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Assessment of quadrivalent characteristics influencing chromosome segregation by analyzing human preimplantation embryos from reciprocal translocation carriers

Ziravard N. Tonyan[1,2], Irina L. Puppo[2,3], Alsu F. Saifitdinova[3,4], Tatyana V. Vavilova[2], Andrey S. Glotov[1]

1 *D. O. Ott Research Institute of Obstetrics, Gynaecology and Reproductology, 3 Mendeleevskaya Line, 199034, Saint Petersburg, Russia* **2** *Almazov National Medical Research Centre, 2 Akkuratova Street, 197341, Saint Petersburg, Russia* **3** *International Centre for Reproductive Medicine, 53/1 Komendantskij prospect, 197350, Saint Petersburg, Russia* **4** *Department of Human and Animal Anatomy and Physiology, Herzen State Pedagogical University of Russia, 48 Moyka River Embankment, 191186, Saint Petersburg, Russia*

Corresponding author: Ziravard N. Tonyan (ziravard@yandex.ru)

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Abstract

Patterns of meiotic chromosome segregation were analyzed in cleavage stage and blastocyst stage human embryos from couples with autosomal reciprocal translocations (ART). The influence of quadrivalent asymmetry degree, the presence of terminal breakpoints, and the involvement of acrocentric chromosomes in the rearrangement were analyzed to evaluate their contribution to the formation of non-viable embryos with significant chromosomal imbalance due to pathological segregation patterns and to assess the selection of human embryos by the blastocyst stage. A selection of viable embryos resulting from alternate and adjacent-1 segregation and a significant reduction in the detection frequency of the 3 : 1 segregation pattern were observed in human embryos at the blastocyst stage. The presence of terminal breakpoints increased the frequency of 3 : 1 segregation and was also associated with better survival of human embryos resulting from adjacent-1 mode, reflecting the process of natural selection of viable embryos to the blastocyst stage. The demonstrated patterns of chromosome segregation and inheritance of a balanced karyotype in humans will contribute to optimizing the prediction of the outcomes of in vitro fertilization programs and assessing the risks of the formation of unbalanced embryos for ART carriers.

Keywords
Chromosome segregation, PGT-SR, quadrivalent asymmetry, reciprocal translocation, segregation, terminal breakpoints

Introduction

Autosomal reciprocal translocations (ART) are balanced structural rearrangements resulting from the interchange among two or more non-homologous chromosomes with an exchange of the fragments (Gardner and Amor 2018). ARTs can contribute to karyotype evolution by altering the structure and organization of chromosomes in germ cells and early embryonic cells (Imai et al. 1986). It is estimated that the overall frequency of reciprocal translocations in *Homo sapiens* Linnaeus, 1758 is 1 : 500 (Ogilvie et al. 2005). Carriers of balanced ART have a normal phenotype, but the risk of producing genetically unbalanced gametes is increased due to malsegregation of chromosomes during meiosis. As a result, ART can lead to recurrent miscarriage, infertility, or the birth of a child with multiple congenital malformations caused by a chromosomal abnormality (De Braekeleer and Dao 1990).

Derivative chromosomes and their normal homologous form a special structure called quadrivalent in the first meiotic division in ART carriers. Quadrivalent chromosomes segregate in one of the following modes: 2 : 2, 3 : 1, or 4 : 0. Alternate segregation is the type of 2:2 segregation, which is characterized by the inheritance by each of the daughter cells of two normal or two derivative chromosomes. It is the only segregation pattern that does not result in the formation of unbalanced gametes. Adjacent-1 and adjacent-2 are two more types of 2 : 2 segregation, leading to the formation of a zygote with partial trisomy for one of the translocated segments (TS) and monosomy for the other TS or with partial trisomy for one of the centric segments (CS) and monosomy for the other CS, respectively. The 3 : 1 mode results in trisomy or monosomy for one of the rearranged or normal chromosomes involved in translocation. The 4 : 0 segregation pattern leads to complete trisomy or complete monosomy for both chromosomes involved in the rearrangement (Gardner and Amor 2018).

Different approaches can be used to analyze chromosome segregation in ART carriers and the factors influencing it. The analysis of the genetic content of polar bodies could provide valuable insights into the nature of meiotic segregation (Kuliev and Verlinsky 2004; Magli et al. 2011). However, during early embryonic development, the first polar bodies are typically eliminated (Fabian et al. 2012), which complicates their use for conducting systematic research. Since there is no activation of the embryo genome before the cleavage stage of human embryonic development, which typically occurs on the third day after fertilization (Dobson et al. 2004), significant selection of genetically unbalanced embryos does not occur until the third day of development. This provides researchers with a unique opportunity to study the segregation of rearranged chromosomes in the gametogenesis of ART carriers by

analyzing cleavage stage human embryos. However, it should be noted that according to recent data, activation of the embryonic genome in humans initiates at the single-cell stage (Asami et al. 2022). As the embryo's genome becomes activated, it starts to transcribe and translate its own genetic material, leading to the production of proteins and molecules necessary for further development and differentiation. A key marker of genome activation of the human embryo is the compaction of genetic material, which is crucial for the embryo's growth and the formation of the blastocyst (Hur et al. 2023). Modern techniques in assisted reproductive technology have advanced the ability to culture and grow blastocysts in vitro (Sills and Palermo 2010). However, not all unbalanced embryos reach the blastocyst stage due to the natural selection of genetically imbalanced embryos by the fifth or sixth day of development (Beyer and Willats 2017).

Studies analyzing the contribution of various factors to chromosome segregation in human embryos at different stages of development demonstrated the influence of the quadrivalent asymmetry degree, the presence of terminal breakpoints, and the participation of the acrocentric chromosome in the rearrangement.

For instance, a higher frequency of the formation of genetically normal/balanced embryos was demonstrated in the absence of terminal breakpoints when analyzing cleavage stage embryos (Ye et al. 2012). The same trend was shown in blastocysts, but the presence of terminal breakpoints also predisposed to adjacent-1 segregation (Xie et al. 2022). A study at the prenatal stage of embryo development showed that terminal breakpoints were an independent predictor of the birth of children with congenital malformations (Shilova et al. 2019).

Previous research suggested the impact of a quadrivalent asymmetry degree on the pattern of chromosome segregation. The results of the study conducted on the cleavage stage embryo demonstrated that severe asymmetry predisposes to the production of genetically unbalanced embryos due to 3:1 segregation (Zhang et al. 2014). At the same time, there were no differences in the frequency of balanced embryos observation at the blastocyst stage; however, ART carriers with severe quadrivalent asymmetry displayed the product of adjacent-2 segregation more often (Zhang et al. 2018).

Several studies emphasized that acrocentric chromosomes involved in rearrangements predispose to 3 : 1 segregation and reduce the incidence of adjacent-1 disjunction (Ye et al. 2012; Yilmaz et al. 2012). In a study conducted on the blastocyst stage, acrocentric chromosomes influenced segregation in combination with strong quadrivalent asymmetry, increasing the frequency of adjacent-2 and 4 : 0 segregation.

The differences in the results of studies conducted on cleavage stage and blastocyst stage embryos can be partly explained by the selection of viable genetically balanced embryos or embryos with relatively small chromosomal imbalances. Therefore, the purpose of this work was to analyze the impact of different factors predisposing to the formation of non-viable embryos with significant chromosomal imbalance due to pathological segregation patterns and influencing the selection of embryos between the third and fifth or sixth days of development.

Materials and methods

A retrospective analysis was performed on 39 couples with ART who underwent in vitro fertilization cycles (IVF) with preimplantation genetic testing for structural chromosomal rearrangements (PGT-SR) using fluorescent in situ hybridization (FISH), next generation sequencing (NGS), or array comparative genomic hybridization (aCGH) between 2016 and 2021 at the International Center for Reproductive Medicine. Karyotypes of carriers are presented in Suppl. material 1: table S1. The informed consent form was signed by all the participants. ARTs were confirmed after karyotyping of spouses on peripheral blood lymphocytes. A total of 306 cleavage stage embryos and trophectoderm cells from 93 blastocyst stage embryos were analyzed. The mean age of female ART carriers was 32.3±4 and 34.4±3 in couples who underwent IVF with PGT-SR using FISH or aCGH/NGS methods on the third day and the fifth / sixth day, respectively.

To determine the segregation type in gametogenesis of ART carriers, the combination of fluorescent signals from TS and CS was assessed when analyzing blastomeres of cleavage stage embryos in the case of PGT-SR using the FISH method or by assessing the gain or loss of genetic material when analyzing trophectoderm cells from blastocyst stage embryos in the case of PGT-SR using aCGH or NGS methods.

TS and CS lengths were measured using the UCSC genome browser (assembly GRCh38/hg38) in millions of base pairs to determine the quadrivalent asymmetry degree and the presence of terminal breakpoints. The starting point for measuring the length of the TS was the proximal end of the cytoband. The size of the CS was calculated by subtracting the length of the TS from the length of the entire chromosome.

The quadrivalent asymmetry degree was assessed by calculating the ratio of the length of the longest TS to the shortest TS and the longest CS to the shortest CS. If both ratios were ≥ 2, the quadrivalent was considered severe asymmetric. If at least one of the ratios was less than 2, the quadrivalent was considered mild asymmetric (Zhang et al. 2018).

The ratio of the length of the TS to the length of the entire chromosome arm was measured to determine the presence of terminal breakpoints in ART carriers. A translocation was considered to contain a terminal breakpoint if this ratio was ≤ 0.2 in one or both chromosomes involved in the rearrangement (Ye et al. 2012).

Statistics were calculated using STATISTICA 12 software (Tibco, CA, USA). Fisher's exact test was used to compare differences between groups.

Results

In this article, we analyzed 1) the selection of embryos resulting from different segregation modes by the blastocyst stage; 2) the influence of terminal breakpoints, quadrivalent asymmetry degree, and the involvement of acrocentric chromosomes on the predominant pathological pattern of chromosome segregation in ART carriers; and 3) the influence of the abovementioned factors on the viability of embryos resulting from different segregation patterns.

Comparison of the segregation patterns detection frequency in cleavage stage and blastocyst stage embryos

In total, alternate mode was the most detected segregation pattern and was observed with a frequency of 32% (128/399). Other patterns were detected with comparatively lower frequencies: adjacent-1 and 3 : 1 modes were observed with the same frequency (24% (97/399) and 21% (82/399) respectively); adjacent-2 mode was detected in 13% of embryos (50/399); and 4 : 0 mode was found only in 1% (4/399). The segregation mode was not determined in 9% of embryos (38/399) due to mosaicism or polyploidy, such embryos were excluded from further analysis (Fig. 1A).

When comparing the observation frequency of different types of segregation on days 3 and 5/6 of embryo development, a significantly lower frequency of 3 : 1 segregation was shown in the previous gametogenesis of the carrier ($p<0.0001$) on day 5/6 embryos, which is explained by the selection of embryos due to the larger size of the chromosomal imbalance, which is typical for this type of segregation (Fig. 1B, Table 1). Segregation modes detected in 3 and 5/6 day embryos are presented in Suppl. material 1: table S2.

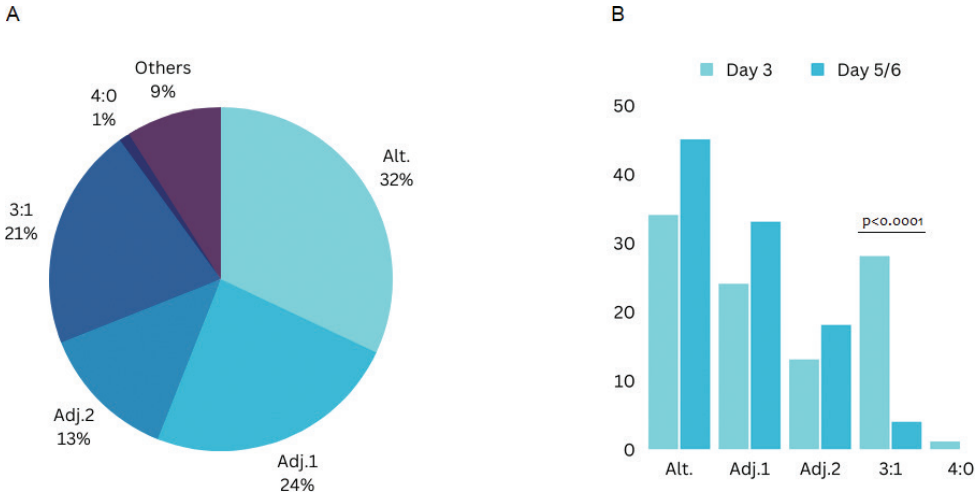

Figure 1. Frequency of observation of different chromosome segregation modes in human embryos **A** from all ART carriers **B** separately in cleavage stage (day 3) and blastocyst stage (day 5/6) embryos. Alt.: alternate mode, Adj.1: Adjacent-1 mode, Adj. 2: adjacent-2 mode.

Table 1. Frequency of segregation modes detection in cleavage stage and blastocyst stage embryos from ART carriers.

Stages of development	Alt., %	Adj.1, %	Adj.2, %	3:1, %	4:0, %
Cleavage	34	24	13	28	1
Blastocyst	45	33	18	4	0
p-value	0.093	0.118	0.281	<0.0001	0.576

Alt.: alternate mode, Adj.1: Adjacent-1 segregation mode, Adj. 2: adjacent-2 segregation mode.

Analysis of factors influencing the chromosome segregation pattern in ART carriers

The direct analysis of gametes is an ideal method for an independent assessment of the influence of factors on the nature of meiotic segregation in ART carriers. Due to the unavailability of gametes for analysis and to exclude the impact of embryo selection by the blastocyst stage the influence of the analyzed factors on the chromosome segregation pattern in ART carriers was assessed on cleavage stage embryos only. Mild quadrivalent asymmetry was determined for 28 ARTs out of 39 (72%). The remaining 11 ART carriers had quadrivalents with severe asymmetry (28%) (Suppl. material 1: tables S1, S3). When assessing the influence of asymmetry degree on the frequency of segregation patterns, no differences were found in cleavage stage embryos (Fig. 2a).

Terminal breakpoints were present in 7 of the 39 translocation (18%) (Suppl. material 1: tables S1, S3). When assessing the influence of the presence of terminal breakpoints on the frequency of segregation patterns, 3 : 1 mode was observed significantly more often in day 3 embryos from ART carriers with terminal breakpoints compared with ART without them (p=0.002) At the same time, genetically balanced embryos were detected with the same frequency (Fig. 2B).

At least one acrocentric chromosome (13, 14, 15, 21, 22) was involved in translocation in 18 of 39 cases (46%) (Suppl. material 1: table S3). When analyzing the frequency of detected segregation patterns in cleavage stage embryos from ART carriers with and without acrocentric chromosomes, it was shown that the involvement of acrocentric chromosomes predisposes to adjacent-2 segregation mode (p=0.046) and reduces the frequency of adjacent-1 mode (p=0,02), without affecting the frequency of balanced embryo formation (Fig. 2C).

Analysis of the influence of asymmetry degree, terminal breakpoints, or acrocentric chromosomes' involvement on the viability of human embryos resulting from different segregation patterns

No statistically significant differences were found regardless of the type of segregation when comparing the frequency of different segregation modes in cleavage stage and blastocyst stage embryos from ART carriers with severe and mild quadrivalent asymmetry, which indicates that quadrivalent asymmetry degree does not affect the viability of human embryos at the initial stages of development (Table 2, Fig. 3A).

When comparing the frequency of segregation modes detected in cleavage stage and blastocyst stage embryos from carriers with terminal breakpoints, a significantly larger number of embryos with alternate (p = 0.05) and adjacent-1 (0.0007) segregation patterns was shown on fifth / sixth days of development.

To assess the effect of acrocentric chromosomes' involvement on the survival of embryos resulting from different segregation modes, the frequency of detection of different patterns was compared on cleavage stage and blastocyst stage embryos in carriers of ARTs involving acrocentric chromosomes. Such a comparison demonstrated the absence of an impact of acrocentric chromosomes on embryo survival by the blastocyst stage, regardless of the previous segregation pattern (Fig. 3C).

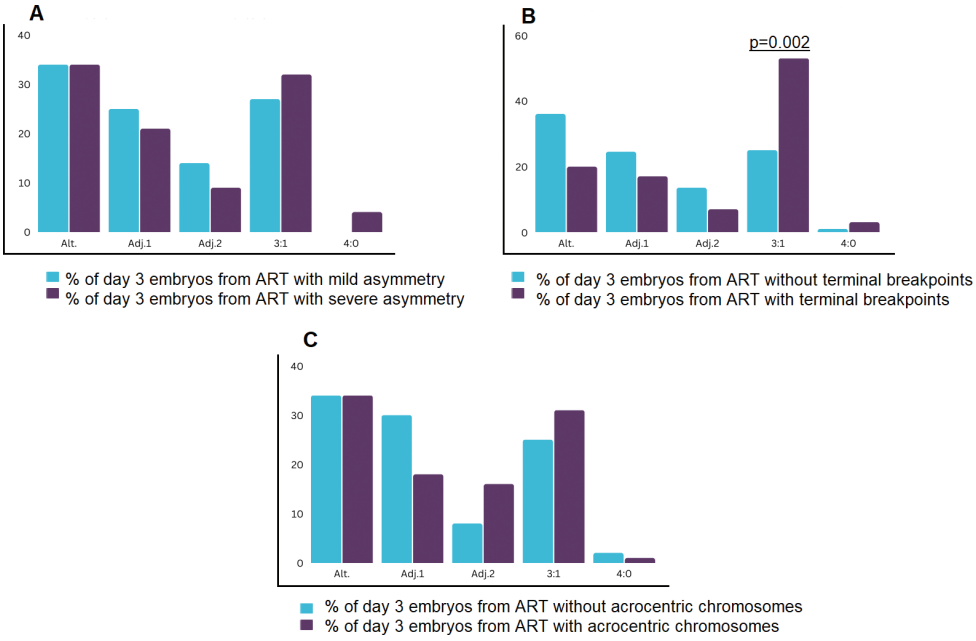

Figure 2. The influence factors on the meiotic chromosome segregation type **A** the quadrivalent asymmetry degree **B** the presence of terminal breakpoints **C** the involvement of acrocentric chromosomes on the meiotic chromosome segregation type. Alt.: alternate segregation mode, Adj.1: Adjacent-1 segregation mode, Adj. 2: adjacent-2 segregation mode.

Table 2. Frequency of segregation modes detection in cleavage stage and blastocyst stage embryos from ART carriers with severe asymmetry degree, presence of terminal breakpoints, or involvement of acrocentric chromosomes.

