c) and diakinesis (d); white arrowheads point to rDNA clusters near centromeric, interstitial and telomeric regions of the chromosomes (c’, d’) and white arrows ITS signals (c”, d”). C/DAPI/CMA3 blocks were located very close to each other, but bright CMA3 signals coincided with active NORs. Heterochromatin (a, e, f) and hybridization areas (c, d) vary in size between homologous chromosomes, which are marked with asterisks (*). The X chromosome is indicated. Scale bars: 10 μm.
of chromosomes with interstitial C-bands revealed clearly seen CMA\textsubscript{3}+ block (for example Fig. 2f, f', f") which coincided with active NORs.

Physical mapping by FISH with the 18S rDNA and telomeric probes was performed in eight individuals from four analyzed populations (Ud: CH7949, CH8048, CH8088; Ul: HE89, HE96, CH8252; Si: CH621; Ni: HE97). Generally, all examined specimens demonstrated similar rDNA signals located in the centromeric, interstitial and telomeric regions and usually were connected with C-positive regions. The acrocentric/ subacrocentric 3\textsuperscript{rd} chromosome pair carried major rDNA located near the centromeric region and interstitial minor 18S rDNA clusters. Additionally, low-intensity/small clusters on the 2\textsuperscript{nd} chromosome pair and the X chromosome, both near the centromeric regions were observed (Figs 2c, c', d, d', 3c, c', d, d', 4). Heteromorphism of rDNA-signals (marked with an asterisk), similar to C-heterochromatin bands (Figs 1a, a', 2a), was observed in terms of the size/ strength or presence/ absence on the homologous chromosome depending on the karyomorphs in the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} chromosome pairs. Some interstitial C-positive regions in the sex chromosome contained rDNA (Figs 1c', d', 2c',d', 4).

FISH analyses with the (TTAGG\textsubscript{n}) probe generated signals in telomeres of all chromosomes but the size of the clusters on different arms of some chromosomes and between individuals, with different karyomorphs varied as well (Figs 1c", d"; 2c", d"). In addition to the typical telomeric, of the so-called interstitial telomeric sequences (ITSs) in the inner parts of all chromosomes were observed at both mitotic metaphase (Figs 1c", 2c") and diakinesis/ metaphase I (Figs 1d", 2d"). The signals within secondary constrictions were much larger than those at the chromosome ends. The 2\textsuperscript{nd} chromosome pair in the karyomorph A exhibited heteromorphic signals of ITS in both the bi-armed chromosomes in the centromeric region (Fig. 1c", d"). Whereas bright hybridization signals were detected in the centromeric region in the bi-armed homologue and interstitially located in the acrocentric homologue of the 2\textsuperscript{nd} pair in karyomorph B (Fig. 2c", d"). Besides that, additional week ITS signals, present in a low copy number, in subterminal/ medial position in autosomes and sex chromosome were observed (Figs 1c", d", 4).

In some individuals with a chromosome number close to the diploid count, intra-individual variability cells with 14 (male) and 16 (female) chromosomes were observed, probably corresponding to tetraploid levels. Thus, based on the analysis of 50 metaphase cells per individual, about 11% oogonial (Ud: CH8073, CH8138, CH8147, Ni: CH8135, Ul: CH8250) and about 5% spermatogonial cells (Ud: CH7949, CH8048, CH8088, CH8145, Ul: HE89, HE96, HE105, CH8251, CH8251, Ni: CH8134) had tetraploid chromosome numbers (Fig. 3a, c, d). However, in somatic gastric caeca mitotic metaphase in both male (Ud: CH7949, CH8088, Ul: CH8252, Si: CH8621) and female (Ud: CH8146, CH8147, Ul: CH8289, Ni: CH8135) cells with intra-individual variability of chromosome numbers were not observed.

Cytogenetic preparations of the females did not show cells in meiotic division. Male meiosis was classified as synaptic and chiasmate because the chromosomes were generally paired during early pachytene stage and bivalents exhibited a chromosomal configuration indicating crossing over (Fig. 3f upper right corner). In anaphase I, the
X chromosome segregated to one pole and in metaphase II, there were 3 (3+0) or 4 (3+X) chromosomes, confirming the regular segregation of all chromosomes during the first meiotic division. (Fig. 3f, f’). However, in some specimens from different populations: Ud (CH8088, CH8089, CH8144, CH8138, CH8147, CH8247),
Ni (HE97) and Ul (HE96, CH8246) sporadically in pachytene spermatocytes, the chromosomal elements showed asynapsed interstitial and/or terminal chromosomal segments (Fig. 3g, j) and in the prophase I a multivalent–like chain or end-to-end association comprising three autosomal elements (Fig. 3h, i). Furthermore, lightly stained, less condensed chromatin in some regions on the bivalents (chromosome pair 3rd) or one of homologous chromosomes in mitotic metaphase, deletions, or fragments of chromosomes/elements were observed in both male (Fig. 3d, j) and female (Fig. 3e) cells. Additionally, in these populations, very rarely mitotic spermatogonal cells showed intra-individual variability since one autosome was lacking.