Types of segregation in ART carriers depending on the presence of factors	Cleavage stage embryos, %	Blastocyst stage embryos, %	p-value
% of embryos with severe quadrivalent asymmetry degree			
Alternate	28	16	0.1861
Adjacent-1	25	29	0.797
Adjacent-2	20	0	0.087
3 : 1	31	25	1
4 : 0	75	0	–
% of embryos with the presence of terminal breakpoints			
Alternate	6	18	0.0513
Adjacent-1	6	26	0.0007
Adjacent-2	6	7	1
3 : 1	20	50	0.208
4 : 0	25	0	–
% of embryos with the involvement of acrocentric chromosomes			
Alternate	55	37	0.0829
Adjacent-1	43	28	0.2474
Adjacent-2	71	67	0.747
3 : 1	60	75	1
4 : 0	25	0	–

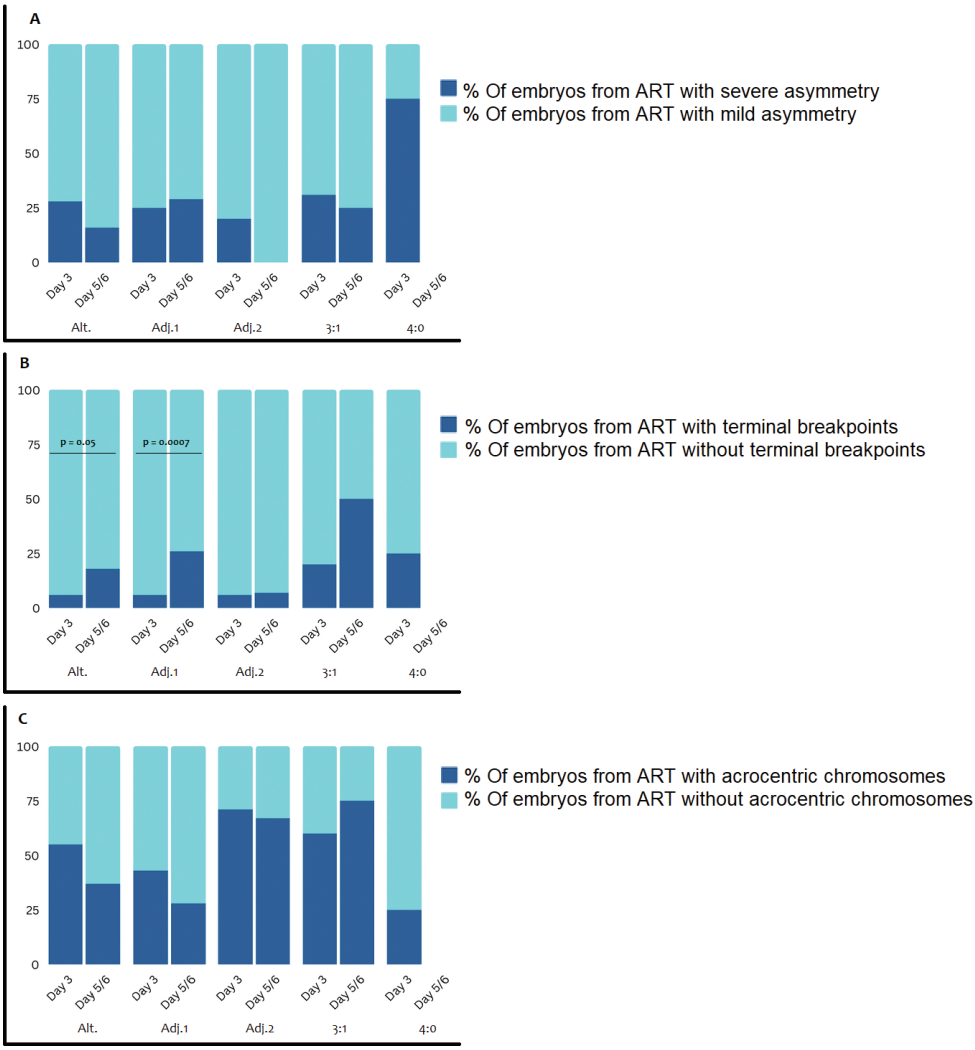

Figure 3. A the influence of factors on human embryos survival by the blastocyst stage depending on the type of meiotic chromosome segregation **A** the quadrivalent asymmetry degree **B** the presence of terminal breakpoints **C** the involvement of acrocentric chromosomes. Alt.: alternate segregation mode, Adj.1: Adjacent-1 segregation mode, Adj. 2: adjacent-2 segregation mode.

Discussion

Our findings on the spectrum of segregation patterns in ART carriers demonstrate the prevalence of an alternate type leading to the formation of gametes forming human genetically normal/balanced embryos (32%). However, the risk of producing genetically unbalanced gametes in carriers remains high, leading to reproductive failure or the birth of offspring with congenital defects (Huang et al. 2019). Moreover, the frequency of observation of pathological segregation patterns is specific to carriers of a particular

rearrangement with its individual quadrivalent characteristics. Previous studies suggested factors that potentially affect segregation, such as quadrivalent asymmetry degree, the presence of terminal breakpoints, and the involvement of acrocentric chromosomes. Therefore, in the present study, we analyzed the factors influencing the nature of meiotic chromosome segregation in ART carriers and their effect on the survival of human embryos resulting from different segregation patterns by the blastocyst stage.

Based on the obtained results, the number of human embryos resulting from 3 : 1 segregation in the gametogenesis of ART carriers is significantly reduced by the blastocyst stage, which is most likely due to the significant size of chromosomal imbalance associated with this segregation mode. This finding is consistent with the result of a previous study, which demonstrated a significant decrease in the number of genetically unbalanced embryos resulting from 3 : 1 segregation by the fifth / sixth days of development (Beyer and Willats 2017). At the same time, this study also demonstrated a significant increase in the number of euploid / balanced blastocyst stage embryos resulting from alternate segregation, as well as embryos with small genetic imbalances caused by adjacent-1 mode. In the present study, there is also a minor trend towards an increase in the frequency of detection of alternate and adjacent-1 modes; however, the differences were not statistically significant (0.09 and 0.1, respectively), which can be explained by the relatively smaller sample size (Fig. 1B, Table 1). Selection of embryos formed as a result of adjacent-1 segregation in gametogenesis by the 3rd day of development can be explained by the fact that chromosomal imbalance in this segregation mode is limited to partial monosomy and trisomy of TSs, which are often relatively small compared to CSs. At the same time, the size of the chromosomal imbalance in 3 : 1 segregation is relatively larger compared to adjacent-1 mode, since in this case the imbalance is presented as tertiary or interchange trisomy / monosomy. The early work of Daniel and Cohen allowed the determination of the size of chromosomal imbalance, represented as haploid autosome length (%HAL), potentially compatible with implantation and fetal development. For this, the authors proposed the Chromosome imbalance size-viability Model (Daniel 1979) and Surface of viable unbalances (Cohen 1994). According to the model proposed by Daniel, the size of the viable imbalance does not exceed 2% HAL for monosomy and 4% HAL for trisomy. In Cohen's modification, these values are 5% and 3% HAL for trisomy and monosomy, respectively. The size of the potentially viable chromosomal imbalance for all the embryos formed as a result of all possible segregation patterns in the analyzed families is presented in Suppl. material 1: table S4. The table shows that the majority of embryos formed after adjacent-1 segregation have a chromosomal imbalance size that is potentially compatible with implantation, which can lead to the birth of a child with congenital anomalies. However, it should be noted that the proposed models for estimating the size of the chromosomal imbalance have not yet been evaluated at the preimplantation stage.

These findings once again confirm the natural selection of human genetically normal/balanced embryos by the blastocyst stage, but at the same time raise the question of factors predisposing to pathological segregation patterns in ART carriers, leading to the formation of genetically unbalanced embryos incompatible with further

development. To address the question, the impact of such factors as quadrivalent asymmetry degree, the presence of terminal breakpoints, and the involvement of acrocentric chromosomes on the preferential segregation pattern was analyzed. The assessment was performed on cleavage stage embryos in order to exclude the influence of the natural selection of embryos by the blastocyst stage. According to our results, the only factor analyzed that affects the nature of chromosome segregation in the gametogenesis of ART carriers is the presence of terminal breakpoints predisposing to the 3 : 1 mode. This result confirmed the finding about the incidence of a 3 : 1 pattern in ART carriers with terminal breakpoints, which is significantly higher compared to translocations without them in cleavage stage embryos (Ye et al. 2012).

When comparing the frequency of detection of various types of segregation in cleavage stage and blastocyst stage embryos with severe and mild asymmetry degrees, as well as with and without the involvement of acrocentric chromosomes, no statistically significant differences were revealed regardless of the segregation type, which indicated the absence of influence of these factors on the viability of human embryos at the initial stages of development (Fig. 3A, B).

The opposite trend was demonstrated when comparing the frequency of detection of segregation modes in cleavage stage and blastocyst stage embryos resulting from ARTs with terminal breakpoints and without them. Significantly more embryos consistent with the alternate ($p = 0.0513$) and adjacent-1 ($p = 0.0007$) segregation patterns, resulting from translocation with terminal breakpoints, were observed on the fifth / sixth days of development (Fig. 3B). This observation can be explained by the small size of the chromosomal imbalance in the gametes formed as a result of adjacent-1 segregation, which is characterized by partial monosomy or trisomy of TS. The small size of the TS due to the presence of terminal breakpoints leads to the formation of zygotes with a minor chromosomal imbalance, which determines the survival of such embryos by blastocyst stage.

Study limitations

A limited number of chromosomes were analyzed in cleavage stage embryos; therefore, selection by blastocyst stage could partly be due to aneuploidy of chromosomes not analyzed. However, selection for chromosomes not involved in the rearrangement should not have affected the frequency of detection of segregation types. The limitations of the study also include the small sample size.

Conclusion

Despite the high frequency of alternate segregation in ART carriers, they are at increased risk of reproductive failure or the birth of offspring with congenital defects due to pathological chromosome segregation in gametogenesis. A selection of viable human embryos is observed in the blastocyst stage due to the presence of terminal breakpoints on the chromosomes involved in ART, making PGT-SR of blastocyst stage

embryos preferable to the cleavage stage. The presence of terminal breakpoints on the chromosomes involved in the rearrangement promotes the survival of human embryos resulting from adjacent-1 segregation mode with a small size of chromosomal imbalance, increasing the risk of the birth of a child with multiple congenital malformations caused by a chromosomal abnormality.

Competing interests

The authors have declared that no competing interests exist.

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ORCID

Ziravard N. Tonyan https://orcid.org/0000-0001-9050-5886
Irina L. Puppo https://orcid.org/0000-0001-8538-3845
Alsu F. Saifitdinova https://orcid.org/0000-0002-1221-479X
Tatyana V. Vavilova https://orcid.org/0000-0001-8537-3639
Andrey S. Glotov https://orcid.org/0000-0002-7465-4504

Supplementary material 1

Supplementary information

Authors: Ziravard N. Tonyan, Irina L. Puppo, Alsu F. Saifitdinova, Tatyana V. Vavilova, Andrey S. Glotov

Data type: xlsx

Explanation note: **table S1.** Calculation of quadrivalent asymmetry degree and the presence of terminal breakpoints in translocation carriers. **table S2.** Segregation patterns detected in 3- and 5/6 days human embryos. **table S3.** Segregation patterns in 3- and 5/6 days human embryos depending on individual quadrivalent characteristics. **table S4.** Size of chromosomal imbalance and potential viability of all human embryos analyzed.

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RESEARCH ARTICLE

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New insights into the chromosomes of stoneflies: I. Karyotype, C-banding and localization of ribosomal and telomeric DNA markers in *Skwala compacta* (McLachlan, 1872) (Polyneoptera, Plecoptera, Perlodidae) from Siberia

Alexander Bugrov[1,2], Tatyana Karamysheva[1,3], Olesya Buleu[1,2,3]

1 *Novosibirsk State University, Pirogova Str. 2, Novosibirsk 630090, Russia* **2** *Institute of Systematics and Ecology of Animals, Russian Academy of Sciences, Siberian Branch, Frunze str. 11, 630091, Novosibirsk, Russia* **3** *Institute of Cytology and Genetics, Russian Academy of Sciences, Siberian Branch, Pr. Lavrentjeva 10, 630090, Novosibirsk, Russia*

Corresponding author: Alexander Bugrov (bugrov04@yahoo.co.uk)

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Abstract

This study provides data on chromosome number (2n♂♀=26), sex determination mechanism (XY♂/XX♀), C-banding pattern, distribution of clusters of telomeric TTAGG repeats and 18S ribosomal DNA in the karyotype of the stonefly *Skwala compacta* (McLachlan, 1872). For the first time in the history of stoneflies cytogenetics, we provide photos of the chromosomes of the Plecoptera insects. The karyotype of males and females of *S. compacta* consists of 12 pairs of autosomes. Three pairs of large autosomes and four pairs of medium-sized autosomes are subacrocentric. The remaining pairs of autosomes are small, with unclear morphology. Pericentromeric C-bands were revealed in all autosomes. The sex chromosomes are also subacrocentric. The short arms of X and Y chromosomes are entirely heterochromatic and are rich in ribosomal DNA sequences. In the X chromosome this arm is larger than in the Y chromosome. It is likely that this arm associated with the nucleolar organizer (NOR). Telomeric DNA $(TTAGG)_n$ repeats were detected in the terminal regions of all chromosomes.

Keywords

18S rDNA repeats, C-banding, FISH, Plecoptera, karyotypes, telomeric $(TTAGG)_n$ DNA repeats

Introduction

Plecoptera or stoneflies are amphibiotic insects distributed worldwide, except for Antarctica (Zwick 2000). Currently, about 3,700 species from 17 families of the stoneflies have been described (Fochetti and Tierno de Figueroa 2008; DeWalt et al. 2023).

To date, the stoneflies remain one of most poorly cytogenetically studied groups among the Polyneoptera. The karyotypes of only sixteen Plecoptera species from Europe, North America and Japan have been described (Nakahara 1919; Junker 1923; Itoh 1933; Matthey and Aubert 1947). These studies have resulted in information on karyotypes and sex determination mechanisms in this group of insects. For more than 70 (!) years, there has been no new information on the karyotypes of these insects. In reviews of sex chromosome evolution often refer to stoneflies as insects with highly diverse karyotypes and chromosomal sex determination systems (White 1973; Blackman 1995; Blackmon et al. 2017). Our research group has devoted several years to studying the evolution of sex chromosomes in grasshoppers (Bugrov and Grozeva 1998; Bugrov et al. 2001; Bugrov et al. 2016; Jetybayev et al. 2017; Buleu et al. 2020), and therefore we could not help but pay attention to the information about the intriguing variety of cytological mechanisms of sex determination in stoneflies. Taking into account the above, we set out to study the karyotypes of *Skwala compacta* (McLachlan, 1872) using cytogenetic methods that have not previously been used in the practice of cytogenetic analysis of this group of insects.

The first paper in our planned series of studies is devoted to the description of the karyotype of the stonefly *S. compacta* from the Izdrevaya River in the vicinity of Novosibirsk.

To study the karyotype of *S. compacta*, we used the C-banding method to determine the localization and size of heterochromatic blocks in chromosomes and fluorescence *in situ* hybridization (FISH) with telomeric $(TTAGG)_n$ and 18S rDNA probes to detect the localization of functionally important regions in autosomes and sex chromosomes. The choice of these molecular markers is determined by knowledge of their important functional role in the genome and information on the localization of telomeric DNA and ribosomal DNA in the chromosomes of many insect species (Frydrychová et al. 2004; Cabrero and Camacho 2008; Sharakhov 2015; Kuznetsova et al. 2019).

Material and methods

Material collection

Nymphs of the *S. compacta* of different ages were collected during the spring and autumnal season (2020–2022) in Izdrevaya river flowing within the city Novosibirsk (GPS coordinates 55.0018°S/N, 83.2156°W/E). The material for studying the karyotype of this species were testes and ovarioles of about 100 larvae.

Methods

Chromosome preparations, C-banding and FISH

Prior to chromosome preparation, *S. compacta* larvae were stored in a refrigerator at 2–4 °C. Chromosome preparations were made from testes and ovaries of the larvae (Fig. 1). For this purpose, 0.1% colchicine solution was injected into the abdomens of *S. compacta* larvae. After 4–6 hours, the gonads were removed and placed in 0.9% sodium citrate solution for 15–20 minutes, and then fixed in freshly prepared ethanol : glacial acetic acid fixative (3:1) for 10–15 minutes. Fixed gonads were dissected using needles on pre-cleaned glass slides in a drop of 60% acetic acid. Finally, the cells were spread on the slide on heat plate at 65 °C.

C-banding of chromosome preparations was performed according to Sumner's protocol (1972) with minor modifications. Slides were treated with 0.2 N HCL for 15–30 min, then rinsed with distilled water and dried at room temperature. Then slides were incubated in saturated $Ba(OH)_2$ solution at 60 °C for 3–5 min, rinsed with water and placed into 2×SSC at 60 °C for 60 min. After washing in distilled water, slides were stained with 2% Giemsa solution in Sorensen's phosphate buffer 30 to 60 min.

Fluorescence *in situ* hybridization (FISH) with telomeric $(TTAGG)_n$ DNA and 18S rDNA probes was performed following the protocol of Pinkel et al. (1986) with modifications described in Rubtsov et al. (2000).

Telomeric repeats $(TTAGG)_n$ were generated by non-template PCR with primers 5'-TAACCTAACCTAACCTAACC-3' and 5'-TTAGGTTAGGTTAGGTTAGG-3'.

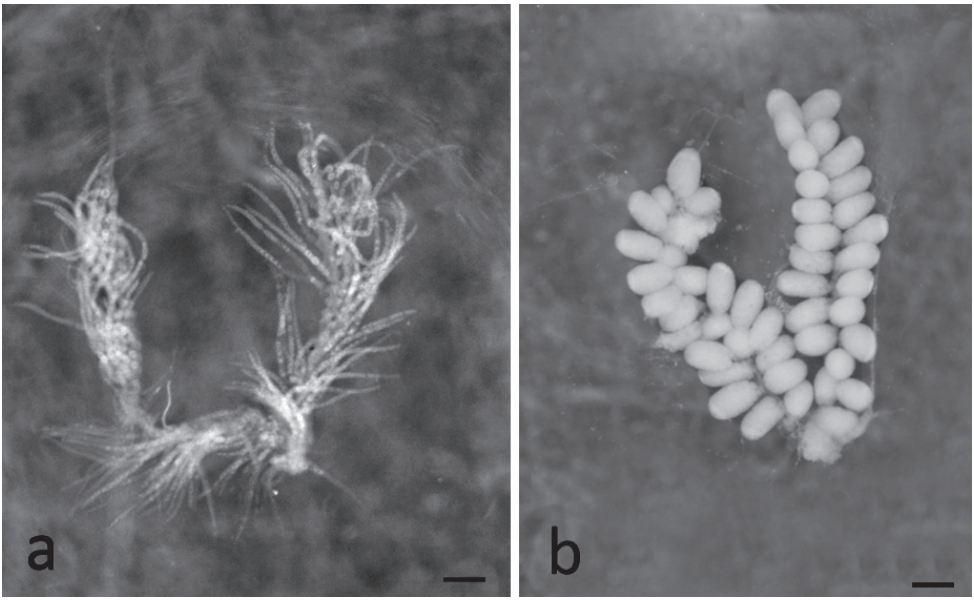

Figure 1. Ovaries (**a**) and testes (**b**) of larvae *Skwala compacta*. Scale bar: 1 mm.

Further labelling with Tamra-dUTP (Biosan, Novosibirsk, Russia) was performed in 33 additional PCR cycles as described previously (Sahara et al. 1999).

The rDNA probe was obtained as previously described by Jetybayev et al. (2017). Unlabelled ribosomal DNA probe was generated by polymerase chain reaction (PCR) according to Jetybayev et al. (2017). The fragments of the 18S rDNA were labelled in additional PCR cycles with Fluorescein-12-dUTP (Biosan, Novosibirsk, Russia) and mixed into a single ribosomal DNA probe.

Microscopic analysis was performed at the Centre for Microscopy of Biological Objects of SB RAS (Novosibirsk, Russia). Chromosomes were examined with an Axio-Imager M1 (Zeiss, Germany) fluorescence microscope equipped with filter sets #49,#46HE, #43HE and a ProgRes MF (MetaSystems GmbH, Germany) CCD camera. The ISIS5 software (METASystems GmbH, Germany) package was used for image capture and analysis.

Results

The karyotype of males and females of *Skwala compacta* consists of 12 pairs of autosomes. Three pairs of large autosomes (L1–L3) and four pairs of medium-sized autosomes (M4–M7) are subacrocentric. The remaining pairs of autosomes (S8–S12) are small, with unclear morphology. Pericentromeric C-bands were revealed in all autosomes (Figs 2, 3).

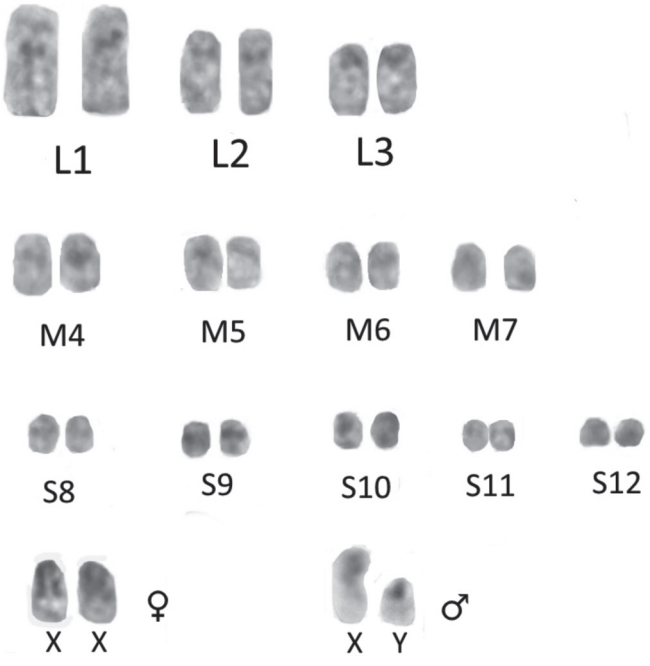

Figure 2. Joint karyogram of oogonial metaphase and spermatognial metaphase of *Skwala compacta*. L – large, M – medium, S – small autosomes.

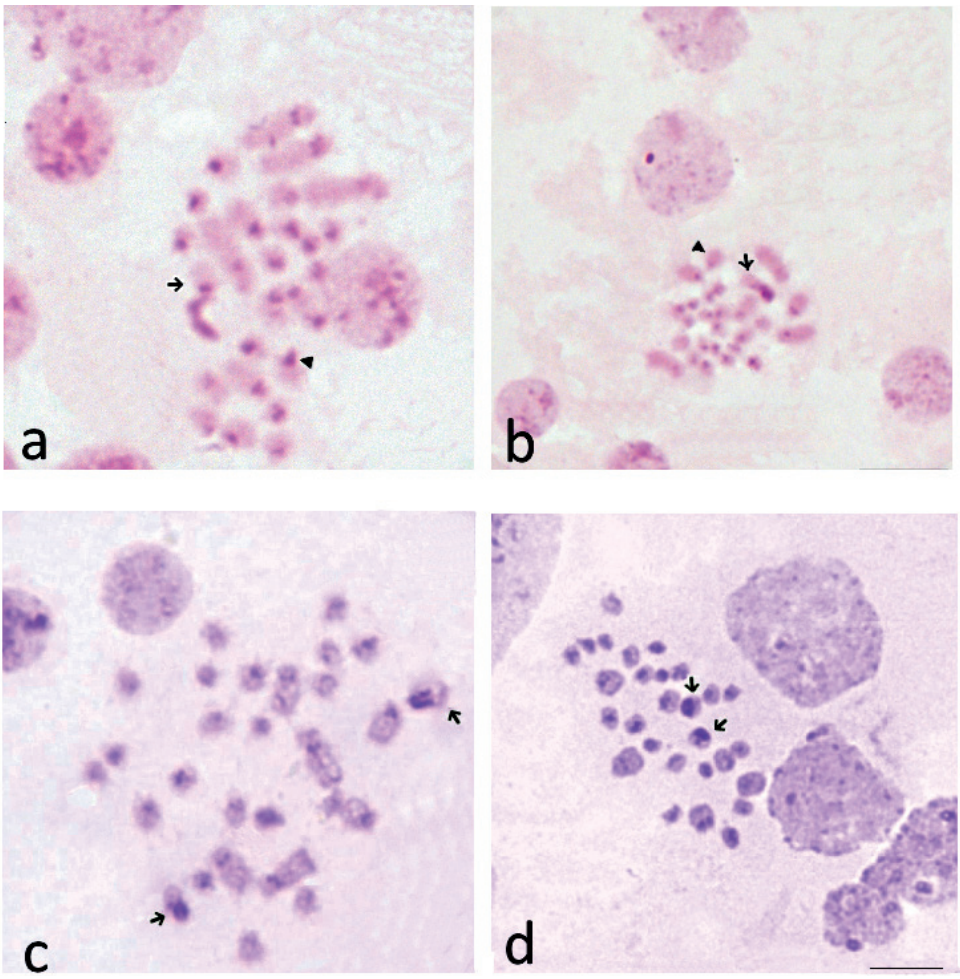

Figure 3. C-banded spermatogonial prometaphase (**a**), spermatogonial metaphase (**b**) and oogonial prometaphase (**c**), oogonial metaphases (**d**) of *Skwala compacta*. Arrows – indicate X chromosomes. Arrowheads – indicate Y chromosomes. Scale bar: 5 μm.

In the male karyotype, in addition to 12 pairs of autosomes, there are two heterosomes, which differ in morphology and size. The large heterosome is two-armed (Figs 2, 3a, b). One arm is entirely heterochromatic. The second arm is predominantly euchromatic, with a C-block localized in the proximal region. In spermatogonial prometaphase the size of heterochromatic arm can vary (Fig. 3a, b). The second heterosome is subacrocentric. According to the size and ratio of euchromatic and heterochromatic regions, one arm of this heterosome is morphologically homologous to the large arm of the large heterosome. The smaller arm of this heterosome is completely heterochromatic (Figs 2, 3a, b).

In the female karyotype, there are 13 pairs of chromosomes, one of which has a large heterochromatic arm in each homologue. Heterochromatic arms in these

chromosomes can vary in size at different stages of oogonial metaphase, as is the case in the large male heterosome during spermatogonial metaphase (Fig. 3c, d).

A comparative analysis of the morphology and behavior of the heterochromatic regions of the large heterosome in males and the mentioned pair of chromosomes in females suggests that these are sex chromosomes. Based on this comparative analysis of the heterosomes, it can be concluded that the mechanism of chromosomal sex determination in *S. compacta* is XY in male and XX in female.

At prophase of male meiosis, chromosomes form 13 bivalents (Fig. 4). The twelve bivalents are symmetrical. The large and medium size autosomes form 1–2 chiasmata, and the small bivalents form only one chiasma (Fig. 4).

Sex chromosomes are usually joined by the terminal regions of the long arms (Fig. 4a), although in some cases, the connection between them is not visible (Fig. 4b). During the prophase of meiosis, the X- and Y-chromosomes are always located next to each other suggesting conjugation between them is conserved.

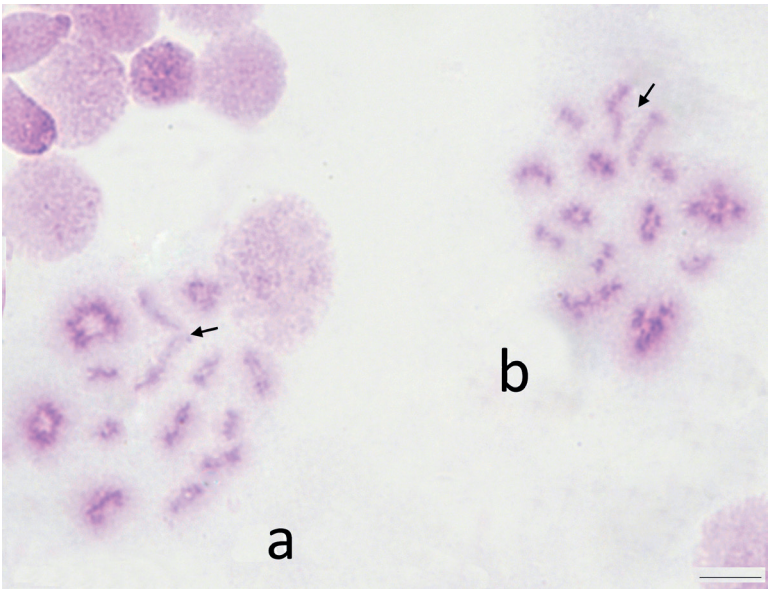

Figure 4. Diakinesis of male meiosis of *Skwala compacta*. Arrows – indicate sex chromosomes bivalent. Scale bar: 5 μm.

Telomeric DNA $(TTAGG)_n$ repeats were detected in the terminal regions of all chromosomes (Fig. 5a).

18S rDNA gene clusters were detected only on X and Y chromosomes (Fig. 5b, c). In the X-chromosome, the rDNA cluster is large, occupying the entire short arm and the proximal part of the long arm. This is clearly visible in the early stages of spermatogonial metaphases (Fig. 5c). In the Y chromosome, the rDNA cluster occupies the entire short arm and the proximal part of the long arm (Fig. 5b, c). The rDNA clusters on interphase cells are clearly visible (Fig. 5d).

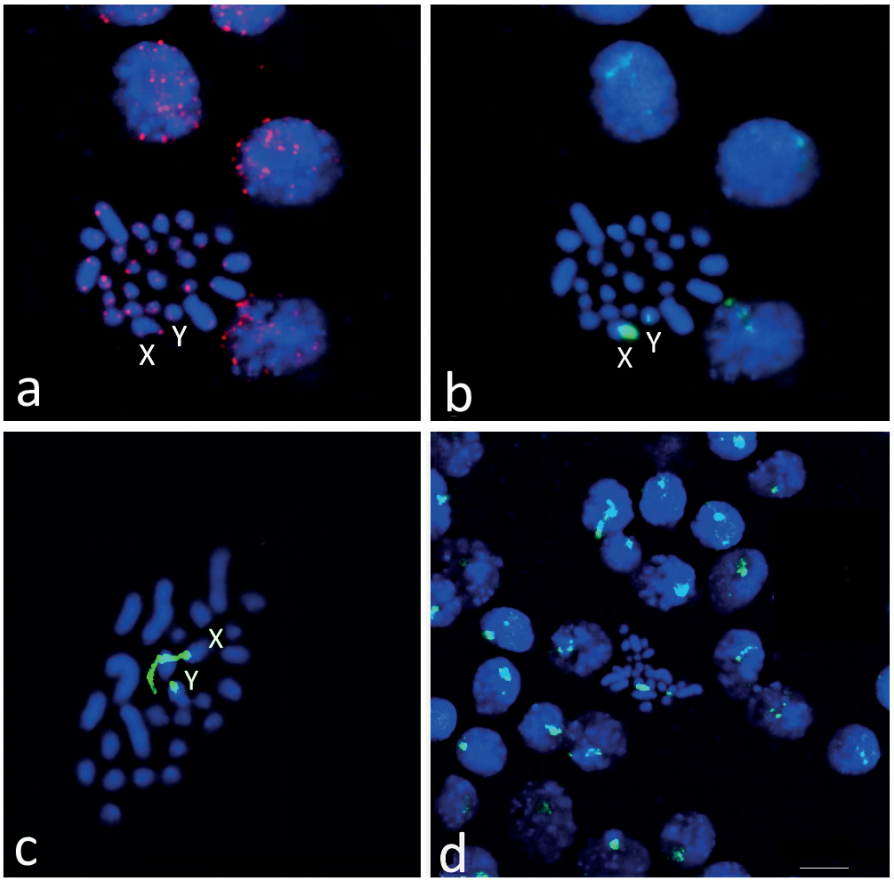

Figure 5. FISH with the telomeric $(TTAGG)_n$ probe (red signals) and the ribosomal DNA probe (green signals) on the chromosomes of male *Skwala compacta*. Same spermatogonial metaphase (**a, b**), early spermatogonial metaphase (**c**) and cells in the interphase and spermatogonial metaphases stages (**d**). Chromosomes were counterstained with DAPI (blue). Scale bar: 5 μm.

Discussion

To date, karyotypes of only 16 species of Plecoptera belonging to the families Perlidae and Perlodidae have been described (Table 1). The number of chromosomes in karyotypes of Plecoptera species varies from 2n♂=10 in *Perla immarginata* (Nakahara 1919) to 2n♂=33 in *Perlodes intricatus* (Matthey and Aubert 1947). In most cases karyotypes were examined in males only. The seven of the fifteen previously studied male stoneflies have 26 chromosomes, and sex is defined as $♂X_1X_20$. In females, 14 chromosomes are sometimes indicated in the haploid set. Only in *Paragnetina immarginata* the mechanism of XY sex determination is described (Table 1).

S. compacta studied by us belongs to the group of species with 2n = 26 and an XX/XY (female/male) mechanism for sex determination. The analysis of the mechanisms of sex chromosome determination in stoneflies shows that in most cases only males were studied, and the mechanism in females was reconstructed from sex chromosomes of males.

Table 1. Karyotype features of the Plecoptera species[1].

Species	2n	n	Sex chromosomes	References
Perlidae				
Acroneuria jezoensis Okamoto (*Calineuria jezoensis* (Okamoto, 1912))	25♂ 26♀	12, 13♂	X0♂	Itoh 1933
Perla abdominalis Guérin-Méneville, 1838	26♂	–	X_1X_20♂	Matthey and Aubert 1947
Perla cephalotes Curtis, 1827 (*Perla baetica* Rambur, 1842 *Dinocras cephalotes* (Curtis, 1827))	26♂	12, 14♂	X_1X_20♂	Matthey and Aubert 1947
Perla bipunctata Pictet, 1833	21♂	11, 10♂	X0♂	Matthey and Aubert 1947
Paragnetina immarginata (Say, 1823)	10♂	5♂	XY♂	Nakahara 1919
Perla marginata (Panzer, 1799)	22♂ 24♀	10, 12♂	X_1X_20♂	Junker 1923
Perla maxima (Scopoli, 1763) (*Perla marginata* (Panzer, 1799))	19♂	9, 10♂	X0♂	Matthey and Aubert 1947
Perlodidae				
Isoperla grammatica (Poda, 1761)	26	12, 14♂	X_1X_20♂	Matthey and Aubert 1947
Isoperla rivulorum (Pictet, 1841)	26♂		X_1X_20♂	Matthey and Aubert 1947
Isogenus (*Dictyogenus*) *imhoffi* Pict.	26♂	14	X_1X_20♂	Matthey 1946
Isogenus (Dictyogenus) alpinum (Pictet, 1841) *(Dictyogenus alpinum* (Pictet, 1841))	26♂	14♂	X_1X_20♂	Matthey 1946
Isogenus (Dictyogenus) fontium (Ris) (*Dictyogenus fontium* (Ris, 1896))	26♂	13♂	X_1X_20♂	Matthey and Aubert 1947
Perlodes intricata (Pictet, 1841)	33♂	–	–	Matthey and Aubert 1947
Perlodes jurassicus Aubert, 1946	31♂	17♂	$X_1X_2X_3$♂	Matthey 1946
Perlodes microcephalus (Pictet, 1833)	27♂	15♂	$X_1X_2X_3$♂	Matthey 1946
Skwala compacta McLachlan, 1872	26♂	13♂	XY♂/XX♀	This paper

[1] The current valid names of Plecoptera species are given in parentheses according to the Plecoptera Species File. https://plecoptera.speciesfile.org.

For 10 out of the 16 species studied, a ♂X_1X_20 mechanism for sex determination is given, whereas only *Acroneuria jezoensis* (Itoh 1933) and *Perla marginata* (Junker 1923) have a reliably described female karyotype. Our data on *S. compacta* show the importance of studying both males and females to correctly determine the sex chromosome mechanism in a particular species. Based on the presence of two heterosomes in males of *S. compacta*, we could interpret their sex chromosome mechanism as X_1X_20 (2n=26), and, thus, the female mechanism as $X_1X_1X_2X_2$ (2n=28) and only the analysis of the female karyotype (2n=26) allowed us to reliably determine the mechanism in this species as XX/XY.

Other variants of chromosomal sex determination identified in stoneflies based on the analysis of male meiosis alone are as the following: ♂X0 (three species); ♂X_1X_20 (ten species); ♂$X_1X_2X_3$ (two species) and ♂XY (one species) (Table 1). Therefore, all these data need to be verified with the obligatory study of the karyotypes of females.

The evolution of chromosomal sex determination is probably the most intriguing problem in comparative cytogenetics of the Plecoptera. Analyzing the primary data on karyotypes of stoneflies, the famous cytogeneticist M. J. White emphasized: “A most interesting series of sex chromosome mechanisms exist in the Stone-flies (Plecoptera),

but its evolutionary history can hardly be guessed at, on the basis of the available evidence" (White 1973, p. 674).

However, he also emphasized that the behavior of the sex chromosomes in this group during the first meiotic division appears to be very peculiar, whether or not there is a 'multiple' mechanism: "Certain species of Stone-flies such as *Perla maxima*, *P. bipunctata* and *Acroneuria jezoensis* are simply X0 in the males (Aubert and Matthey 1943; Matthey 1946; Matthey and Aubert 1947; Itoh 1933), the X is a large metacentric element which is negatively heteropycnotic and lies in one-half of the first meiotic spindle" (White 1973, p. 674–675).

Indeed, compared to other groups of Polyneoptera, in which sex chromosomes in meiosis are either positively heteropyknotic (Acridoidea) or do not differ in compaction from autosomes (Tettigonioidea) (White 1940), in *S. compacta* we studied, the sex chromosomes are also negatively heteropyknotic during meiotic prophase.

Since White's time, the peculiarities of chromosomal sex determination in the stoneflies have been discussed numerous times (White 1941; White 1973; Blackman 1995; Blackmon et al. 2017), but always in speculative tones because, new data simply have not been forthcoming since 1947 (Matthey and Aubert 1947).

Without new comparative material, we cannot yet discuss the ways in which sex determination mechanisms are formed. Therefore, we decided to focus on obtaining new information on the karyotypes of stoneflies, using methods that have not been previously applied to the study karyotypes of this group of insects.

At this stage, to study the karyotypic features of one of the most common species of stoneflies in Siberia, *S. compacta*, we tested various methods of preparing chromosome slides from different tissues of larvae and adults (testes and ovaries, Malpighian tubules, pyloric glands of the stomach and neuroblasts of the brain). The method of preparing slides from cell suspension prepared from germarium of testes and ovaries of this species proved to be the most effective (see section Methods).