Discussion

The result obtained here is in accordance with the previous study about diploid chromosome numbers in *Gonatoxia helleri* (Hemp et al. 2016a). It is probably a young species with specific karyotypic macrostructure, a very interesting case with the lowest number of chromosomes (2n♂ = 7, X0 type of sex determination) compared to other species of the genus *Gonatoxia* (2n♂ = 29, 27, X0) and Phaneropterinae as a whole. Such a low number of chromosomes, in addition to morphological differences, allowed describing the analyzed individuals as a new species in this genus (Hemp et al. 2016a). To date, a similar karyotype has been described only in the Australian *Yutjuwalia sallyae* Rentz, 2001 (Listroscelidinae), with 7 metacentric chromosomes in the male, but without additional information (Ueshima 2001). Most East African bush-crickets of Phaneropterinae, e.g. *Parapyrrhicia* Brunner von Wattenwyl, 1891, *Eurycorypha* Stål, 1873, and *Plangia* Stål, 1873 have uniform karyotypes (Hemp et al. 2013a, 2015a, 2017) with a diploid chromosome number (2n♂) 31 or 29 and the X0 type of sex determination.

The main characteristic of the karyotype of *G. helleri* is the presence of very large autosomes compared to the other species of this genus, based on the analysis of the main relative lengths of the autosomes (Warchałowska-Śliwa et al. in preparation). This reflects the derivation from multiple rearrangements. Even under the assumption that fusions or inversions are frequent in orthopteran chromosomal evolutionary history (e.g. White 1973, Hewitt 1979, Warchałowska-Śliwa 1998) leading to a reduced diploid number, the karyotype of *G. helleri* is an extreme example compared to other phaneropterines and *Gonatoxia* species (Warchałowska-Śliwa et al. in preparation). In specimens analyzed in this paper, probably Robertsonian translocations (centric fusion) and tandem fusions and/or inversion were the reason of the observed morphological arrangements in the 1st chromosome pair in all investigated populations of *G. helleri*. However, in the 2nd chromosome pair, such types of chromosomal rearrangements occurred only in the Sigi (Si) and Nilo (Ni) populations of the species. In some individuals, the 2nd chromosome pair [Udzungwa Mts (Ud) and Uluguru Mts (Ul)] and the 3rd chromosome pair (Ud, Ul, and Ni) were polymorphic with respect to the morphology (bi-armed or acrocentric) of homologous chromosomes. Unfortunately, not much can be said about the variability of the karyotype in individuals from the Sigi population due to the insufficient number of individuals analyzed. Robertsonian trans-
locations are the basic mechanism of rearrangements of chromosomes in the evolution of the orthopteran karyotype, especially in Acrididae grasshoppers (e.g. Taffarel et al. 2015) and some katydids Tettigoniiniae, Saginae and Bradyporiniae (Warchałowska-Śliwa et al. 2009, 2013a; Grzywacz et al. 2017). These rearrangements are not usually found in Phaneropterinae, as here tandem fusions dominate in karyotype evolution (e.g. Warchałowska-Śliwa 1998). Due to such extensive reorganization, it is very difficult to determine the order and evolutionary causes of these specific rearrangements that led to the extreme karyotype evolution of *G. helleri*, compared to the probable ancestral number of chromosomes (*2n♂ = 29*) in the genus *Gonatoxia*.

Physical mapping involving fluorescence *in situ* hybridization of specific segments of genomic DNA is extremely useful in terms of making an insight into structural rearrangements within the genome. Repetitive sequences change rapidly during evolution, providing excellent markers for the identification of chromosomes, chromosome segments and the resulting evolutionary chromosome rearrangements (e.g. in crickets: Palacios-Gimenez et al. 2015). Generally, Phaneropterinae usually carry one rDNA/NOR (per haploid genome), found in the pericentromeric region or rarely located interstitially (e.g. Grzywacz et al. 2011; Warchałowska-Śliwa et al. 2011, 2013b; Grzywacz et al. 2014a, b). In other orthopterans, a high number and variation of rDNA was observed mainly in the grasshopper taxa *Podisma sapporensis* Shiraki, 1910 and *P. pedestris* (Linnaeus, 1758) (VelTsos et al. 2009, Grzywacz et al. 2019), *Eyprepocnemis plorans* (Charpentier, 1825) (Cabrero et al. 2003), or *Abracris flavolineata* (De Geer, 1773) (Ferretti et al. 2019). Compared to phaneropterines and other tettigonids, the chromosomal distribution of major 18S rDNA in *G. helleri* described in this paper is unique because: (i) it occurs in a high number of sites with different intensity of hybridization signals, (ii) it is located pericentromerically, interstitially and near the telomeric regions, (iii) clusters of various size or heteromorphic structures occur in the same chromosome pair. The 3rd chromosome pair (most variable in the amount of heterochromatin) carried large/high intensity clusters near the pericentromeric region, whereas in the 2nd chromosome pair and the X chromosome this cluster was very small. Additionally, interstitial regions with a very low intensity of hybridization signals of 18S rDNA were seen located in different chromosomes (Fig. 4). Generally, our results demonstrate a coincidence between the location of rDNA loci, C-positive (Fig. 4) and GC-rich heterochromatin regions as well as active NORs (occurring in secondary constrictions and small ones in bivalents and the X chromosome). It should also be noted that heterochromatin amplification or loss could be responsible for the variation in the morphology among karyomorphs in all three pairs of chromosomes. In some grasshoppers a large variety of localization of 18 rDNA and tDNA clusters is not associated with structural rearrangements of chromosomes in the karyotype but to the evolution/massive amplification of repetitive DNA (Bugrov et al. 2016, Buleu et al. 2019).

Both conventional heterochromatin staining and rDNA-FISH revealed size heteromorphism/polymorphism between homologous chromosomes, indicating either recent or rapid evolution in this species. The presence of individuals with heteromorphic pairs may be a result by unequal meiotic cross-over, tandem duplication of ribosomal
sequences and related to sister chromatid exchange or translocation rearrangements or homologous recombination (e.g., Cabral-de-Mello et al. 2011; Warchałowska-Śliwa et al. 2013a,b; Grzywacz et al. 2014a, b).