This approach is a modification of the technique for obtaining chromosome preparations from grasshopper embryos (Bugrov et al. 2001). The technique used makes it possible to achieve a satisfactory spread of cells on glass, which allows to use different methods of chromosome staining depending on the task of the study.

Using this method it was possible to obtain information on the number and morphology of chromosomes of the model species, and, for the first time for the order Plecoptera as a whole, to identify the localization of constitutive heterochromatin (C-blocks) in chromosomes (see section Results).

The use of the C-banding staining method allowed us not only to reveal the relative size and localization of C-heterochromatin in the chromosomes of the studied species, but also to show that one of the arms of the X chromosome is completely heterochromatic, the length of which strongly depends on the degree of spiralization during spermatogonial mitosis (Fig. 3). It should be especially emphasized that the other chromosomes do not exhibit this feature during mitosis.

Fluorescence in situ hybridization (FISH) with telomeric $(TTAGG)_n$ sequences revealed strong hybridization signals colocalized with the ends of metaphase

chromosomes (Fig. 5a). Our data are in full agreement with the findings of a study of telomeric repeats in the stonefly *Perla burmeisteriana* Claassen, 1936 (Frydrychová et al. 2004). We can only regret that the authors of this study could not obtain information on other karyotypic features of the studied species and limited themselves to the observation that spermatogonia of this species are "with large numbers of chromosomes" (Frydrychová et al. 2004, p. 173).

This type of localization of telomeric repeats is typical for insect chromosomes (Frydrychová et al. 2004; Kuznetsova et al. 2019). Only in some cases, telomeric repeats appear in an interstitial position, indicating possible inversions and translocations of chromosomes in the karyotypic evolution of a particular group of insects (Jetybayev et al. 2012; Kuznetsova et al. 2019).

The localization of rDNA on stonefly chromosomes has not been previously studied. We identified clusters of rDNA only in the heterochromatic arms of the X and Y chromosomes. It is likely that these arms, rich in rDNA sequences, and are regions of the nucleolus organizer (NOR).

This is also evidenced by strong variations in the relative sizes of these heterochromatic arms at different stages of the cell cycle (Fig. 3). Thus, at the early stage of spermatogonial metaphase, heteromorphism in a pair of sex chromosomes is clearly manifested due to a different degree of amplification of rDNA. Such heteromorphism, for example in amphibians, is often considered as a feature that allows such chromosomes to be considered sex chromosomes (Mahony 1991).

In conclusion, the mechanism of sex determination in stoneflies is the most intriguing problem in the cytogenetics of this group of insects.

As our study has shown, this problem can be alleviated by the use of modern chromosomal analysis techniques.

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ORCID

Alexander Bugrov http://orcid.org/0000-0002-7259-3103
Tatyana Karamysheva https://orcid.org/0000-0002-5140-4350
Olesya Buleu https://orcid.org/0000-0003-3913-9950

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Chromatin diminution as a tool to study some biological problems

Andrey Grishanin[1,2]

1 *Papanin Institute for Biology of Inland Waters, Russian Academy of Sciences, 152742 Borok, Yaroslavl Prov., Russia* **2** *Department of Biophisics, Faculty of Natural and Engineering Sciences, Dubna State University, Universitetskaya 19, 141980, Dubna, Moscow Prov., Russia*

Corresponding author: Andrey Grishanin (andreygrishanin@mail.ru)

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Abstract

This work reveals the opportunities to obtain additional information about some biological problems through studying species that possess chromatin diminution. A brief review of the hypothesized biological significance of chromatin diminution is discussed. This article analyzes the biological role of chromatin diminution as it relates to the *C*-value enigma. It is proposed to consider chromatin diminution as a universal mechanism of genome reduction, reducing the frequency of recombination events in the genome, which leads to specialization and adaptation of the species to more narrow environmental conditions. A hypothesis suggesting the role of non-coding DNA in homologous recombination in eukaryotes is proposed. *Cyclops kolensis* Lilljeborg, 1901 (Copepoda, Crustacea) is proposed as a model species for studying the mechanisms of transformation of the chromosomes and interphase nuclei structure of somatic line cells due to chromatin diminution. Chromatin diminution in copepods is considered as a stage of irreversible differentiation of embryonic cells during ontogenesis. The process of speciation in cyclopoids with chromatin diminution is considered.

Keywords

C-value enigma, differentiation, evolution, genome reorganization, recombination, speciation

Introduction

All organisms exhibit genome variability produced by mutations, recombinations, deletions, insertions, mobile elements, etc. A small number of animals additionally exhibit a special, hard-coded form of genome modification called chromatin diminution, DNA elimination, programmed DNA elimination (PDE). As a result of this complex, genetically determined process chromosomes, and fragments of chromosomes undergo elimination. The phenomenon of chromatin diminution has a long history and was discovered by Theodor Boveri (1887). Suffice it to mention that only a year after the discovery of chromatin diminution, Waldeyer (1888) introduced the term chromosome into scientific use. Interest in chromatin diminution revitalized in the second half of the 20th and early 21st centuries (Beermann 1959, 1977, 1984; White 1959; Stich 1962; Painter 1966; Kunz et al. 1970; Geyer-Duszynska 1959, 1961; Bantock 1970; Kloetzel 1970; Ammermann 1971; Ammermann et al. 1974; Wyngaard and Chinnappa 1982; Tobler et al. 1985; Tobler 1986; Bennet 1987; Etter et al. 1991; Prescott 1992; Grishanin et al. 1996; Dorward and Wyngaard 1997; Wyngaard and Rasch 2000; Kloc and Zagrodzinska 2001; Rasch and Wyngaard 2006; Wang and Davis 2014; Zagoskin and Wang 2021). Studies of chromatin diminution (elimination) attracted the attention of an increasingly wide range of scientists, and information about this amazing phenomenon began to quickly accumulate. Review articles devoted to chromatin diminution (elimination) shows how comprehensive research on this phenomenon has become (Raikov 1976; Ammermann 1985; Prescott 1992; Tobler et al. 1992; Goday and Pimpinelli 1993; Grishanin et al. 2006b; Grishanin 2014; Wang and Davis 2014; Dedukh and Krasikova 2021; Drotos et al. 2022; Kloc et al. 2022).

Chromatin diminution in metazoans is the removal of chromosomal material (mostly heterochromatin) from the cells of the somatic line in early embryogenesis; chromatin diminution (programmed DNA elimination) in Protozoa is the removal of entire chromosomes or of sequences interspersed among genic loci in the somatic nucleus. The process of chromatin diminution is species-specific. The diploid number of chromosomes after diminution processes can remain the same or change. The biological role of chromatin diminution remains unknown. Despite being studied for over 100 years, chromatin diminution, in the author's opinion, is an example of one of the most underestimated biological phenomena. The purpose of this work is to show that chromatin diminution is not only interesting as a biological phenomenon, but also provides researchers with a unique opportunity to work with species in which genome size changes during ontogeny, in some cases by more than 90%, which provides additional advantages when studying various biological structures and processes.

Biological roles of chromatin diminution

The biological role of chromatin diminution remains open. Theodor Boveri was the first to suggest a biological role for the eliminated chromatin. He suggested that the

eliminated chromatin has important functions for germline cells, since centrifugation of *Parascaris equorum* Goeze, 1782 (Ascaridida, Nematoda) embryos in the early stages of development initiates diminution in all cells of the embryo, including germline cells (Boveri 1887). Therefore, according to the Boveri hypothesis, chromatin diminution is necessary to determine the direction of development. Evidence that chromatin diminution in parasitic nematodes involves the loss of unique genes from the germline cells and represents the first molecular evidence for Boveri's hypothesis (Etter et al. 1991, 1994; Spicher et al. 1994; Huang et al. 1996). Sigrid Beermann (1977) suggested, considering the chromatin diminution process either as an extreme case of chromatin inactivation, or as a rare variant of chromosomal polymorphism, which leads to the development of heterochromatic blocks in some species of Cyclopoida (Copepoda, Crustacea). One function of DNA eliminated during chromatin diminution may be to control transcription in germline cells, to regulate meiosis, and to regulate replication and transcription processes (Ammermann 1985). Goday and Pimpinelli (1993) consider chromatin diminution as a mechanism for regulating quantitative changes in gene products during ontogenesis. Others scientists suggest that the role of the eliminated DNA is the regulation of recombination processes and the formation of bivalents during meiosis (Müller and Tobler 2000; Staiber and Wahl 2002). The retention of satellite DNA in the germline of *Ascaris* Linnaeus, 1758 may contribute to meiotic homologous recombination, genome evolution, or serve as chromatin spacers, scaffolds, or impact 3D genome organization (Shatskikh et al. 2020). The detection in the eliminated fraction of the *Ascaris lumbricoides* Linnaeus, 1758 (Ascaridida, Nematoda) genome of the gene encoding the ALEP-1 ribosomal protein supports the idea that chromatin diminution is an alternative way of regulating gene activity (Tobler et al. 1992). Others hypothesize that nematodes use chromatin diminution to silence germline-expressed genes in the soma and for sex determination for some species of Strongylidae (Rhabditida, Chromadorea) (Albertson et al. 1979; Streit et al. 2016). Standiford (1988), researching oogenesis in *Acanthocyclops vernalis* Fischer, 1853 (Crustacea, Copepoda) suggested the hypothesis that rDNA sequences are lost during chromatin diminution. The subsequent research of chromatin diminution reported gene deletion during this phenomenon in many taxa (Zagoskin and Wang 2021). For example, ribosomal RNA (rRNA) genes are eliminated in *Cyclops kolensis* Lilljeborg, 1901 (Copepoda, Crustacea) (Zagoskin et al. 2010). It is assumed that a large number of copies of rRNA genes is required only in gametogenesis and in the early stages of development. For later developmental stages, a large number of ribosomal DNA copies may not be necessary. It is also possible that chromatin diminution removes only inactive copies of rDNA. Moreover, the number of rDNA copies can be adjusted according to the genome size using chromatin diminution, since the number of rDNA copies positively correlates with the size of the eukaryotic genome (Prokopowich et al. 2003). The hypothesis of Goday and Pimpinelli (1993) connects the elimination of chromatin in presomatic cells of nematodes with an increase in the ploidy of individual somatic cells of the adult organism and considers chromatin diminution as a mechanism for regulating gene expression by regulating chromatin amount or gene dosage during ontogeny.

The eliminated DNA of the parasitic nematode *Ascaris* contains genes (1000 genes in total) that are predominantly expressed in the germline (Wang et al. 2020). However, considering that genes make up only a small part of the eliminated sequences, it can be concluded with high probability that the removal of genes is not the main goal of chromatin diminution (Zagoskin and Wang 2021).

As a result of chromatin diminution in *Ascaris* chromosomes, both preserved and eliminated chromosomes acquire new telomeres (Wang et al. 2020). It is also proposed that a decrease in genome size due to chromatin diminution leads to a decrease in cell size and a shortening of the cell cycle, which in turn causes a decrease in body size and the achievement of sexual maturity at an earlier age (Gregory and Hebert 1999; Gregory 2001; Wyngaard et al. 2005). A hypothesis stating that the elimination process ensures the maintenance of a functional somatic genome and concomitantly allows extremely rapid and profound changes in the germ line genome is presented, thereby allowing the development of new germ line specific functions and providing a selective advantage for the chromatin diminution in nematodes during subsequent evolution (Bachmann-Waldmann et al. 2004).

In some species, representatives of the order Diptera (Cecidomyiidae, Sciaridae, Chironomidae), elimination of individual chromosomes or entire chromosome sets is observed in the process of sexual differentiation; elimination of chromosomes is preceded by their heterochromatinization (Geyer-Duszynska 1959, 1961; White 1959; Hartl and Brown 1970; Fux 1974; Matuszewski 1982; Jazdowska-Zagrodzinska et al. 1992).

The most complete list of existing hypotheses about the biological significance of programmed DNA elimination (chromatin diminution) suggests the following functions: gene silencing and regulation, nucleotypic effects, mutation rate reduction, and energetic benefits (Grishanin 2014; Wang and Davis 2014; Dedukh and Krasikova 2021; Drotos et al. 2022; Kloc et al. 2022).

Causes and consequences of changes in the structure of interphase nuclei during chromatin diminution in *Cyclops* Müller, 1785 (Copepoda, Crustacea)

The study of chromatin diminution in *Cyclops strenuus strenuus* Fisher, 1851 showed that throughout the prediminution interphase, the nucleus of somatic cells has a weak uniform color. Only 20 minutes before the start of division, numerous lumps of condensed chromatin appear in the nucleus, distributed along the periphery of the nucleus (Beermann 1977). A similar pattern was observed in *C. kolensis* (Grishanin 1995). The nuclei of embryonic cells of *C. kolensis* in the early interphase of the first cleavage divisions have a relatively weak homogeneous color; heterochromatinized structures and chromocenters are absent, which is manifested on preparations stained both by the Feulgen method and studied using the electron microscope. After chromatin diminution, chromatin remains scattered throughout the nucleus, but is interspersed

with more condensed heterochromatic segments. The chromocenters become detectable, and part of the chromocenters adjoin the nuclear membrane (Grishanin 1995). A similar picture is common when describing the interphase nucleus of a eukaryotic cell, when embryonic cells have homogeneous, diffuse chromatin, while in differentiated cells chromatin is dispersed throughout the entire volume and alternates with areas of highly condensed chromatin (Bostock and Sumner 1978; Koryakov and Zhimulev 2009).

Thus, before chromatin diminution, *C. kolensis* embryonic cells have a typical structure of interphase nuclei of embryonic cells, while after chromatin diminution, the structure of interphase nuclei irreversibly changes and more closely resembles the structure of interphase nuclei observed in multicellular eukaryotic cells after differentiation.

As is known maternal genes of the eggs determine the pattern of embryonic formation before fertilization and during initial cleavage divisions, after which the genes localized in the nuclei of embryonic cells play a role in the developmental process (Jaeger 2018). The similarity of the structure of *C. kolensis* somatic cells after diminution with differentiated cells of an adult organism may be due to changes in the structure of interphase nuclei in early embryogenesis in *C. kolensis* due to the transition from the regulation of maternal genes in the early stages of cleavage division to the regulation of nuclear genes of embryonic cells. These facts suggest that chromatin diminution as a stage of embryo development coincides with the stage of Maternal to Zygotic Transition, at which Zygotic Genome Activation occurs. With regard to the process of chromatin diminution itself, the question arises: what path does the initiation of chromatin diminution processes take? Is it through some factors present in the cytoplasm of an unfertilized egg, or do these factors appear in the presomatic cells of the embryo due to Zygotic Genome Activation. We hypothesized that if the chromatin diminution mechanism is triggered by nuclear genes, then suppression of the nuclear genome at the early stages of embryogenesis before the manifestation of the morphogenetic function of the nuclei should stop the chromatin diminution process; if the course of the diminution process is determined by cytoplasmic determinants, then inactivation of the nuclear genome will not affect the progress of the chromatin diminution process. This assumption was confirmed by data from an experiment on irradiation of *C. kolensis* embryos with high doses of radiation blocking the functioning of the nuclear genome of the embryos (Grishanin and Chinyakova 2021). The results of the experiment showed that mechanisms regulating the morphogenetic function of *C. kolensis* nuclei are triggered after the 4th cleavage division, during which chromatin reduction occurs.

It has been established that during the course of chromatin diminution, a decrease in the size of the nuclei in the somatic line cells occurs (Beermann 1977; Grishanin et al. 1996; Gregory and Hebert 1999; Gregory 2001). According to the existing models of the organization of the eukaryotic interphase nucleus, all chromosomes occupy their strictly defined chromosomal territories, the functional activity of which

is determined by the structure of these territories (Koryakov and Zhimulev 2009; Cremer and Cremer 2010). The ordered spatial arrangement of the intranuclear subcompartments of the interphase nuclei are generally evolutionarily conservative and genetically determined (Patrushev and Minkevich 2007). Interphase chromosomes are attached to the nuclear matrix, which is a network of protein fibrils to which chromatin strands are attached in areas called the Matrix Attachment Regions (MAR). For species with chromatin diminution, one might expect not only that the process of genome reduction is programmed, but that the structure of interphase nuclei, including the structure of the nuclear matrix, should also be rearranged as a result of chromatin reduction. In particular, a sharp decrease in genome size should coincide with a change in the number of permanent and functionally dependent sites for binding to the matrix of DNA molecules. A rearrangement of interphase nuclei structure after chromatin diminution can explain the results of certain experiments, which showed that the frequency of chromosome aberrations during post-diminution cleavage divisions in *C. kolensis* is 30–50 times less than during pre-diminution cleavage divisions (Grishanin and Akifyev 2005). A sharp decrease in the frequency of chromosome aberrations in embryos after chromatin diminution compared with embryos before chromatin diminution does not fit into the framework of the classical theory of chromosome aberrations induction (Savage 1989; Akifyev et al. 1990). Based on this theory, the frequency of chromosome aberrations in embryonic cells in *C. kolensis* before and after chromatin diminution should decrease in accordance with the reduction of the genome, in other words 15–16 times. However, the frequencies of chromosome aberrations in germ cells of *C. kolensis* before and after chromatin diminution differ by 50 times. The patterns discovered by Grishanin and Akifyev (2005) could be explained by the results obtained by Akifyev et al. (1995), according to which chromosome aberrations are formed in the minor part of the genome associated with Matrix Attachment Region, which is the most mutable part of the genome. The 94% of DNA removed from the somatic cell chromosomes in *C. kolensis* over the course of chromatin diminution is expected to include a significant number of Matrix Attachment Regions. If one assumes that most chromosome aberrations form in the part of the genome associated with nuclear matrix, then when this part of the genome is removed, the number of chromosome aberrations in cells should also decrease (Fig. 1). Hence, it can be assumed that chromatin diminution causes a 50-fold decrease in the number of points of contact between the nuclear DNA of *C. kolensis* presomatic cells and the nuclear matrix, as a result of which the frequency of chromosome aberrations is reduced by the same 50-fold.

Thus, chromatin diminution in copepods can be considered as a stage of irreversible differentiation of embryonic cells during ontogenesis. The reduction of 94% of the nuclear genome in *C. kolensis* makes it impossible to return the cells of the somatic line to the potencies of the germ line cells. ChroTeMo, a tool (Tkacz et al. 2016) for chromosome territory modelling, may be of great interest to those who study species with chromatin diminution.

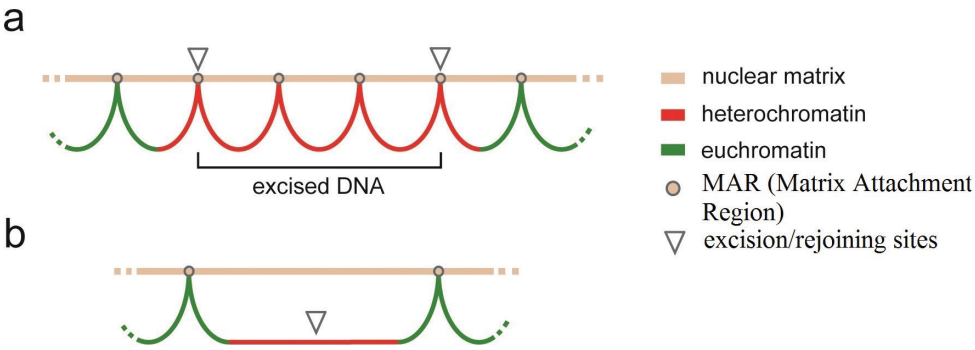

Figure 1. Hypothetical Matrix Attachment Region cutting scheme. Scheme of the *Cyclops kolensis* chromosome, in which, as a result of chromatin diminution, its part associated with the nuclear matrix (matrix attachment region, MAR) is cut out **a** chromosome before chromatin diminution **b** chromosome after chromatin diminution.