Another universal probe is the telomeric sequence \([\text{tDNA}, (\text{TTAGG})_n]\) that itself is an ideal marker for the identification of chromosome ends (Kuznetsova et al. 2020) and a marker for chromosome rearrangements, being conserved in many groups of insects (Vítková et al. 2005). Interstitial telomeric sequences (ITSs) reflect remnants of multiple chromosome fusions of ancestral chromosomes e.g. in moths (Lepidoptera, Noctuidae: Rego and Marec 2003), giant water bugs (Hemiptera: Chirino et al. 2017) or grasshoppers (Jetybayev et al. 2012, Grzywacz et al. 2019). The individuals examined in this study showed differences in the intensity and position of the hybridization signals of the \((\text{TTAGG})_n\) probes in both autosomes and sex chromosomes (Fig. 4). Variation in the intensity of signals in chromosomes, including sex chromosomes,
result from differences in the length of target TTAGG sequences. Sometimes the lack of hybridization signals in some ends of chromosomes suggests a low number of copies of telomeric repeats (López-Fernández et al. 2004).

The karyotype described here for *G. helleri* is different from that described for other species of this genus since we found a reduction in the number of acrocentric pairs (Hemp et al. 2016a), and probably an inter-population variation (more individuals should be analyzed). In this species several sites with ITS were identified in addition to the terminal/true telomeric sequences. The presence of ITSs near the pericentromeric and interstitial and/or near telomeric region (Fig. 4) suggest that this karyotype /these karyomorphs could be the result of telomere-telomere fusions of the chromosomes, inversions (intra-chromosomal rearrangements), unequal crossing over, or the insertion of telomeric DNA into unstable sites during the repair of double-strand breaks (e.g. Bolzán and Bianchi 2006). ITS repeats in the pericentric C-positive block of the bi-armed chromosome pairs in the 2nd and the smaller 1st chromosome pairs of *G. helleri* created through centric fusion has never been recorded in any phaneropterine so far. Thus, it cannot be excluded that these centric fusions are not the result of Robertsonian rearrangements (i.e. fusion-fission cycle) but are true fusions that left remnants of telomeric DNA in the arms of the acrocentric chromosomes involved in the fusion. A similar observation was described in the grasshopper species *Chorthippus jacobsoni* (Jetlybayev et al. 2012). In this case, the chromosome break points are localized near the centromere, which are less frequently involved in the formation of dicentric chromosomes. Additionally, it is also worth noting that the acrocentric X chromosome with some interstitial ITSs thin C-bands (Figs 1c’, d’, 4) and sometimes secondary NORs (Figs 1a, a’, e, 2a, b, e) might have undergone sequential inversions and/or end-to-end fusions during the chromosome evolution of *G. helleri*.

Some individuals of *G. helleri* exhibited multivalent chromosome associations during meiosis I, asynapsed and/or heterosynapsed chromosome segments and bivalents with distinctly associated regions (gaps and less condensed chromatin) in postpachytene nuclei. These findings indicate that certain chromosome regions were non-homologous and carried heterozygous chromosomal rearrangements. Various degrees of heterosynapsis/asynapsis have also been described in other organisms, which were heterozygous for paracentric or pericentric inversions in grasshoppers or scorpions (e.g. Díez and Santos 1993, Mattos et al. 2013). Variation as the chromosome polymorphism observed in *G. helleri* indicates that the meiotic segregation of these chromosomes has not led to the production of gametes with unbalanced chromosomes and consequent fertility loss. It suggests that some of the chromosome mutations had no negative impact on the carriers and were neutral or may have even increased fitness (Menezes et al. 2013). In *G. helleri*, based on the orientation of the chromosomes in metaphase I and the haploid complement observed in metaphase II, the chromosomes analyzed in the present work probably underwent balanced segregation. A fascinating intra-individual variability of chromosome numbers in some mitotic spermatocytes/oogonia and in meiotic cells was observed as tetraploid cells. This observation could indicate the occurrence of endopolyploidy. The absence of polyploid cells in metaphase II nuclei can be assumed
that the “aberrant cells” degenerated during the cellular cycle as errors in chromosome segregation (Mattos et al. 2013). The formation of polyploid mitotic cells can be probably associated with problems in cytokinesis that disrupt chromosome segregation.

Based on the results presented in this paper, we suggest that the change in chromosome numbers associated with multiple chromosomal rearrangements and observed heterozygous chromosomes may have presented a precondition to colonize new habitats and might be a case of adaptive radiation in *G. helleri*. Generally, the occurrence of chromosomal changes may be the result of ancestral allopatry, sympatry, and/or hybridization (meiotic and mitotic instability), demographic processes associated with colonization (founder effect), environmental fragmentation or a combination of these factors, and it may also point to recent speciation processes and hybridization (e.g. Dion-Côté et al. 2017; Gould et al. 2017). An excellent example for the role of chromosomal rearrangement in speciation in orthopteran insects is the Australian genus of morabinae grasshoppers *Vandiemenella* Key, 1976, specifically the *viatica*-species group. All taxa of this genus show extensive chromosomal variation, parapatric distribution patterns, and narrow zones at their boundaries. A number of population genetic and phylogenetic studies showed “extensive non-monophyly of chromosome races and suggest that geographical isolation leading to the fixation of chromosomal variants in different geographic regions, followed by secondary contact, resulted in the present day parapatric distribution of chromosomal races” (review Kawakami et al. 2011).

*Gonatoxia helleri* is the only *Gonatoxia* species able to inhabit almost the complete offer of ecological niche forests in eastern Africa, while most other species of this genus are restricted to certain forested types. Many or maybe even most bush-cricket taxa probably were first forest dwellers and later adapted to open land habitats in Africa (Voje et al. 2009) due to the beginning aridification of Africa about 8 Mya (deMenocal 2004; Cohen et al. 2007). It cannot be excluded that inter-population differences in the genome may be a sign that species in high elevations of the Uluguru Mts and Udzungwa Mts have a different karyomorph compared to lowland populations of *G. helleri* along the coast, East and West Usambara (Nilo and Sigi populations). On the other hand, there may already have been a selection of different populations because they are now isolated from each other. Our results suggest that forest dwelling *Gonatoxia* species with a restricted area of occurrence, such as *G. furcata* and *G. immaculata*, are more basal taxa, and species which are wide-spread and inhabiting a broad ecological niche, such as *G. maculata* and *G. helleri*, might be evolutionary more recent species. *Gonatoxia helleri* is probably the most plastic and adaptive species as well as has the most dramatically rearranged genome and inter-specific differences even within one population and might be the youngest species of the genus.

In conclusion, the cytogenetic analysis of *G. helleri* provides a new example of chromosomal evolution by multiple rearrangements. Several rearrangements, probably including primary (insertion, deletion or duplication, peri- or paracentric inversion, and intra- or interchromosomal reciprocal translocation) or secondary translocations were responsible for the formation of the karyotype and karyomorphs in *G. helleri*. The bi-armed chromosomes of the 1st pair occurring in individuals from all populations...
probably originated by Robertsonian fusion, whereas the other two pairs in the set are still subject to continuous rearrangements. At this moment, the insufficient number of individuals analyzed from the Nilo Forest Reserve and the Sigi Trail populations in the East Usambaras does not allow to determine possible differences of individuals within and between the populations. Nevertheless, we suggest that these chromosome mutations had no negative impact on the fitness of carriers.

The present study demonstrates that molecular cytogenetic techniques as useful tools for understanding chromosomal organization and evolutionary history in the genus *Gonatoxia*. The chromosome number is lower and the degree of chromosomal polymorphism is greater in *G. helleri* than previously reported in bush-cricket. Our results suggest that this species may be a valuable new model system for further studying the potential role of morphological rearrangements of chromosomes in speciation. We determine the possibility that chromosomal rearrangements might be a driver of adaptive radiation enabling a species to broaden its ecological niche and thus higher adaptability to changing climatic conditions. The adaptive significance of chromosomal rearrangements for *G. helleri* and the origin of such low diploid chromosome numbers require additional genetic analyses, especially the development of multiple cytogenetic markers and molecular studies.

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Chromosomal evolution in the bush-cricket Gonatoxia helleri


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Comparative cytogenetic of six species of Amazonian Peacock bass (Cichla, Cichlinae): intrachromosomal variations and genetic introgression among sympatric species

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Abstract
Cytogenetic data for the genus Cichla Bloch et Schneider, 1801 are still very limited, with only four karyotype descriptions to date. The sum of the available cytogenetic information for Cichla species, points to a maintenance of the diploid number of 48 acrocentric chromosomes, considered a typical ancestral feature in cichlids. In the current study, we performed molecular and classical cytogenetic analyses of the karyotype organization of six species of Cichla, the earliest-diverging genus of Neotropical cichlids. We cytogenetically analysed Cichla kelberi Kullander et Ferreira, 2006, Cichla monoculus Agassiz, 1831, Cichla piquiti Kullander et Ferreira, 2006, Cichla temensis Humboldt, 1821, Cichla vazzoleri Kullander et Ferreira, 2006 and Cichla pinima Kullander et Ferreira, 2006, including three individuals that showed mixed morphological characteristics, likely from different species, suggesting they were hybrid individuals. All individuals analysed showed 2n = 48 acrocentric chromosomes, with centromeric heterochromatic blocks on all chromosomes and a terminal heterochromatic region on the q arm of the 2nd pair. Mapping 18S rDNA gave hybridization signals, correlated with the nucleolus organizer regions, on the 2nd pair for all analyzed individuals. However, we found distinct patterns for 5S rDNA: interstitially at the proximal position on...
6th pair of four species (C. kelberi, C. pinima, C. piquiti and C. vazzoleri), and on the distal of the 4th pair in two (C. monoculus and C. temensis). Accordingly, we present here new data for the genus and discuss the evolutionary trends in the karyotype of this group of fish. In addition, we provide data that supports the occurrence of hybrid individuals in the Uatumã River region, mainly based on 5S rDNA mapping.

Keywords
5S rDNA, FISH, Heterochromatin, Hybridization, karyotype

Introduction

The genus Cichla Bloch et Schneider, 1801 belongs to the subfamily Cichlinae that, jointly with Retroculus Eigenmann et Bray, 1894, makes up the tribe Cichlini, and is the earliest-diverging lineage of Neotropical cichlids (Leo Smith et al. 2008). This taxon is widely distributed within the Amazon, Tocantins, and Orinoco River basins, and in the smaller rivers draining the Guianas to the Atlantic Ocean. Most Cichla species follow an allopatric distribution pattern, although some species are sympatric or even syntopic (Kullander and Ferreira 2006). However, some species, such as C. monoculus Agassiz, 1831, C. kelberi Kullander et Ferreira, 2006 and C. piquiti Kullander et Ferreira, 2006, have been introduced into other areas, where they are well established, due to their generalist habit. Cichla are very emblematic fish in South America, with high economic and ecological importance, especially since they are predators in Amazonian rivers and used widely for sport fishing (Nascimento et al. 2001; dos Santos et al. 2016; Diamante et al. 2017).