Chromatin diminution and C- value enigma

History of the problem

The problem of non-coding DNA (*C*-value paradox, *C*-value enigma) was formulated in the middle of the 20th century and relates to the fact that the most DNA of eukaryotic genomes is non-coding (Mirsky and Ris 1951; Dawkins 1976; Gregory 2001, 2005). While genomes of species belonging to the same genus, e.g., *Drosophila melanogaster* Meigen, 1830 and *Drosophila virilis* Sturtevant, 1916 (Moriyama et al. 1998), can differ in size by more than two-fold, there are no grounds or evidence that point to a significant difference in the number of genes between such species. Some amoebas have 200 times more nuclear DNA than humans, which does not indicate the presence of a larger number of genes in amoeba than in humans. Thus, the phenomenon of genome redundancy in eukaryotic organisms requires an explanation for the more than 200,000-fold differences in genome size that are not related to the complexity of the organism or the number of its genes. Many hypotheses have been proposed for the biological role of non-coding DNA. Some have not stood the test of time; others are still being discussed at the present time. So, the very first hypothesis of Callan (1967) was not supported, postulating that each gene consists of a series of tandem repeats, which are periodically checked for one copy to eliminate mutational divergence. This hypothesis did not stand the test of molecular genetics, since it was later found that eukaryotic genes are mainly represented by unique sequences. Some advocated for the idea of the regulatory function of non-coding DNA (Britten and Davidson 1971), believing that gene loci can be organized into operon-like structures (Georgiev 1970). Since the beginning of the 1970s, the opinion began to spread among biologists that the non-coding DNA has no function. The term "junk" in relation to non-coding DNA, was introduced by Ohno (1972). Ohno suggested

that non-coding DNA does not affect the fitness of organisms, is a useless part of the genome, and is simply passively transferred by chromosomes to the next generation. The non-coding DNA came to be called "selfish" (Dawkins 1976). According to the authors of the "selfish" DNA hypothesis (Dawkins 1976; Doolittle and Sapienza 1980; Orgel and Crick 1980) an increase in the number of copies of sequences of the non-coding fraction of the genome with certain adaptive properties does not affect the phenotype and is not subject to selection. "Selfish" DNA can enhance their own transmission at the expense of other genes in the genome, even if this has no effect on organismal fitness. As a result, these authors believed that non-coding ("selfish") DNA does not affect the adaptive properties of the whole organism. The prevailing point of view among molecular biologists is that non-coding DNA is selectively neutral (Charlesworth et al. 1994; Elder and Turner 1995; Kreitman 1996). Such DNA does not carry coding and regulatory functions, and although it is a certain metabolic burden for the organism, it is still not eliminated by selection and accumulates in the course of evolution as a result of mutational pressure. This concept is essentially similar to the "junk" DNA hypothesis. Petrov (2001) suggested that genome size fluctuations can occur under the influence of various factors: transposable genetic elements, degradation and excision of pseudogenes; the presence of "harmless" insertion sites, which equates events associated with changes in the structure of the genome occurring in these cases to neutral mutations. However, Petrov (2001) considers the change in the rate of appearance of small insertions and deletions (indel) to be the main factor in the variability of the size of the eukaryotic genome. If the frequency of spontaneous insertions and their size is greater than that of deletions, then, according to Petrov, this should create constant pressure in the direction of increasing the size of the genome. Ultimate control, according to Petrov, belongs to natural selection. With the weakening of selection, fluctuations in the size of the genome can be affected by other "factors", for example, genetic drift, which can rebuild the genotypic structure of the population in a short time (the size of the genome in this case should be considered a phenotypic trait). This idea is consistent with concept of "skeletal" DNA known for more than 40 years (Cavalier-Smith 1978, 1985) and shared with certain reservations by some authors (Wyngaard and Gregory 2001; Kozlovski et al. 2003). According to the Cavalier-Smith concept, DNA not only encodes genetic information DNA but also has a structural function, and plays the role of a "nucleoskeleton" that determines the size of the nucleus, so the non-coding amount of DNA is determined by selection, since the larger the cell, the larger the nucleus should be. This correlation has been found for eukaryotes (Horner and Macgregor 1983; Olmo 1972; Gregory and Hebert 1999; Gregory et al. 2000) but not others (Pagel and Johnstone 1992). According to Gregory (2003), DNA plays not only a qualitative role in evolution, being a genetic material, but also a quantitative one, since changes in genome size should be considered as mutational events leading to phenotypic variations that can be influenced by natural selection. It should be noted that neither Gregory nor other authors reflecting on this topic believe that the nucleotypic hypothesis (Petrov 2001; Gregory 2003) is sufficiently substantiated and consistent.

Several authors have shown a correlation between genome size and various ecological or physiological parameters including the body's resistance to cold and dryness in some plant species (Bennet 1987; Macgillivray and Grime 1995; Wakamiya et al. 1996), and the metabolic rate in certain species of mammals and birds (Vinogradov 1995). Vinogradov (1998) proposed the presence of buffer functions in non-coding DNA, providing passive energy-independent cell homeostasis, and would explain the dependence of the metabolic rate on the amount of non-coding DNA. The non-coding DNA is hypothesized to protects genes from the effects of physical and chemical mutagens (Hsu 1992). Of particular interest is a study on *Drosophila* Fallén, 1823, which showed a decrease in the viability of individuals as a result of the deletion of part of the satellite DNA (Wu et al. 1989). At various times, it was suggested that non-coding DNA is involved in the regulation of the functioning of unique genes, in particular, with the help of RNA interference (Fire et al. 1998). A hypothesis was proposed suggesting a protective function of non-coding DNA (Patroushev and Minkevich 2007).

The *C*-value paradox poses another question for biologists to answer: why organisms occupying a lower position on the phylogenetic tree, being ancestral forms or contemporaries of ancestral forms, have a significantly larger genome than more evolutionarily advanced or more specialized species. Mirsky and Ris (1951) drew attention to the fact that more specialized species have a smaller genome. Convincing evidence has been provided that animals and plants considered primitive or ancestral life forms, have more nuclear DNA than specialized species or species considered evolutionarily advanced (Ginatulin 1984; Hinegardner 1976). For example, psilophytic and fern-like plants contain up to 100 pg per haploid genome (1*C*), while the genomes of evolutionarily more advanced flowering plants contain less than 10 pg DNA per nucleus. While 90% of all modern fish species have a genome size in the range of 0.5–2 pg, the genome size of some species of Polypteridae have the range 3.69–7.25 pg, Salmonidae have the range 1.98–4.9 pg (www.genomesize.com). The genome size of the more primitive fish Chondrichthyes and Lepidosireniformes, which lived on the planet more than 400 million years ago, is much larger: in the former it is within the range of 1.58 – 14.8 pg, in the latter from 40 to 132.83 pg (www.genomesize.com). The genome size of caudate amphibians (Proteidae, Urodela) has a genome size from 25 to 120.6 pg per 1C (www.genomesize.com). Most bird species specialized for flight contain (0.91–1.93 pg DNA per 1C) 1.5–2 times less nuclear DNA than reptiles (1.26–5.44 pg per 1C), a genome size of Mammalia have in the range 1.63- 6.3 pg (www.genomesize.com). It cannot be expected that less specialized or ancestral species possess a large number of genes. The difference in the size of genomes depends on the amount of non-genic DNA. Therefore, non-coding DNA must perform a very specific function.

The study of the chromatin diminution process allows us to shed light on the fate of eliminated DNA (primarily constitutive heterochromatin), which was classified as non-coding or "junk", and on the fate of some unique sequences that are also removed from the nuclear genome of somatic cells as a result of chromatin diminution. The idea of linking non-coding DNA to chromatin diminution belongs to Alexei Akifyev (Akifyev 1974; Akifyev et al. 2002; Akifyev and Grishanin 2005). He wrote: "Many years of dissatisfaction in understanding the biological role of non-coding DNA in eu-

karyotes, its actually dead-end state, from our point of view, is due to the fact that there was no directed search for that genetic process that would allow one to judge the actual functions of non-coding DNA and determine goals for further research." According to Akifyev, the search for the biological role of non-coding DNA should be sought by studying the phenomenon of chromatin diminution.

A unique objective for solving the *C*-value enigma can be a representative of freshwater copepods, *C. kolensis*, in which, during the 4th cleavage division, 94% of the DNA is excised from the chromosomes of somatic line cells, while germ-line cells retain their nuclear DNA unchanged throughout ontogeny. The diploid number of chromosomes remains unchanged (Grishanin et al. 1996; Drotos et al. 2022). In the somatic line, the remaining 6% of the genome is sufficient to perform all necessary functions of an adult organism. The eliminated 94% of DNA in *C. kolensis* can undoubtedly be considered as non-coding DNA for somatic cells, since the absence of this part of the genome in them does not interfere with the normal course of ontogenetic processes.

According to the selectively neutral hypotheses (Charlesworth et al. 1994; Elder and Turner 1995; Kreitman 1996), non-coding DNA has no coding or regulatory functions. It follows that the fraction of non-coding DNA, at least in fairly evolutionarily old species, should be dominated by sequences with a fairly high degree of divergence. In the eliminated DNA of *C. kolensis*, which we consider as non-coding for cells of the somatic line, there is a complex organization of various repeating sequences, due to the characteristic alternation of repeats and spacers, the complex structure of many repeats, the presence of slightly divergent, and often 100% identical to the consensus direct and inverted repeats present both in the same fragment and in different regions of the *C. kolensis* genome, many fragments (repeats) consist of submotifs, that is, they have a mosaic structure (Degtyarev et al. 2004). A comparative analysis of the consensus sequences of one of the eliminated DNA repeats *C. kolensis* showed that this repeat is present in the genome of both Moscow and Baikal populations of *C. kolensis* and is conserved (97–98% homology), is not eliminated completely in the course of chromatin diminution and is present in the genome of somatic cells of both populations (the degree of homology of the nucleotide sequence before and after diminution is 100% for the Moscow population and 99.1% for the Baikal population) (Grishanin et al. 2006c). It can be assumed that such strict conservation of non-coding sequences is determined by their role in the function of germline cells and does not allow us to consider the eliminated part of the genome as "junk" or "parasitic".

The assumption that the role of non-coding DNA is in gene repression, which occurs during heterochromatinization of non-coding DNA, involving neighboring areas of euchromatin in this process (Zuckerkandl 1997), is unlikely from the perspective of data on copepods. Indeed, the elimination of 94% of DNA from cells of the somatic line of *C. kolensis* argues against this hypothesis, since morphogenesis begins after chromatin diminution is completed. Elimination of 94% of the genome of somatic cells of *C. kolensis* allows us to conclude that the eliminated DNA does not have significant coding and regulatory functions. Considering the fact that the full-length genome is preserved in germline cells, we hypothesize that some eliminated sequences, removed during the process of chromatin diminution from genome of somatic line cells, but retained in genome of germline cells, are necessary for the normal course of meiosis and maturation of germ cells.

There is a need to dump non-coding DNA

Consider a model assuming the role of non-coding DNA in homologous recombination in *C. kolensis.* Suppose that the function of non-coding DNA is to increase the speed and frequency of recombination processes, the purpose of which is to increase the qualitative diversity of offspring that fall under the action of selection. At the same time, the more recombinant variants of the genome will be obtained, and the faster recombination events will take place, the more diverse offspring will be obtained. Given the interference rule, according to which two exchanges rarely occur in close proximity to each other, it can be assumed that the lower the density of genes in the genome, the higher the rate of recombination processes. It can be assumed that the role of non-coding DNA is to increase the distances between genes and their parts (exons and introns), as well as regulatory and structural elements (enhancers, silencers, insulators, MARs, etc.), in order to ensure the greatest freedom during recombination processes. The greater the distance between the coding regions of the genome, as well as coding and regulatory sequences, the greater the number of introns in the genes, and their magnitude, the less likely there will be violations of the structure of genes during recombination processes.

The more often recombination events that take place, the more different gene variants will appear during the rearrangement of coding and regulatory sections of the genome, and the more variants of structural and regulatory proteins will appear in this individual. In addition, non-coding DNA, creating a spatial three-dimensional structure in the interphase nucleus, largely determines the genome's likelihood to undergo ectopic recombination. Thus, a genome "diluted" with non-coding DNA makes it possible to quickly search for a wide variety of gene variants. The evaluation of these variants is carried out through the phenotype of an individual during the implementation of various genome variants in the interaction of the organism with the external environment. A successful variant of the genome should be stabilized; therefore, a decrease in the rate of recombination processes due to genome reduction can be considered as a mechanism for reducing genome variability. In other words, with the specialization and adaptation of the species to narrow ecological conditions, the need to find the optimal variant of the genome decreases. A large genome makes it difficult to fix the optimal variant of linear and spatial relationships of various parts of the genome, which allows the species to interact within this ecological niche in the most successful way. A non-coding genome during the fluctuation of the environment provokes further changes during recombination processes, and the loss of the optimal structure is found by it under the conditions of the ecological niche to which it has adapted. There is a need to dump non-coding DNA. This is achieved by genome reduction in somatic and germline cells. All the mechanisms necessary for such a process in cells exist: restriction by endonucleases and crosslinking of free ends by ligases. Genetic regulation of the main events of meiosis is well studied. If meiosis is disrupted, then sterility occurs in one or both sexes (Huang and Roig 2023). If, during recombination, important genes that should be involved during meiosis, but do not participate in the subsequent ontogenetic development of somatic cells, fall into the region of non-coding DNA intended for deletion, it becomes possible to preserve the original genome only in cells of the germline and reduce part of the genome in cells

of the somatic line, which we observe in species with chromatin diminution (Fig. 2). Consequently, the origin of the chromatin diminution phenomenon can be considered as an incomplete process of genome reduction in both somatic and germline cells. In this case, chromatin diminution is an instrument of genome reduction in the course of evolution only in cells of the somatic line. Although the evolutionary advantages of a species with chromatin reduction are very conditional compared to a species without this phenomenon, nevertheless, this complex process of genome reorganization appeared during evolution. Despite the risk of losing important genetic information, species with chromatin diminution radically solve the problem of genome size reduction by removing, predominantly heterochromatin, from the genome of somatic line cells. Therefore, the chromatin diminution should not be considered as a rare phenomenon in the phylogeny of a small number of species, but as a universal mechanism of genome reduction, which may have been quite common among eukaryotes throughout their evolution. In addition, the removal of non-coding DNA during chromatin diminution can lead to a change in the sequence of exons and to a change in the level of gene expression. This point of view is consistent with the explanation of morphological evolution not due to the accumulation of point mutations, but due to the redistribution of genes, I.e. due to the rearrangement of DNA sequences and their exchange between members of the population (Doolittle and Sapienza 1980; Dover 1982).

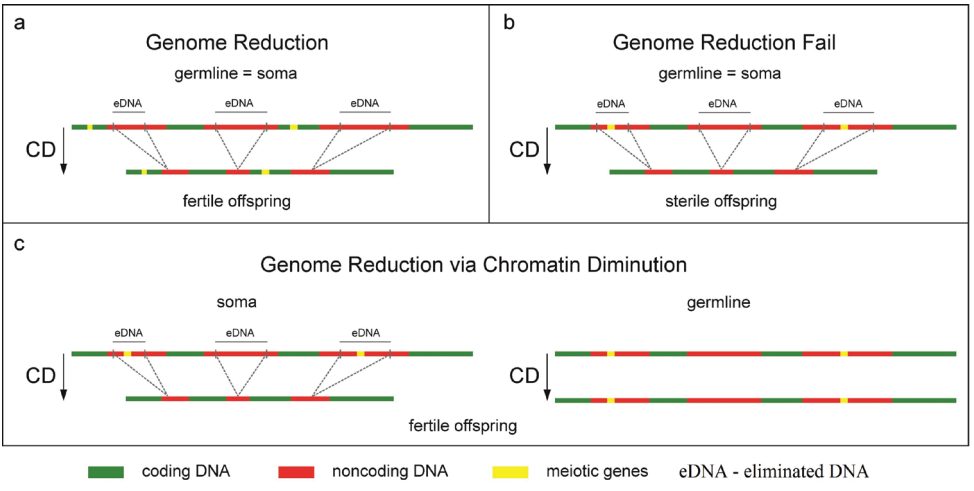

Figure 2. Hypothetical scheme of the origin of Chromatin Diminution (CD) **a** genome reduction during evolution occurs in germ line cells and somatic line cells; genes responsible for meiosis are located in the part of chromosomes that is not subject to genome reduction **b** genome reduction during evolution occurs in germ line cells and somatic line cells; during genome reduction the genes responsible for meiosis are located in the part of chromosomes that is subject to reduction; as a result, the offspring become sterile due to the absence of genes controlling meiosis **c** genome reduction occurs only in somatic line cells while preserving the original genome in germ line cells; during chromatin diminution the genes responsible for meiosis are located in parts of the chromosome that are subject to reduction during chromatin diminution; but they are retained in the chromosomes of germline cells. The offspring are viable. Chromatin diminution does not affect development processes.

Chromatin diminution as a factor of genetic isolation

The appearance of chromatin diminution in the ontogenesis of a species of the genus *Cyclops* may also become a factor contributing to genetic isolation and further contribute to speciation. Due to the relatively short life cycle of freshwater copepods, genetic isolation can occur quite quickly (Dodson et al. 2003; Grishanin et al. 2005, 2006a). Investigating chromatin diminution in *C. kolensis*, we drew attention to the differences between the Russian and Germany populations of this species in a number of cytogenetic features and the chronology of diminution processes. According to cite author and year, chromatin diminution in individuals of the Moscow and Baikal populations of *C. kolensis* occurs during the 4th embryonic division, and according to Ulrich Einsle, chromatin diminution in *C. kolensis* is observed during the 5th embryonic division (Einsle 1993; Grishanin et al. 1996, 2006b). Granules of eliminated chromatin in the anaphase of diminution division of embryonic cells of individuals of the German *C. kolensis* population accumulate in the equator region, whereas specimens of the Russian *C. kolensis* population such granules accumulate mainly at the poles of the division spindle (Einsle 1993; Grishanin and Akifyev 2000). It is obvious that such signs as the presence or absence of chromatin diminution in ontogenesis, differences in the diploid number of chromosomes among the studied *Cyclops* species, differences in a number of characteristics of the chromatin diminution process (chronology of chromatin diminution, distribution of granules of eliminated chromatin in the anaphase of diminution division and other features of chromatin diminution) are inherited and rigidly determined in ontogenesis.