Representatives of the genus Cichla are easily distinguished from all other Neotropical cichlids by the shape of the dorsal fin, and the presence of 1 to 4 dark vertical bars along the body. However, the species are very similar, and while their color patterns still provide the best species diagnostic characters, in some cases these may complicate accurate identification, since key characters may show ontogenetic changes (Kullander and Ferreira 2006).

According to Kullander and Ferreira (2006), the genus comprises 15 morphologically distinct species, and recently another species has been described (Cichla cataractae Sabaj et al. 2020) from the Essequibo River basin, where it is endemic). However, Willis et al. (2012), based on multilocus data, recognized only eight species. The species often have restricted natural distributions, but to variable extents. For example, while C. monoculus is found all over the Amazon River and low tributary course, C. temensis Humboldt, 1821 is found only in black water rivers, whereas C. piquiti and C. kelberi are restricted to the Tocantins River.

Cytogenetic data concerning the family Cichlidae points to a remarkable trend in the maintenance of the diploid number 2n = 48, mostly in the acrocentric form (Thompson 1979). However, as more species were karyotyped, a huge chromosomal diversity was observed in the derived clades (ranging from 32 to 60 chromosomes), but with predominance of 2n = 48 in most lineages, which is considered an ancestral trait for this group (Feldberg et al. 2003; Gross et al. 2009; Poletto et al. 2010; da Costa et al. 2019). For the genus Cichla, only C. monoculus, C. temensis, C. kelberi and C. piquiti have had their karyotypes described, all exhibiting a diploid number com-
posited of 48 acrocentric chromosomes, as the species from earliest-diverging Cichlinae tribes (Retroculini, Astronotini and Chaetrobranchini) (Feldberg et al. 2003; Alves-Brinn et al. 2004; Poletto et al. 2010; Mourão et al. 2017).

Interestingly, Alves-Brinn et al. (2004), based on cytogenetic data, reported the occurrence of hybridization between *C. monoculus* and *C. temensis* in the Uatumá River (Balbina Hydroelectric Dam). In addition, interspecific hybridization and introgression between species has been much discussed in relation to the adaptive advantages and increase of genetic variability (Willis et al. 2012). For some authors, hybridization may be related to diversification and speciation, or the extinction of populations or species (Mourão et al. 2017). Under either species concept, the phylogenetic breadth of introgression in this group is clear, with both sister species and species from different mtDNA clades exhibiting genetic introgression (Willis et al. 2012).

In the current study, we used different classical and molecular cytogenetic markers to characterize *Cichla* species, from different river drainages within the Amazon basin and investigate the likely existence of hybrid individuals, where more than one species occurs, such as at Uatumá River (Balbina Hydroelectric Dam).

**Material and methods**

In the current study, we sampled 50 individuals of the genus *Cichla* from five locations in the Brazilian Amazon basin (Table 1, Figs 1, 2) under ICMBIO (Instituto Chico Mendes de Conservação da Biodiversidade) permit number: 28095-1. Voucher specimens were deposited in the Fish Collection of the National Institute of Amazonian Research (Instituto Nacional de Pesquisas da Amazônia – INPA) (Table 1). Dr. Efrem Ferreira and Dr. Jansen Zuanon, following description of Kullander and Ferreira (2006), identified the *Cichla* species included in the current study. However, three individuals had mixed characteristics of more than one species, and were thus considered possible hybrids by specialists.

Chromosomal preparations were obtained from the kidney, following the protocol of Gold et al. (1990). The active nucleolus-organizing region (NOR) was detected with

**Table 1.** The *Cichla* species included in the current study, collecting localities, the number of individuals analyzed, and Voucher number. ♂ = male, ♀ = female. AM = Amazonas State, PA = Pará State, MT = Mato Grosso State.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of individuals</th>
<th>Collecting localities</th>
<th>Coordinates</th>
<th>Voucher</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. kelberi</em></td>
<td>4 ♂ 3 ♀</td>
<td>Araguaia River – São Félix, MT</td>
<td>11°39’03.9&quot;S, 50°52’59.4&quot;W</td>
<td>MZUSP125273</td>
</tr>
<tr>
<td><em>C. monoculus</em></td>
<td>5 ♀</td>
<td>Anavilhanas (Negro River), AM (Black water)</td>
<td>2°33’28.4&quot;S, 60°46’29.7&quot;W</td>
<td>INPA-ICT059045</td>
</tr>
<tr>
<td><em>C. monoculus</em></td>
<td>3 ♀</td>
<td>Uatumá River (Balbina Hydroelectric Dam) AM, Black water</td>
<td>1°55’02.2&quot;S, 59°28’23.7&quot;W</td>
<td>INPA-ICT059046</td>
</tr>
<tr>
<td><em>C. monoculus</em></td>
<td>1 ♀</td>
<td>Tapajós River – Santarém, PA (Clear water)</td>
<td>2°24’53.0&quot;S, 54°46’48.3&quot;W</td>
<td>INPA-ICT059047</td>
</tr>
<tr>
<td><em>C. monoculus</em></td>
<td>4 ♂ 1 ♀</td>
<td>Catalão Lake, AM (Mix of white and black water)</td>
<td>3°10’30.8&quot;S, 59°56’30.3&quot;W</td>
<td>INPA-ICT059044</td>
</tr>
<tr>
<td><em>C. pinima</em></td>
<td>7 ♂ 6 ♀</td>
<td>Tapajós River (Mix of white and clear water)</td>
<td>24°21’16.4&quot;S, 54°70’23.16&quot;W</td>
<td>INPA-ICT059045</td>
</tr>
<tr>
<td><em>C. piquti</em></td>
<td>2 ♂ 2 ♀</td>
<td>Araguaia River – São Félix, MT</td>
<td>11°38’01.7&quot;S, 50°40’11.3&quot;W</td>
<td>MZUSP125272</td>
</tr>
<tr>
<td><em>C. temensis</em></td>
<td>2 ♂ 2 ♀</td>
<td>Uatumá River (Balbina Hydroelectric Dam), AM, Black water</td>
<td>1°55’02.2&quot;S, 59°28’23.7&quot;W</td>
<td>INPA-ICT059043</td>
</tr>
<tr>
<td><em>C. vazaleieri</em></td>
<td>2 ♂ 3 ♀</td>
<td>Uatumá River (Balbina Hydroelectric Dam, AM, Black water)</td>
<td>1°55’02.2&quot;S, 59°28’23.7&quot;W</td>
<td>INPA-ICT059048</td>
</tr>
<tr>
<td>Hybrids</td>
<td>3 ♂</td>
<td>Uatumá River (Balbina Hydroelectric Dam, AM, Black water)</td>
<td>1°55’02.2&quot;S, 59°28’23.7&quot;W</td>
<td>INPA-ICT059047</td>
</tr>
</tbody>
</table>
silver nitrate impregnation (Ag-NOR), following Howell and Black (1980), while constitutive heterochromatin was detected following Sumner (1972). DNA was extracted using the Wizard Extraction Kit (Promega), following manufacturer’s recommendations, and quantified using a NanoVue Plus spectrophotometer (GE Healthcare).

Amplification of 18S and 5S rDNA used the Polymerase Chain Reaction (PCR) with primers 18S F(5’ -CCG CTT TGG TGA CTC TTG AT-3’) and R(5’ -CCG AGG ACC TCA CTA AAC CA-3’) (Gross et al. 2010), and the primers 5S F(5’ -TAC GCC CGA TCT CGT CCG ATC-3’) and R(5’ -CAG GCT GGT ATG GCC GTA AGC-3’) (Martins and Galetti 1999).

All PCRs were performed with a final volume of 25 μL, containing genomic DNA of each species (200 ng), 10× buffer with 1.5 mM MgCl₂, DNA polymerase (5 U/μL), dNTPs (1 mM), primers (5 mM) and Milli-Q. The reaction profile for 18S rDNA was 1 min. at 95 °C, 35 cycles of 1 min. at 94 °C, 1 min. at 56 °C and 1 min. and 30 s at 72 °C, followed by 5 min. at 72 °C. The reaction profile for 5S rDNA amplification was 1 min. at 95 °C, followed by 30 cycles of 1 min. at 94 °C, 1 min. at 59 °C and 1 min. and 30 s at 72 °C. The final extension was 5 min. at 72 °C. PCR products were checked on 1% agarose gel, quantified on a NanoVue Plus spectrophotometer (GE Healthcare). PCR products were labeled with digoxigenin (Dig-Nick Translation mix; Roche) and biotin (Bio-Nick Translation mix; Roche), and used as probes for the fluorescent in situ hybridization technique (FISH).

Hybridizations were performed according to the protocol described by Pinkel et al. (1986), with a stringency of 77% (2.5 ng/μL) for 18S rDNA, 5S rDNA, 5% formamide, 10% dextran sulfate and 2xSSC at 37 °C for 18 h), post-hybridization washes were made with formamide 15% and 2xSSC Tween 0.5%. Chromosomes were counterstained with DAPI (2 mg/mL) using the Vectashield (Vetor) mounting medium. Telomeric segments were generated using non-templated PCR with primers (TTAGGG)5 and (CCCTAA)5 (Ijdo et al. 1991).
We analyzed at least 30 metaphase per individual to confirm the diploid number and karyotype structure. Images were captured using an Olympus BX51 epifluorescence microscope, and processed using Image-PRO MC 6.0 softwares. Chromosomes

Figure 2. Cichla species and individuals considered morphologically hybrid (A–C). C. kelberi SL = 170.0 mm; C. pinima SL = 190.5 mm; C. piquiti SL = 200.0 mm; C. temensis SL = 210.0 mm; C. vazzoleri SL = 250.0 mm; C. monocuscus (Uatumã River) SL = 160.0 mm; C. monocuscus (Catalão Lake, Negro River) SL = 150.0 mm; C. monocuscus (Anavilhanas, Medium Negro River) SL = 180.0 mm; C. monocuscus (Santarém, Tapajós River) SL = 180.0 mm; Hybrid A SL = 280.0 mm; Hybrid B SL = 230.0 mm; Hybrid C SL = 320.0 mm.
were measured using the Image J program, arranged in descending order of chromosome size, and classified according to Levan et al. (1964).

All methodological procedures in the current study were performed in accordance with the guidelines of the Ethics Committee of the National Institute of Amazonian Research (Instituto Nacional de Pesquisas da Amazônia – INPA), protocol: CEUA No. 009/2018.