A large-scale rearrangement of the genome has occurred apparently in the species *Cyclops insignis* Claus,1857 as evidenced by the German population which has chromatin diminution (Einsle 1993) and the Russian population which lacks chromatin diminution (Grishanin et al. 2004); otherwise, has no visible morphological differences are evident. In this case, the mechanisms of speciation may be associated with the exclusion of those required stages of the diminution processes that must occur in presomatic cells in species that possess chromatin diminution, and the chromatin diminution process itself might thus be a driver of genetic isolation between populations that differ in how chromatin diminution is achieved, or between species, one of which has chromatin diminution and the other does not (Akifyev and Grishanin 1998, 2005; Grishanin 2014). The lack of morphological differences may be because cyclopoids, and especially the *Cyclops* genus, are characterized by morphological stasis. Analyzing the molecular structure of eliminated *C. kolensis* sequences (Akifyev et al. 2002) we assumed that eliminated DNA may play a role in the genetic isolation mechanism preventing the synapsis of homologous chromosomes in meiosis of interspecific *Cyclops* hybrids.

Consider a hypothetical scheme of speciation. The parental species (presumably *Cyclops* sp.) has a genome containing a large amount of non-coding DNA (Fig. 3). The genomes of his somatic and germline cells are the same. Let us assume that during the evolution of a species a reduction of the genome is programmed. Genome reduction can take place in its descendants in two ways: population A, in which genome reduction occurred only in somatic line cells, and population B, in which genome reduction

took place in somatic and germline cells (Fig. 3). At the same time, let's assume that in population B due to inversion the linear order of the arrangement of functionally significant DNA sections, which the chromosomes of both the somatic and germ line possess, has changed and the exon sequences changed from 1-2-3-4 to 2-1-4-3. Individuals with inverted chromosomes will be denoted as population B1 (Fig. 3). Individuals of population A, in which genome reduction took place only in cells of the somatic line, retained the original linear order of the arrangement of functionally significant sections of the genome. When crossing individuals of population A with individuals of population B or B1, they will give different pictures of the chromosomes conjugation in meiosis. In hybrids of individuals of populations A and B, partial conjugation will take place in meiosis (Fig. 3). In hybrids of individuals of populations A and B1 conjugation in meiosis will be impossible, as a result of which meiosis will be disrupted, and such hybrid individuals will be infertile (Fig. 3). In other words, a genetic barrier will arise between hybrid individuals of populations A and B1. Therefore, we can assume that chromatin diminution and genome reorganization may lead to genetic isolation of individuals (populations) of cyclops species.

The phenomenon of gonomery in fresh-water Copepoda species can be considered as an example of an intermediate stage of genome evolution in species with the phenomenon of chromatin diminution. Sigrid Beermann (1977) found polymorphism in the amount of heterochromatin in females of *C. strenuus strenuus* and *Cyclops furcifer* Claus, 1857. Dimorphism in the content of heterochromatin in *C.s. strenuus* also causes a difference between the sexes. If the females are *C.s. strenuus*, as a rule, are heterozygous for the "enrichment" of chromosomes with heterochromatin, then males are always homozygous for this trait and contain only large chromosomes with interstitial heterochromatin. In heterozygous females, chromosomes enriched with "heterochromatin" from a set of large chromosomes and a set of small chromosomes, which consist primarily of euchromatin diverge in separate groups during anaphase of cleavage divisions before chromatin diminution (Beermann 1977). About half of the eggs have one set of short chromosomes and one set of long ones, while the other half of the eggs contain only long chromosomes. The difference in length is approximately equally distributed between all chromosomes. A smaller amount of eliminated chromatin is formed in heterozygous embryos, a larger amount of eliminated chromatin in homozygous ones. Removal of a part of chromatin from the chromosomes of *C.s. strenuus* as a result of chromatin diminution leads to a decrease in the size of chromosomes. Chromosomes enriched with heterochromatin from a set of large chromosomes change more strongly than chromosomes from a set of small chromosomes, which consist primarily of euchromatin. In species of *C.s. strenuus* and *C. furcifer* chromatin diminution completely eliminates the significant difference in size between homologous chromosomes. In other words, chromosomal polymorphism is limited only to germline cells. Regardless of the distribution of eliminated chromatin in all three species *C.s. strenuus* and *C. furcifer* after chromatin diminution there are always 22 pairs of identical chromosomes in diploid somatic cells. Gonomery and chromatin diminution was also found in *Mesocyclops longisetus* Forbes, 1891 (Copepoda) (Rasch and Wyngaard 2008).

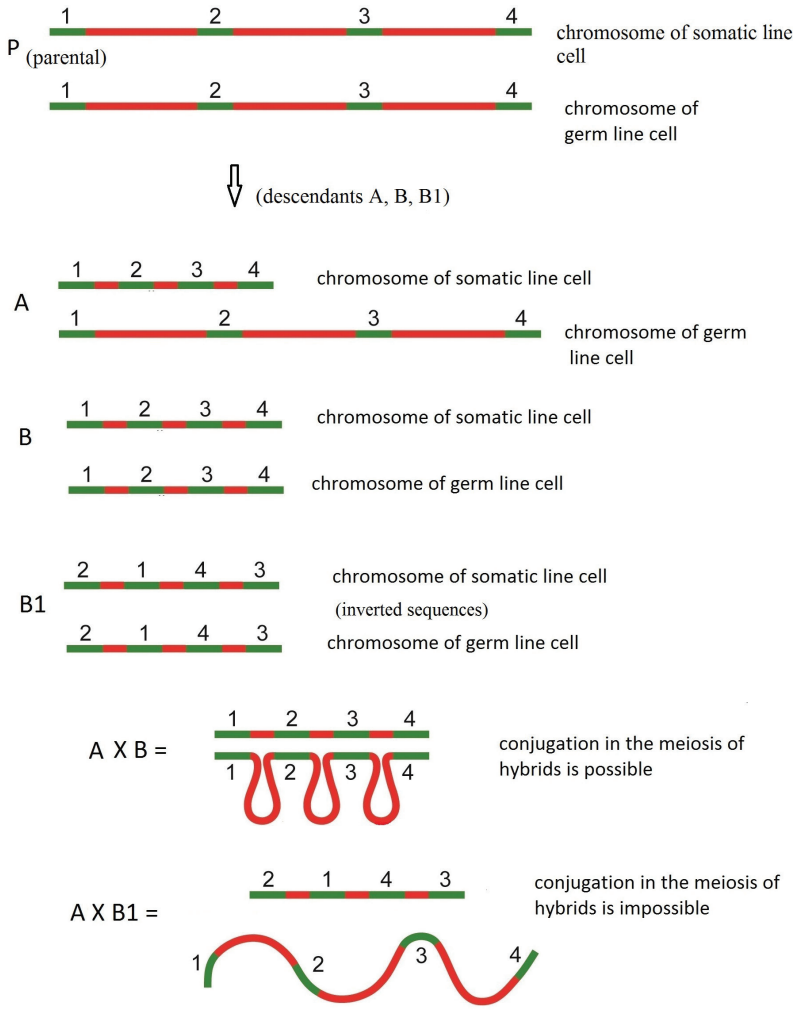

Figure 3. Hypothetical scheme in which reproductive isolation is determined by the appearance of chromatin diminution in one of the populations. Genome reduction of parental species (P) can take place in its descendants in two ways: population A, in which genome reduction occurred only in somatic line cells, and population B, in which genome reduction took place in somatic and germline cells. In population B due to inversion the linear order of the arrangement of functionally significant DNA sections has changed (population B1). In hybrids of individuals of populations A and B, partial conjugation will take place in meiosis. In hybrids of individuals of populations A and B1 conjugation in meiosis will be impossible and such hybrid individuals will be infertile.

Chromosomes from the set of small chromosomes in females of *C.s. strenuus*, in which there is no eliminated chromatin, can be considered as a genome in which a reduction of heterochromatin (part of non-coding DNA) has occurred. Thus, we can consider species with gonomery as an example of genome evolution, during which genome reduction is observed not only in somatic line cells, but also in germ line cells.

Conclusions

Reduction of 94% of DNA in the somatic cell line as a result of chromatin diminution in *C. kolensis*, allowed us to consider the eliminated DNA as non-coding for cells of the somatic line, since the absence of this part of the genome in them does not interfere with the normal course of ontogenesis. At the same time, it suggests that the eliminated DNA does not carry any significant coding and regulatory functions in the somatic line.

Studies of chromatin diminution in *C. kolensis* have shown that as a result of chromatin diminution, a change in the structure of interphase nuclei occurs, which is characteristic of the interphase nucleus of a differentiated eukaryotic cell. The results obtained led to the conclusion that the process of chromatin diminution is an alternative form of regulation of cell differentiation into the somatic and germ lines.

Studies of different species of *Cyclops* have shown that the reason for the appearance of chromatin diminution in ontogenesis is not related to the need to remove non-coding DNA from the genome of somatic cells, as can be seen when comparing *C. insignis* from Moscow, Russia, which does not have chromatin diminution, and *C. insignis* from Germany, which has chromatin diminution (Grishanin et al. 2004).

The genome reduction is a tool aimed at reducing the speed of the evolutionary process of a species by reducing the frequency of recombination events, which leads to a decrease in the diversity of genotype variants in offspring when the necessary level of adaptability to environmental requirements is achieved. The chromatin diminution can be considered as one of the options for this process, when genome reduction in germ line cells is impossible due to localization of sequences there that are presumably important for the processes of meiosis and early stages of embryogenesis, but not necessary for subsequent development.

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ORCID

Andrey Grishanin https://orcid.org/0000-0002-6368-5807

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SHORT COMMUNICATION

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The role of cellular polyploidy in the regeneration of the cirrhotic liver in rats and humans

Natalia N. Bezborodkina[1], Vsevolod Ya. Brodsky[2], Boris N. Kudryavtsev[3]

1 *Zoological Institute, Russian Academy of Sciences, Universitetskaya emb.1, St Petersburg 199034, Russia* **2** *Koltzov Institute of Developmental Biology, Russian Academy of Sciences, 26 Vavilov str., Moscow 119334, Russia* **3** *Saint-Petersburg State University, University ave 26, St Petersburg 198504, Russia*

Corresponding author: Natalia N. Bezborodkina (Natalia.Bezborodkina@zin.ru)

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Abstract

Polyploidy is a condition in which a cell has multiple diploid sets of chromosomes. Two forms of polyploidy are known. One of them, generative polyploidy, is characteristic of all cells of the organism, while the other form develops only in some somatic tissues at certain stages of postnatal ontogenesis. Whole genome duplication has played a particularly important role in the evolution of plants and animals, while the role of cellular (somatic) polyploidy in organisms remains largely unclear. In this work we investigated the contribution of cellular polyploidy to the normal and the reparative liver growth of *Rattus norvegicus* (Berkenhout, 1769) and *Homo sapiens* Linnaeus, 1758. It is shown that polyploidy makes a significant contribution to the increase of the liver mass both in the course of normal postnatal development and during pathological process.

Keywords

Hepatocytes, human, polyploidy, rat, reparative growth

Introduction

Polyploidy, expressed in a multiple increase of the number of chromosomes in cells, is represented in multicellular organisms by two forms. The generative form of polyploidy, which is inherited in a series of generations, arises as a result of genomic mutation in meiosis during the formation of gametes. It is characterised by an increase in

the number of chromosome sets in all cells of the organism, including germline cells, and has played a prominent role in plant and animal evolution (Madlung et al. 2013; Van de Peer et al. 2021; Mezzasalma et al. 2023). In animals, whole genome duplication is much rarer than in plants. Nevertheless, this form of polyploidy is quite common in invertebrates and also occurs in vertebrates at the bottom of the evolutionary ladder, mainly fish and amphibians (David 2022). Generative polyploidy is almost totally absent in mammals, which is thought to be associated with the genetic mechanism of sex determination (Ohno 1970; Wertheim et al. 2013). The only instance of a tetraploid mammal is the red vizcacha rat *Tympanoctomys barrerae* (Lawrence, 1941) from Argentina (Otto and Whitton 2000).

Though lacking generative polyploidy, mammals are characterised by somatic polyploidy, which develops only in individual tissues or cells. In the case of somatic polyploidy, polyploid cells may constitute a significant part of the cell population of some mammalian organs, such as the liver or the heart (Brodsky and Uryvaeva 1985; Kudryavtsev et al. 1997; Donne et al. 2020; Kirillova et al. 2021; Anatskaya and Vinogradov 2022). As a rule, the ploidy of cells in these organs does not exceed the octoploid level. However, in some cases, especially in pathology, the ploidy of hepatocyte and cardiomyocytes can significantly exceed the diploid level: 32-fold, 64-fold and more. The number and ploidy levels of cells in tissues have a strong individual variability and are not inherited. Polyploid cells in somatic tissues are formed anew each time at certain stages of postnatal ontogenesis by incomplete mitoses. The alternation of acytokinetic mitoses forming binucleate cells and bimitoses (2c → 2c×2 → 4c → 4c×2 → 8c → etc.), where "c" is the amount of DNA in cell nuclei corresponding to its amount in the diploid set of chromosomes, leads to the emergence of hepatocytes of increasingly higher ploidy levels (Brodsky and Uryvaeva 1985). Multipolar mitoses also play a certain role in the formation of polyploid hepatocytes (Duncan et al. 2010). The details of the polyploidisation process may differ in different mammalian species, but the pool of mononucleate diploid hepatocytes is always its starting point.

An increase in cell size is considered to be the most noticeable manifestation of polyploidy at the cellular level. In plants, this increase often leads to gigantism. In contrast to plants, in animals polyploids are usually similar to diploids in body size and, as a consequence, have fewer cells (Kudryavtsev et al. 1988). It is also believed that during the development of polyploidy in animals the proliferative activity of cells decreases and so does the ratio of cell surface area to cell volume. The latter may result in a decrease in the metabolic rate in various organs. However, it has been established that various indicators of cell metabolism change in accordance with the gene dosage under both normal and pathological conditions (Brodsky and Uryvaeva 1985; Bezborodkina et al. 2016). It is assumed that polyploidisation of hepatocytes arose in the course of evolution as a genetic mechanism of cell adaptation to the damaging effect of various xenobiotics consumed with food (Duncan 2013; Sladky et al. 2020; Sladky et al. 2022).

At the organismic level polyploidy, due to increased heterozygosity, is a powerful tool of speciation, helping new species to conquer new habitats previously inaccessible to their diploid ancestors. The role of polyploidy at the tissue level remains largely

obscure. In this work, we investigated this problem by evaluating the contribution of polyploidy in the normal and reparative liver growth of *Rattus norvegicus* (Berkenhout, 1769) and *Homo sapiens* Linnaeus, 1758, and comparing it with other cellular growth mechanisms, proliferation and hypertrophy.

Methods

The DNA content in hepatocyte and their dry mass were determined according to a previously described combined cytochemical method for quantifying several components in the same cell (Bezborodkina et al. 2016).

The relative contribution of proliferation (Q_1), polyploidisation (Q_2) and hypertrophy (Q_3) of hepatocytes during the normal and the reparative growth of rat and human liver were calculated using the following formulae (Bogdanova et al. 1990):

$$Q_1 = \frac{M \times (P_1/P_2) - 1}{M - 1}$$

$$Q_2 = \frac{M \times m_1 \times (g_2 - g_1)}{(M - 1) \times P_2}$$

$$Q_3 = \frac{M \times g_2 \times (m_2 - m_1)}{(M - 1) \times P_2}$$

where: M – repetition factor of liver parenchyma mass change during the study period (6 months). Based on the data on the value of mitotic index, duration of mitosis in hepatocytes and the level of parenchyma necrotisation during repeated exposure to CCl_4, it was calculated that the loss of parenchyma mass during 6 months of exposure exceeds the initial mass approximately 5-fold. In case of physiological regeneration the loss of parenchyma mass due to cell death during the same period is equal to its initial mass (Sakuta and Kudryavtsev 1996); P_1 and P_2 – dry mass of hepatocyte before the beginning of poisoning of rats with CCl_4 and at the end of the experiment, respectively; m_1 and m_2 – average dry mass calculated per diploid hepatocyte before the beginning of poisoning of rats with CCl_4 and at the end of the experiment, respectively; g_1 and g_2 – average ploidy of hepatocytes divided by 2.

Results

Cirrhosis of various aetiologies is a widespread human and animal disease in which functioning liver parenchyma is replaced by useless connective tissue. As a consequence, the number of hepatocytes, which perform the multiple functions of this organ, decreases during the development of cirrhosis by 28% ($P < 0.001$) in rats and twofold

($P < 0.001$) in humans. These profound changes in the architectonics and metabolism elicit a powerful regenerative response of the liver expressed in a greater proliferation of hepatocytes and their hypertrophy, increasing as compared to the norm by ~ 25% ($P < 0.01$) both in rats and in humans.

Cytophotometric analysis of hepatocyte distribution by ploidy classes in rats with CCl_4-cirrhosis of the liver showed that the composition of the cell population of the liver parenchyma in this group of animals significantly differs from the norm (Table 1).

The parenchyma of the cirrhotic rat liver is characterised by a decrease in the ratio of binucleate hepatocytes with diploid nuclei (2c×2-cells) and an increase in the relative number of cells with a high ploidy. As a result, the average ploidy level of hepatocytes of rats of the experimental group increases by 14.8% ($P < 0.01$) as compared to the norm (Table 1).

In contrast to rats, in humans the modal class of hepatocytes is represented by mononucleate diploid (2c) cells. The average ploidy of hepatocytes of the normal human liver was 2.21±0.05c, while in patients with LC it increased by 15.8% (Table 1).

Data on the changes in the liver parenchyma mass during LC development, DM of hepatocytes and their ploidy in the normal and the cirrhotic liver of rats and humans make it possible to quantify the contribution of proliferation (Q_1), polyploidization (Q_2) and hypertrophy (Q_3) of cells to normal and reparative liver growth.

The data presented in Table 2 indicate that in rats cell hypertrophy (about 18%) plays a significant role in the increase of liver mass during LC development. However, the main contribution to the reparative growth of the liver is made by cellular processes associated

Table 1. Distribution of rat and human hepatocytes by ploidy classes in the cell populations of the normal (control) and the cirrhotic liver (LC) ($X \pm S_x$).

		Proportion of hepatocytes of different ploidy classes, %						
		2c	2c×2	4c	4c×2	8c	8c×2	Average cell ploidy, c
Rat	Control (n = 5)	0.63±0.24	3.62±0.48	81.84±3.14	9.38±2.90	3.53±1.79	–	4.46±0.15
	LC (n = 5)	2.86±0.91[1]	2.13±1.45	68.51±3.95[1]	12.68±2.94	12.37±2.82[1]	1.45±0.62	5.12±0.17[1]
Human	Control (n = 7)	89.57±2.28	4.70±1.47	5.73±1.38	–	–	–	2.21±0.05
	LC (n = 7)	75.19±5.23[1]	16.22±3.81[1]	7.07±1.89	1.07±0.58	0.45±0.17	–	2.56±0.17[1]

[1] Significantly different from the value in the control at $P < 0.05$.

Table 2. Relative contribution (%) of proliferation (Q1), polyploidisation (Q2) and hypertrophy (Q3) of hepatocytes to changes in rat and human liver mass during the development of liver cirrhosis (LC).