Results

The six species analyzed (C. kelberi, C. monoculus, C. pinima, C. piquiti, C. temensis and C. vazzoleri) all had a diploid number equal to 48 acrocentric chromosomes, and a fundamental number (FN) equal to 48. The NORs (Ag-NORs and 18S rDNA) were located in a distal position on the q arms of pair n° 2 in all species (Figs 3, 4). Cichla monoculus was the sole species sampled in more than one location, and it showed no difference when compared to data in Alves-Brinn et al. (2004) and Schneider et al. (2013) (data not shown). The 5S rDNA site was located interstitially at the proximal position of pair n° 6 in four species (C. kelberi, C. pinima, C. piquiti and C. vazzoleri), and on distal portion of pair n° 4 in two (C. monoculus and C. temensis) (Fig. 4).

The six species had centromeric heterochromatic blocks on all chromosomes and a terminal heterochromatic region on pair 2, which corresponds to the same position as the NORs. However, some blocks were species-specific: terminal blocks were observed on the q arm of C. kelberi pairs 1, 3 and 4; pair 3 of C. pinima and C. temensis; pairs 1 and 3 of C. piquiti; and in C. vazzoleri pairs 3 and 6 (Fig. 3). C. monoculus, which was sampled in four different locations, showed variable constitutive heterochromatin patterning, where individuals from the Uatumã River (Balbina Hydroelectric Dam) also had terminal blocks on the q arms of the chromosomal pairs 1, 6, 9, 12, 15, 19. Catalão Lake individuals appeared to have terminal pale blocks on all pairs, with conspicuous ones on 1, 5, 6, 8, 10 chromosomal pairs. This also occurred for individuals from Anavilhanas, but in these, the blocks were more conspicuous in practically all chromosomes, and still had interstitial markings on pairs 1, 3 and 6. Individuals from the Tapajós River had terminal blocks on pairs 1, 3, 5, 11, 14, 15, and interstitials on pairs 14 and 15 (Fig. 5).

The three individuals morphologically considered hybrids also had 2n = 48 acrocentric chromosomes and FN = 48, Ag-NOR and 18S rDNA on the second pair at terminal position on the q arm, collocated with a conspicuous heterochromatic portion (Figs 6, 7). Constitutive heterochromatin was present in the centromeric region of all chromosomes in the three individuals and the first pair had an interstitial block. Additionally, individual A (Fig. 6b) had terminal blocks on pairs 1 and 5; individual B (Fig. 6e) had terminal blocks on most chromosomes and interstitials on pairs 3 and 6; individual C (Fig. 6h) had terminal pale blocks on pairs 3 and 10.

5S rDNA was detected interstitially on one pair 4 homolog, and on one pair 6 homolog in two hybrid individuals (A and C). Individual B showed 5S rDNA sites on both pair 4 chromosomes (Fig. 7).
Comparative cytogenetic analysis of Cichla species

For all analysed species, hybridization with telomeric probes showed, as expected, only markings on the terminal portions of both arms (data not shown).

Discussion

For Cichlidae species, a diploid number equal to 48 acrocentric-like chromosomes is considered an ancestral feature (Thompson 1979), and chromosomal evolution in this family was thought to be conserved from the karyotype macrostructure point of view (Feldberg et al. 2003). However, as more Cichlidae species were cytogenetically studied and more accurate techniques were applied (e.g. mapping of different molecular
Figure 4. Karyotypes analyzed with molecular chromosome markers. Double FISH with 18S (red) and 5S (green) rDNA probes. *Cichla monoculus* (a) *C. kelberi* (b) *C. pinima* (c) *C. piquiti* (d) *C. temensis* (e) *C. vazzoleri* (f).

Figure 5. *Cichla monoculus* karyotype from different locations with conventional Giemsa staining. C. banding: a Uatumã River b Catalão Lake (Negro River) c Anavilhanas (middle Negro River) d Tapajós River.
Comparative cytogenetic analysis of Cichla species

Figure 6. Karyotype of hybrid individuals A, B and C (as in Fig. 2) respectively, with conventional Giemsa staining (a, d, g), C-banding (b, e, h) and Ag-NOR (c, f, i).

chromosomal markers), several karyotypic formulas and configurations have been found (Gross et al. 2009; Poletto et al. 2010; Schneider et al. 2013), suggesting that this fish group experienced multiple non-robertsonian chromosomal rearrangements during its evolution, since the $2n = 48$ is retained in most Cichlinae lineages.

In the current study, analyzes focused on the genus *Cichla*, which represents one of the most basal lineages of Neotropical cichlids (Leo Smith et al. 2008). To date, all species karyotyped possess a complement of 48 acrocentric-like chromosomes, with very similar karyotypes between species, including the NOR pattern, which is usually found on the 2nd pair (Alves Brinn et al. 2004; Schneider et al. 2013; Mourão et al. 2017; current study). In addition, some studies examining morphological-mitochondrial divergences (Andrade et al. 2001; Willis et al. 2010), as well as chromosome features (Alves Brinn et al. 2004; Oliveira et al. 2006), and electrophoretic esterase comparisons (Teixeira and Oliveira 2005) have inferred hybridization in natural and in artificial or disturbed environments/populations.