	Q_1	Q_2	Q_3
Rat	66	16	18
Human	111.2	–7.3	–3.9

Note: In calculating the contribution(s) of cell proliferation, polyploidy and hypertrophy, it was assumed that the ratio of the liver parenchyma mass of the cirrhotic liver to that of the normal liver (M) is 4.0 in rats and 0.37 in humans, taking into account cell renewal during LC development.

with DNA synthesis, accompanied by an increase in the number of cells. Proliferation associated with normal mitotic cell divisions accounts for 66% and polyploidization, for 16%. Determination of the contribution(s) of hepatocyte proliferation, polyploidy and hypertrophy to reparative liver growth in humans showed that reparative growth of the human liver during the development of cirrhosis was solely due to mitotic divisions of small diploid hepatocytes (Table 2). An intense proliferation of 2c-hepatocytes during LC may indicate the transformation of the liver parenchyma into hepatocellular carcinoma (Wang et al. 2017; Matsumoto et al. 2021; Sladky et al. 2021; Matsumoto 2022).

Conclusions

Thus, our data indicate that somatic polyploidy plays a significant role in the normal (postnatal) and reparative growth of the rat and the human liver. At the same time, normal mitotic divisions of mononucleate diploid hepatocytes make the most significant contribution to the increase in the liver mass during postnatal ontogenesis and during regeneration.

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ORCID

Natalia N. Bezborodkina https://orcid.org/0000-0002-7435-742X
Boris N. Kudryavtsev https://orcid.org/0000-0002-1236-822X

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RESEARCH ARTICLE

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Interphase nuclei, karyotypes and nuclear DNA amounts in five species of *Oenocarpus* (Arecaceae)

Natália Padilha de Oliveira[1], Gabriel de Siqueira Gesteira[1], Maria do Socorro Padilha de Oliveira[2], Lisete Chamma Davide[1]

1 *Departmento de Biologia, Universidade Federal de Lavras, Campus Universitário, Caixa Postal 3037, CEP 37200-000, Lavras, MG, Brazil* **2** *Embrapa Amazônia Oriental, CPATU, 66095-903, Belém, PA, Brazi*

Corresponding author: Natália Padilha de Oliveira (nataliapadilhactrb@gmail.com)

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Abstract

The genus *Oenocarpus* Martius, 1823 (Arecaceae) includes five species commonly used in Amazonia, especially for their fruits. Little is known about the cytogenetic characteristics and DNA amounts of these species, except for *O. bataua* (Martius, 1823). This study characterized and compared the types of interphase nuclei, the chromosome sets, and estimated the nuclear DNA amounts of *Oenocarpus bacaba* (Martius, 1823), *O. bataua*, *O. distichus* (Martius, 1823), *O. mapora* (H. Karsten, 1857) and *O. minor* (Martius, 1823). Standard cytogenetic analyses and estimates of the nuclear DNA amount by flow cytometry were carried out. These are the first reports of chromosome numbers and DNA amounts, except for *O. bataua*, as is the description of the chromatin distribution in interphase nuclei and karyotype for all species. All species presented 2n = 36, confirming the previous report for *O. bataua*. Differences between karyotype formulas and the positioning of secondary constrictions were observed. There were no significant differences for the nuclear DNA amounts among species. The constancy in chromosome number and variations in karyotype formulas suggest the occurrence of chromosome rearrangement as an important mechanism in *Oenocarpus* speciation.

Keywords

C-value, cytogenetic analyses, flow cytometry, karyogram, karyotype asymmetry, palms

Introduction

The family Arecaceae includes approximately 2,400 species in 190 genera, and is considered to be one of the most abundant among the monocotyledons (Röser 1995; Dransfield et al. 2008). Among the typically tropical genera is *Oenocarpus* Martius, 1823, with nine species (Henderson 1995) found throughout the northern part of South America. Five species have significant economic value for Amazonian communities, especially due to the products derived from their fruits, e.g. used as food, tools and utensils, and for construction (Balick 1986; Henderson 1995; Zambrana et al. 2007). Because of their importance, studies that can increase knowledge of their biology, management and sustainable use of their genetic resources, and their domestication are important.

Cytogenetics offers information for the characterization of germplasm banks, as well as for the management of these resources in genetic breeding programs (Stace 2000). The determination of chromosome number and karyotype are the easiest and cheapest activities among all cytogenetic techniques available, and constitute important information for cytotaxonomic studies (Guerra 2008). There are numerous studies of palm cytogenetics, some including karyotypes, banding patterns and comparison of interphase nuclei morphology, based on chromatin distribution and arrangement (Röser 1993, 1994, 1995, 1999, 2000; Röser et al. 1997; Castilho et al. 2000; Corrêa et al. 2009; Abreu et al. 2011; Battistin et al. 2012; Gaiero et al. 2012; Oliveira et al. 2016; Pereira et al. 2017; Kadam et al. 2023; Witono et al. 2024). However, among the five subfamilies of Arecaceae, the one that has the least cytogenetic information for its species is Arecoideae, which includes *Oenocarpus*, with at least five tribes with no information even on the chromosome number (Dransfield et al. 2008). For *Oenocarpus* only *O. bataua* (Martius, 1823) has a chromosome number report: 2n = 36 (Röser et al. 1997).

Studies involving species of this subfamily can contribute to understanding karyotype evolution in the Arecaceae.

The analysis of nuclear DNA amounts by flow cytometry in plant species allows estimation of genome sizes, for comparison with chromosome numbers, ploidy levels and detection of numerical alterations (Bennett and Leitch 1995; Doležel and Bartos 2005). The amount of information for palms has increased recently (Rival et al. 1997; Sandoval et al. 2003; Madon et al. 2008; Abreu et al. 2011; Cepeda-Cornejo et al. 2012; Farias Neto et al. 2016; Jatt et al. 2019; Sharma et al. 2023). However, the most extensive study, which included 83 species in all five subfamilies (Röser et al. 1997), used the microdensitometry methodology of Feulgen (Teoh and Rees 1976).

In this context, this study characterized and compared interphase nuclei morphology and chromosome sets, and estimated the amount of nuclear DNA for *O. bacaba* (Martius, 1823), *O. bataua*, *O. distichus* (Martius, 1823), *O. mapora* (H. Karsten, 1857) and *O. minor* (Martius, 1823). These are the five most useful species, and samples are maintained for study and improvement by Embrapa Eastern Amazon, Amazon, Belém, Pará.

Material and methods

Plant material

Seeds obtained from three accessions of *O. bacaba*, *O. bataua*, *O. distichus*, *O. mapora*, and *O. minor*, kept at the Active Germplasm Bank of Embrapa Eastern Amazon, in Belém, Pará, Brazil, were used in both analyses. Analysis was authorized by the federal institutions **CGEN** (process no. 02000.002611/2012-60) and **IBAMA** (process no. 02001.001558/2006-21). Vouchers are deposited in the **IAN** Herbarium, Belém, and details of each accession are presented in Table 1. After mechanical processing, seeds were set to germinate in BOD at 28 °C with a 12 h photoperiod. Seedlings obtained from each species were kept in a greenhouse at the Federal University of Lavras **UFLA**, Lavras, Minas Gerais, Brazil.

Table 1. Number of individuals and origin of *Oenocarpus* sp. genotypes used on analyses.

Species	Number of indivuduals	Origin
O. bacaba	1	Magazão-AP
	1	Macapá-AP
	1	Porto Grande-AP
O. bataua	1	Irituia-PA
	2	Anajás-PA
O. mapora	3	Abaetetuba-PA
O. distichus	3	Oriximiná-PA
O. minor	3	Terra Santa-PA

Cytogenetic analysis

Root tips were pre-treated with colchicine 0.1% for 5 h at 4 °C, fixed in Carnoy's solution (3:1 alcohol/acetic acid) and stored at -20 °C. Slide preparation used the squashing technique (Guerra and Souza 2002) following cell wall digestion with cellulase/pectinase (100U/200U) for 2 h at 37 °C. Aceto-orcein 1% was used to stain the samples for the analysis of mitotic metaphases, while 10% Giemsa (diluted in phosphate buffer, pH 6.8, following Guerra and Souza 2002) was used to analyze interphase nuclei.

The slides were examined in a bright-field microscope (Leica DMLS), equipped with a digital camera (Nikon Digital Sight DS-Fi1) to digitalize the best nuclei and metaphases. In order to evaluate chromatin organization at interphase, 500 nuclei were analyzed for each species. Ten metaphases were selected to determine the chromosome number for each species, of which five were used for karyotype construction, after obtaining the measurements of the short (s) and long (l) arms of the chromosomes, using the IMAGE TOOL 3.00 program from The University of Texas Health Science Center in San Antonio (http://ddsdx.uthscsa.edu/dig/download.html). The total length of the chromosome (**Cti** = l + s), arm ratio (**AR** = l/s), total length of the haploid set (**TLHS** = ΣCti/2), and relative length of each chromosome (**RL** = Cti/TLHS × 100), and were

estimated. Chromosome morphology was described based on arm ratios, following Levan et al. (1964). Karyograms were obtained using Adobe Photoshop CS2. To compare the mean sizes of the chromosome sets among species, an analysis of variance of a completely randomized design was used and means were compared with the Tukey test at 5%, using the R package in R (R Development Core Team 2011).

For karyotype asymmetry, the intrachromosomal asymmetry (A1), which quantify the relative differences in the centromere position among chromosomes of a complement, and the interchromosomal asymmetry (A2), which quantify the heterogeneity in chromosome size, were calculated following Zarco (1986). Karyotype asymmetry was also calculated following Stebbins (1971), which proposes a classification based on three degrees of difference between the largest and the smallest chromosome of the complement, combined with four degrees regarding the proportion of chromosomes which are acro- or telocentric.

Estimates of nuclear DNA amounts

Nuclear DNA amounts were estimated by flow cytometry, using leaf tissue, following Galbraith et al. (1983). Propidium iodide (1 mg/ml) was used as a fluorochrome and for internal standard, a pretest was conducted, after which *Vicia faba* (Linnaeus, 1753) (2C = 26.9 pg) was chosen because of the quality of graphics obtained. For each species three specimens, the same accessions used in the cytogenetic analysis, were analyzed and three estimates were made for each one of them. The analyses were carried out in a FacsCalibur cytometer (BD Biosciences, San Jose, CA, USA), the histograms were obtained using the software Cell Quest (Becton Dickinson and Company, San Jose, CA, USA) and analyzed with the software WinMDI 2.8. Nuclear DNA amounts (2C) of each accession were estimated as (sample G1 peak mean/ standard G1 peak mean) × standard 2C value. To compare the mean nuclear DNA amounts among species, an analysis of variance of a completely randomized design was used and means were compared with the Tukey test at 5%, using the R package in R (R Development Core Team 2011).

Results

Only semi-reticulate interphase nuclei were found (Fig. 1A–E), which are characterized by the presence of strongly pigmented chromatin structures with irregular edges, known as chromocenters (Guerra 1987).

The chromosome number was also constant among species: 2n = 36 (Fig. 2A–E). However, there was variation in size, morphology and position of secondary constrictions (Figs 3A–E, 4A–E). The karyotype formulas found for the species were the following: *O. bacaba* (2M + 11SM + 5A), *O. bataua* (8M + 10SM), *O. distichus* (4M + 14SM), *O. mapora* (3M + 14SM + 1A) and *O. minor* (3M + 15SM). Total length for the haploid set was higher for *O. mapora*, with 63.7 μm, while *O. bacaba* showed the lowest value, 51.8 μm. However, the analysis of variance did not detect differences among mean values (Table 2).

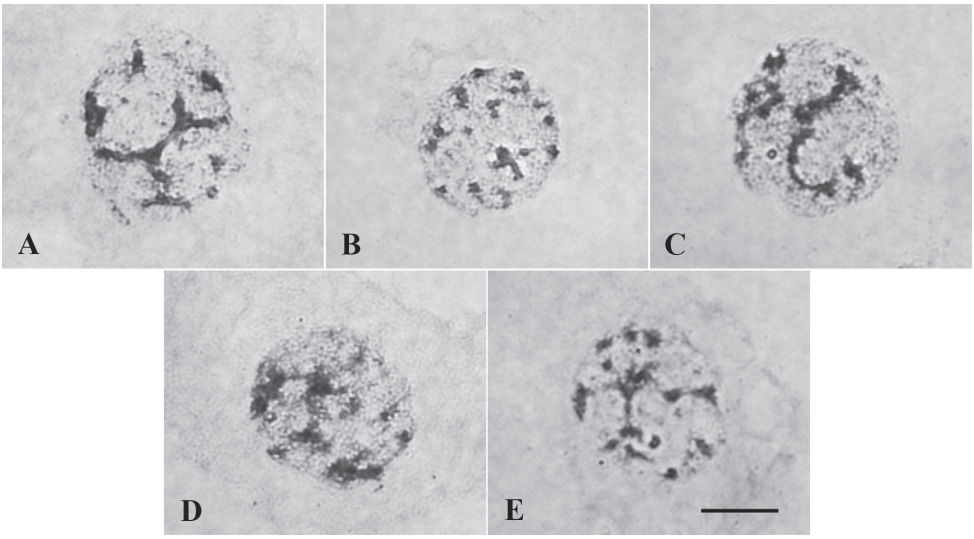


Figure 1. Semi-reticulate interphase nuclei found for *Oenocarpus* spp. **A** *O. bacaba* **B** *O. bataua* **C** *O. distichus* **D** *O. mapora* **E** *O. minor*. Scale bar: 10 µm.

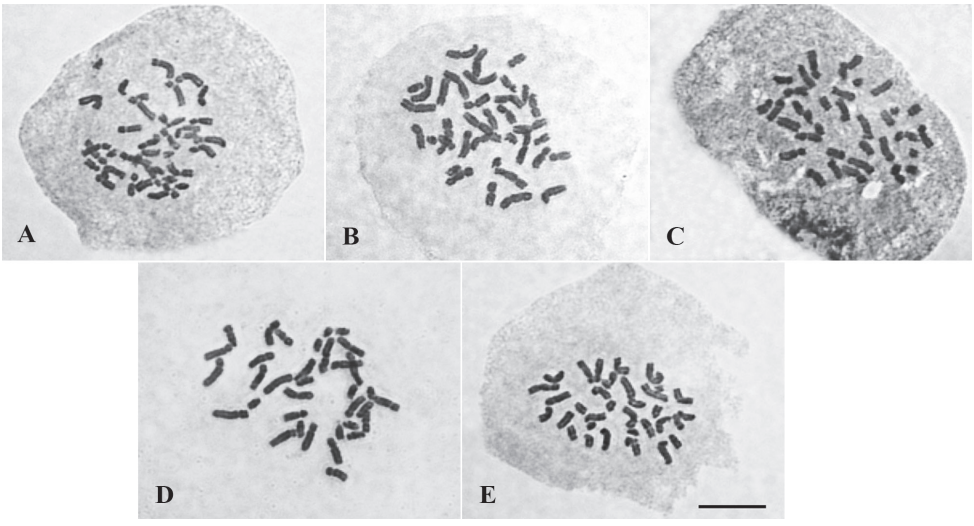


Figure 2. Mitotic metaphases of *Oenocarpus* spp. showing 2n = 36 **A** *O. bacaba* **B** *O. bataua* **C** *O. distichus* **D** *O. mapora* **E** *O. minor*. Scale bar: 10 µm.

Table 2. Mean values of total length of haploid set and DNA amount of *Oenocarpus* sp.

Species	*O. bacaba*	*O. bataua*	*O. distichus*	*O. mapora*	*O. minor*
TLHS (µm)	51.835a	61.823a	54.001a	63.712a	59.053a
2C DNA amount (pg)	6.794a	6.457a	6.554a	6.483a	6.960a

Same letter indicates group formed by Tukey test at 5%.

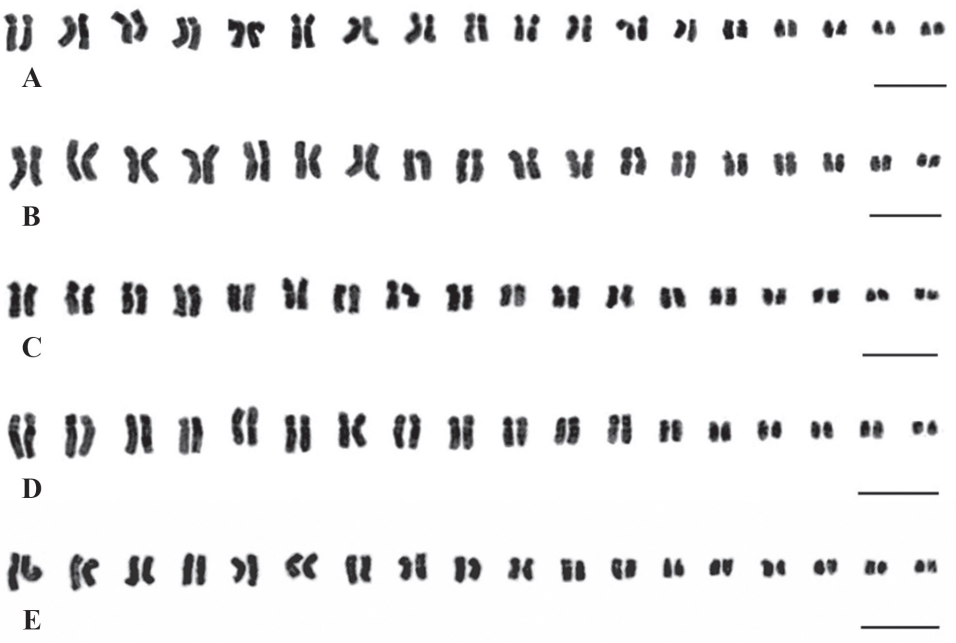
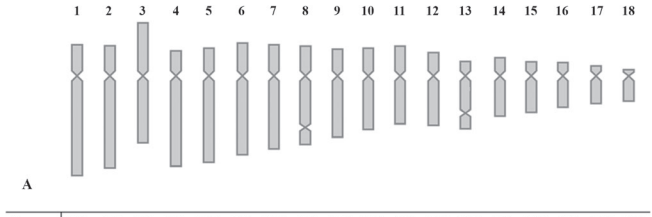


Figure 3. Karyograms of *Oenocarpus* spp. based on the metaphases displayed previously **A** *O. bacaba* **B** *O. bataua* **C** *O. distichus* **D** *O. mapora* **E** *O. minor*. Scale bar: 10 μm.

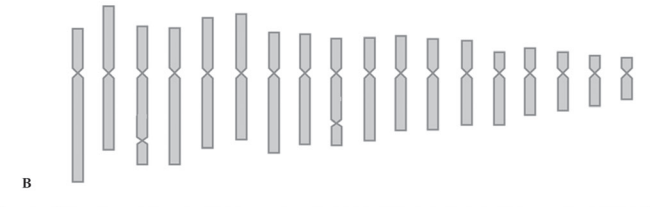
In the karyotypes of the five species two chromosome pairs with secondary constrictions were observed, all located in the terminal portion of the long arm. In *O. bacaba* secondary constrictions occurred in chromosome pairs 8 and 13, and presented 0.60 and 0.53 μm, respectively (Fig. 4A); in *O. bataua* they occurred in pairs 3 and 9, with 0.81 and 0.79 μm, respectively (Fig. 4B); in *O. distichus* in pairs 1 and 4, with 0.95 and 0.82 μm, respectively (Fig. 4C); in *O. mapora* in pairs 3 and 10, with 0.86 and 0.80 μm, respectively (Fig. 4D); and in *O. minor* in pairs 1 and 5, with 0.88 and 0.85 μm, respectively (Fig. 4E).