For constitutive heterochromatin, the distribution pattern can often be used as a species-specific or population marker (Feldberg et al. 2003; Vicari et al. 2006; Benzaquem et al. 2008; Perazzo et al. 2011). In our analyses, for instance, we found four different heterochromatin patterns for *C. monoculus* (Fig. 5). This is one of the most widely-distributed species in the Amazon River basins (Kullander and Ferreira 2006), and the
commonly introduced into dam reservoirs throughout Brazil (dos Santos et al. 2016; Diamante et al. 2017). Heterochromatin is known to play important roles in the chromosomal architecture and karyotype organization, such as assisting in chromosomal segregation, nuclear organization and expression of gene regulation, associated with responses to environmental changes (Grewal and Jia 2007; Varriale et al. 2008; Bühler 2009; Ribeiro et al. 2017; Viana Ferreira et al. 2019). This seems to be the case for the different *C. monoculus* populations analyzed in our study, where individuals from black and acid waters (Negro and Uatumã rivers), white and black mixed waters (confluence of Negro and Solimões rivers), and in the confluence of white and clear waters (confluence of Amazonas and Tapajós rivers), showed intraspecific variability in their heterochromatic patterns, possibly reflecting chromatin adaptation and/or epigenomic responses to changes in the specific environment inhabited by these different populations.

Figure 7. Karyotypes of hybrid individuals A, B and C (as shown in Fig. 2) respectively with molecular chromosomal markers. Double FISH with 18S (red) and 5S (green) rDNA probes.
Interestingly, the heterochromatic patterns of the three probable hybrids was very similar and much closer to the pattern described for the Negro River (C. monoculus from Anavilhanas) with some interstitial blocks. Could it be heterochromatinization? Such heterochromatin variability can also be explained by stressors, such as environmental changes, or even hybridization processes (Richards et al. 2010; Ribeiro et al. 2017), which would explain the heterochromatin distribution differences found in C. monoculus and in the probable hybrids.

Besides the conservation of the karyotype macrostructure, Schneider et al. (2013) reported that 12 out of 13 Cichlinae species analyzed in their study had only one chromosome pair harboring 5S rDNA sites, but at different karyotypic positions, indicating that 5S rDNA sites are a robust molecular chromosomal marker in cichlid species. 5S rDNA is an important cytotaxonomic and evolutionary marker, since it helps provide a better understanding of fish chromosomal diversity (Bellafronte et al. 2005; Teixeira et al. 2009; Vicari et al. 2010). For instance, a study by Ferreira et al. (2016), mapping of 5S rDNA sequences in Bunocephalus coracoideus Cope, 1874, revealed an association between this rDNA site and a multiple sex chromosome system previously unknown in Siluriformes (X,X,X,X,Y,Y,Y,Y). Repetitive 5S and 18S rDNA sequences are the most well-studied in fish, and have been gaining prominence mainly in studies of between-species evolutionary relationships, population characterization and genome structure (Martins et al. 2004; Terencio et al. 2012; Schneider et al. 2013).

In the current study, individuals of all six species, including the hybrids, had 18S rDNA on terminal position of the q arm of the 2nd chromosomal pair (same position as NORs). Meanwhile, 5S rDNA mapping in Cichla species showed two patterns: on the 4th pair (C. monoculus and C. temensis), and on the 6th pair (C. kelberi, C. pinima, C. piquiti and C. vazzoleri). However, in the individuals morphologically considered hybrids, we found two distinct patterns: two of them (hybrids A and C) having 5S rDNA in one homologue of the 4th pair and one homologue of the 6th pair, while the other hybrid (individual B) had 5S rDNA on both homologues of the 4th pair. Since these probable hybrids were captured in the Uatumã River (Balbina Hydroelectric Dam), where C. monoculus, C. temensis and C. vazzoleri all occur (Kullander and Ferreira 2006), we believe that these species might be hybridizing.

Interestingly, the karyotypes of C. pinima and C. vazzoleri (current study) are very similar, except for a heterochromatic terminal block on the q arms of the 6th pair in C. vazzoleri. It is notable that C. pinima was sampled in the Tapajós River and C. vazzoleri in the Uatumã River, very distant locations with no history of sympathy or migration (Ferreira, personal communication). However, according to Willis (2017), C. pinima sensu lato includes C. pinima, C. vazzoleri, C. jariina, Kullander et Ferreira, 2006 and C. thyrorus Kullander et Ferreira, 2006 (sensu Kullander and Ferreira 2006), and reports that the evolutionary relationships in this group are more complex than previously thought. Willis (2017) suggest that this separation into four species does not correspond to its evolutionary history and contemporary dynamics of the genus.

In addition, Willis et al. (2012) reported that genetic introgression is a common phenomenon in Cichla species. Introgression can be defined as the movement of DNA from the genetic pool of one species into that of another species by repeated backcross-
ing of hybrid individuals with one or both parent species. Such hybridization events are expected to occur most commonly in modified habitats, but interestingly, most of the hybridization cases known for *Cichla* species, were found in undisturbed natural environments (Willis et al. 2012), suggesting that introgression forms a natural part of the evolution of many tropical species, so increasing genetic diversity. In this sense, we cannot rule out hybridization and genetic introgression among the likely parental species, especially taking in account that all three probable hybrid individuals used here had male gonads.

**Conclusions**

Our data supports the tendency in the maintenance of the 2n = 48 chromosomes for *Cichla* species, as well as the conservation of the karyotypic formula and simple NOR, but reveals 5S rDNA to be an important cytogenetic marker for this group. In addition, here we provide, for the first time, the karyotype for *C. pinima* and *C. vaz- zoleri*. Furthermore, our data shows that the heterochromatin pattern may differentiate populations of *C. monoculus*, suggesting that this variation might be the result of epigenetic events triggered by different water types.

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**References**


Comparative cytogenetic analysis of Cichla species