The results of the karyotype asymmetry analysis were coincident for the methodologies proposed by Stebbins (1971) and Zarco (1986). The greater symmetry as presented by *O. bataua*, classified in the category 2b (Stebbins 1971), as well as a lower intrachromosomal asymmetry (A1) and a lower variation in size between the chromosomes (A2) (Zarco 1986). The species *O. distichus*, *O. mapora* and *O. minor* were grouped in the same category, 3b (Table 3). In Fig. 5 it is noted that *O. distichus*, *O. mapora* and *O. minor* formed a similar group. The species *O. bacaba* presented higher values for A1 and A2 and was classified in the 3c category, thus representing the most asymmetrical of the five species in both methodologies (Table 3).

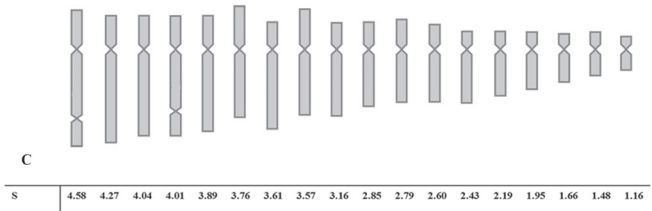
As for the 2C amount of nuclear DNA, the average values found for the species varied between 6.46 pg, in the *O. bataua*, and 6.96 pg, in the *O. minor* (Table 2). The analysis of variance did not detect differences among averages values.



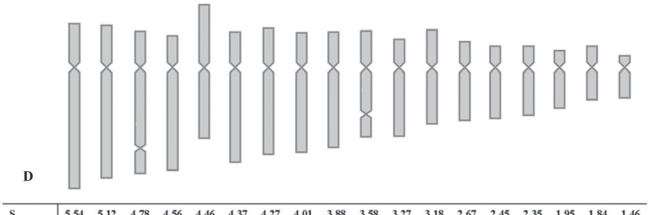
S	4.44	4.14	4.05	3.91	3.87	3.78	3.54	3.34	2.99	2.75	2.65	2.47	2.29	2.00	1.72	1.53	1.28	1.09
RS (%)	8.57	7.98	7.81	7.55	7.45	7.29	6.83	6.45	5.76	5.31	5.11	4.77	4.41	3.86	3.31	2.96	2.48	2.10
MO	A	SM	M	A	A	SM	SM	SM	SM	SM	M	SM	A	SM	SM	SM	SM	A



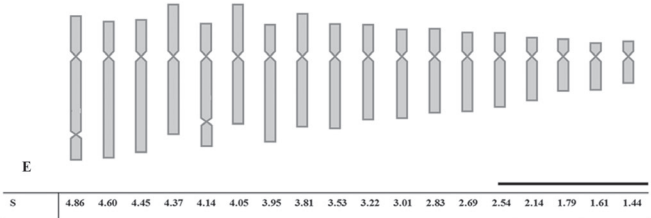
S	5.15	4.84	4.67	4.58	4.39	4.23	4.07	3.73	3.62	3.47	3.21	3.08	2.85	2.48	2.27	2.01	1.73	1.44
RS (%)	8.32	7.83	7.55	7.41	7.11	6.84	6.58	6.04	5.86	5.61	5.19	4.98	4.61	4.01	3.68	3.25	2.81	2.33
MO	SM	M	SM	SM	M	M	SM	SM	SM	SM	M	M	M	SM	M	SM	SM	M



S	4.58	4.27	4.04	4.01	3.89	3.76	3.61	3.57	3.16	2.85	2.79	2.60	2.43	2.19	1.95	1.66	1.48	1.16
RS (%)	8.48	7.91	7.49	7.43	7.21	6.97	6.69	6.60	5.84	5.28	5.16	4.82	4.49	4.06	3.60	3.07	2.75	2.16
MO	SM	SM	SM	SM	SM	M	SM	M	SM	SM	SM	SM	SM	SM	SM	SM	M	M



S	5.54	5.12	4.78	4.56	4.46	4.37	4.27	4.01	3.88	3.58	3.27	3.18	2.67	2.45	2.35	1.95	1.84	1.46
RS (%)	8.69	8.04	7.51	7.16	7.00	6.85	6.70	6.30	6.08	5.61	5.13	4.99	4.18	3.84	3.68	3.06	2.89	2.28
MO	SM	SM	SM	A	M	SM	SM	SM	SM	SM	SM	M	SM	SM	SM	SM	M	SM



S	4.86	4.60	4.45	4.37	4.14	4.05	3.95	3.81	3.53	3.22	3.01	2.83	2.69	2.54	2.14	1.79	1.61	1.44
RS (%)	8.29	7.85	7.60	7.46	7.07	6.91	6.73	6.51	6.02	5.50	5.14	4.83	4.60	4.33	3.66	3.05	2.75	2.46
MO	SM	SM	SM	M	SM	M	SM	M	SM	SM	SM	SM	SM	SM	SM	SM	SM	SM

Figure 4. Idiograms of *Oenocarpus* spp. including length (L), relative length (RL), and morphology (MO) of each chromosome pair **A** *O. bacaba* **B** *O. bataua* **C** *O. distichus* **D** *O. mapora* **E** *O. minor*. Scale bar: 5 μm.

Table 3. Karyotype asymmetry for the five *Oenocarpus* species according to Stebbins (1971) and Zarco (1986).

Species	Karyotype Asymmetry		
	Stebbins	Zarco (A1 e A2)	
O. bacaba	3c	0.5767	0.3693
O. bataua	2b	0.4046	0.3274
O. distichus	3b	0.5361	0.344
O. mapora	3b	0.5497	0.3412
O. minor	3b	0.5241	0.3373

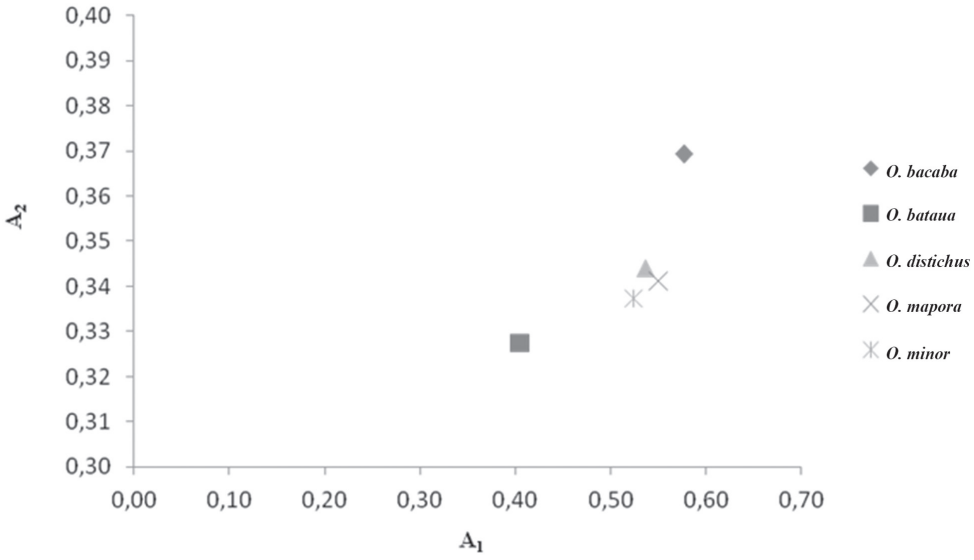


Figure 5. Scatterplot for karyotype asymmetry of the five *Oenocarpus* species based on the intrachromosomal asymmetry index (A1) and the interchromosomal asymmetry index (A2), according to Zarco (1986).

Discussion

Except for *O. bataua*, the chromosome counts obtained in this study, as well as the karyotypes, the morphology of interphase nuclei and nuclear DNA amounts of the species are new. The chromosome number found for the *O. bataua* confirms the prior report (Röser et al. 1997), but the 2C DNA value differs by more than 1 pg from that presented by those authors. It is important to point out that the methodology used by those authors, Feulgen's microdensitometry, estimates the amount of DNA in a different way than flow cytometry used in this study (Röser et al. 1997). In the literature, although correlated results for DNA amounts using both techniques are frequent (e.g. Baranyi and Greilhuber 1996), differences in DNA amounts for the same species when estimated with both techniques have been reported, although there is no agreement as to the explanation for this fact (Schifino-Wittmann 2001). The analysis of the

specimens studied by Röser et al. (1997) using flow cytometry would help to verify whether the difference found in this study is due to the methodology or whether there is intraspecific variation, as found in *Cocos nucifera* (Linnaeus, 1753) by Gunn et al. (2015). Abreu et al. (2011) also found a different 2C value than found by Röser et al. (1997) for *Acrocomia aculeata* (Loddiges ex. Martius, 1823), and the authors suggested that the different methodologies and different origins of the genotypes probably influence the estimation of nuclear DNA amounts.

As for the type of interphase nuclei, in the Arecaceae there are reports on the occurrence of three types of nuclei, reticular, semi-reticular and areticular, and this characteristic has proven to be constant among congener species, and sometimes even in superior taxonomic levels such as tribes (Röser 1994). Our results for these five *Oenocarpus* species confirm the pattern. According to Guerra (2000), the semi-reticular type of nucleus is typical of species with medium sized chromosomes, e.g., 3 to 5 µm, as found in this study.

The number of chromosomes found for the *Oenocarpus* species was the same as that found for other species of the tribe Euterpeae (Battistin et al. 2012; Oliveira et al. 2016). The constancy in the number of chromosomes among closely related species is quite common among groups of Arecaceae (Röser 1994, 1995; Dransfield et al. 2008; Corrêa et al. 2009).

The chromosome number for Arecaceae species varies from 2n = 26 to 2n = 36 (Röser 1999; Dransfield et al. 2008). The number 2n = 36 is the most commonly found in some subfamilies, such as the Coryphoideae (Röser 1994; Dransfield et al. 2008), in which almost all the species present this number. According to the same authors, the subfamily Arecoideae, in turn, is the most diversified, with 2n = 32 chromosomes being the most common number, although the number 2n = 36 is also quite expressive in tribes such as Euterpeae.

Other chromosome numbers have been reported for species of the subfamily Arecoideae, to which the genus *Oenocarpus* belongs. Castilho et al. (2000), Corrêa et al. (2009), Battistin et al. (2012), and Pereira et al. (2017) found 2n = 32 chromosomes for *Elaeis guineensis* (Jacquin, 1763), five species of *Butia* (Beccari, 1916), *Archontophoenix alexandrae* ((F. Muell.) H. Wendland et Drude, 1875), and *Cocos nucifera*, respectively; Abreu et al. (2011) reported the number 2n = 30 for the *Acrocomia aculeata* species; Cepeda-Cornejo et al. (2012) found a variation for different species of *Chamaedorea* (Willdenow, 1806), 2n = 32 for *C. tepejilote* (Liebmann, 1849) and *C. alternans* (Willdenow, 1880), and 2n = 26 for *C. pinnatifrons* (Oersted, 1858) and *C. ernesti-augusti* (Wendland, 1852).

As for the secondary constrictions, in Arecaceae species it is common to find one or two pairs of chromosomes bearing nucleolus organizer regions (NORs), but five pairs occur in *Pseudophoenix vinifera* ((Mart.) Beccari, 1912) (Röser 1994). Those regions have been found more frequently at the end of the short arm of the chromosomes (Röser 1999; Castilho et al. 2000; Pereira et al. 2017). Roa and Guerra (2012) pointed out a tendency for the quantity and location of 45S rDNA sites for angiosperm species in general to be similar to that found palms (Röser 1999). Although the secondary

constrictions verified in *Oenocarpus* were found at the end portion of the chromosomes, they were all detected on the long arm. Oliveira et al. (2016) also found secondary constrictions on the long arm for *E. edulis* (Martius, 1824) and *E. precatoria* (Martius, 1842). It is important to stress that the subfamily Arecoideae has the least cytogenetic information. Therefore, it needs to be verified whether this difference in positioning found for the secondary constrictions is exclusive to *Oenocarpus*, or whether it is a characteristic shared by other genera of this subfamily.

Based on the karyotypes of the species studied here and emphasizing the differences between the positioning of the centromere and the secondary constrictions, it can be inferred that alterations, especially structural rearrangements, such as translocations and pericentric inversions, as well as activities related to the transposable elements, accumulated during the evolution of this genus. According to Stebbins (1971), such rearrangements are important in evolution, as they increase karyotype asymmetry and the differentiation among chromosome sets.

Regarding the nuclear DNA amounts, similar results have been found for other palm species from the same subfamily. *Cocos nucifera*, *Elaeis guineensis* and *Attalea* spp. (Kunth, 1816), all with 2n = 32 chromosomes, and *Acrocomia aculeata* (2n = 30) had their 2C DNA value estimated at 3.76 pg (Sandoval et al. 2003), 3.86 pg (Rival et al. 1997), 3.80 pg (NP Oliveira, unpubl. res.), and 5.81 pg (Abreu et al. 2011), respectively. Nevertheless, much higher 2C values have been found for species from different subfamilies of Arecaceae, *e.g.*, *Iriartea deltoidea* (Ruiz et Pavón, 1798), *Pinanga coronata* (Blume, 1839), and *P. subintegra* (Ridley, 1907), all from the same subfamily Arecoideae to which *Oenocarpus* belongs and with chromosome number 2n = 32, but with 2C values estimated at 24.56, 17.71, and 27.81 pg, respectively; *Trithrinax campestris* (Drude et Griseback, 1879) (2n = 36), and *Caryota urens* (Linnaeus, 1753) (2n = 34), from Coryphoideae, with 17.15 ± 0.07 pg (Gaiero et al. 2012) and 13.22 pg (Röser et al. 1997), respectively.

The nuclear DNA amount in palm species, unlike the number of chromosomes, presents large variation (Röser et al. 1997). Differences of more than 14 times between the smallest and the largest genome size were found, considering only diploid palm species from different genera and subfamilies, which explains the observed variation found for chromosome sizes in the same species (Röser 1994, 2000; Röser et al. 1997). Despite the remarkable diversity found in this family, nuclear DNA amounts seldom vary much within genera, as found here for these five *Oenocarpus* species, and even at higher taxonomic levels (Röser et al. 1997; Röser 2000). Furthermore, the amount of DNA in Arecaceae species seems to follow the same trend as chromosome number, that is, reduction (Röser 2000), but does not seem to be proportional to the chromosome number reduction. Castilho et al. (2000) suggested the amplification of dispersed repetitive DNA sequences as one of the mechanisms responsible for such variation in nuclear DNA amounts. Nevertheless, there is still a lot of research to be done to better understand this diversity.

The five species of *Oenocarpus* follow the majority of the tendencies identified in the Arecaceae family, such as the constancy in chromosome number within the genus

and little variation for nuclear DNA amounts. However, other studies are seeking to understand more clearly the mechanisms involved in the karyotype differentiation of these species, as well as consolidating phylogenetic inferences suggested for this genus.

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ORCID

Natália Padilha de Oliveira https://orcid.org/0000-0001-8413-5495
Gabriel de Siqueira Gesteira https://orcid.org/0000-0002-4106-7346
Maria do Socorro Padilha de Oliveira https://orcid.org/0000-0002-4753-2018
Lisete Chamma Davide https://orcid.org/0000-0003-2719-5584

Supplementary material 1

Size of each chromosome and total length of haploid set (TLHS) for *Oenocarpus* species

Authors: Natália Padilha de Oliveira, Gabriel de Siqueira Gesteira, Maria do Socorro Padilha de Oliveira, Lisete Chamma Davide

Data type: xlsx

Explanation note: Asterisk (*) indicates second constriction.

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RESEARCH ARTICLE

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Molecular cytogenetic characterization of 9 populations of four species in the genus *Polygonatum* (Asparagaceae)

Yan-Fang Wei[1], Xiang-Hui Jiang[2], Rong Song[3], Chao-Wen She[1]

1 *College of Life Sciences and Chemistry, Hunan University of Technology, Zhuzhou, Hunan, 412007, China* **2** *Key Laboratory of Research and Utilization of Ethnomedicinal Plant Resources of Hunan Province, Huaihua University, Huaihua, Hunan, 418008, China* **3** *Institute of Agricultural Environment and Ecology, Hunan Academy of Agricultural Sciences, Changsha, Hunan, 410125, China*

Corresponding author: Chao-Wen She (shechaowen@aliyun.com)

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Abstract

To characterize the chromosomes of the four species of *Polygonatum* Miller, 1754, used in traditional Chinese medicine, *P. cyrtonema* Hua, 1892, *P. kingianum* Collett et Hemsley, 1890, *P. odoratum* (Miller, 1768) Druce, 1906, and *P. sibiricum* Redouté, 1811, and have an insight into the karyotype variation of the genus *Polygonatum*, fluorescence *in situ* hybridization (FISH) with 5S and 45S rDNA oligonucleotide probes was applied to analyze the karyotypes of 9 populations of the four species. Detailed molecular cytogenetic karyotypes of the 9 populations were established for the first time using the dataset of chromosome measurements and FISH signals of 5S and 45S rDNA. Four karyotype asymmetry indices, CV_{CI}, CV_{CL}, M_{CA} and Stebbins' category, were measured to elucidate the asymmetry of the karyotypes and karyological relationships among species. Comparison of their karyotypes revealed distinct variations in the karyotypic parameters and rDNA patterns among and within species. The basic chromosome numbers detected were x = 9, 11 and 13 for *P. cyrtonema*, x = 15 for *P. kingianum*, x = 10 and 11 for *P. odoratum*, and x = 12 for *P. sibiricum*. The original basic chromosome numbers of the four species were inferred on the basis of the data of this study and previous reports. All the 9 karyotypes were of moderate asymmetry and composed of metacentric, submetacentric and subtelocentric chromosomes or consisted of two of these types of chromosomes. Seven populations have one locus of 5S rDNA and two loci of 45S rDNA, and two populations added one 5S or 45S locus. The karyological relationships among the four species revealed by comparison of rDNA patterns and PCoA based on x, $2n$, TCL, CV_{CI}, M_{CA} and CV_{CL} were

basically accordant with the phylogenetic relationships revealed by molecular phylogenetic studies. The mechanisms of both intra- and inter-specific dysploidy in *Polygonatum* were discussed based on the data of this study and literature.

Keywords
Cytotaxonomy, fluorescence *in situ* hybridization, karyotype, karyotype asymmetry, *Polygonatum*, ribosomal RNA genes (rDNA)

Introduction

The genus *Polygonatum* Miller, 1754, as the largest genus in the tribe Polygonateae (Asparagaceae), comprises ca. 70 species (Chen and Tamura 2000). The genus is distributed throughout the temperate regions of the Northern Hemisphere with ca. 50 species in east Asia (from Himalaya to China and Japan), 5 species in Europe and 3 species in North America, and main diversification centered in southwest China and northeast Asia (Chen and Tamura 2000; Meng et al. 2014; Wang et al. 2022). *Polygonatum* is also one of the most important medicinal taxa in Asia. At least 37 species and 1 variety of *Polygonatum* plants have been used as traditional medicine and functional food with the rhizome being the most commonly used part of the plant (Zhao et al. 2018). In traditional Chinese medicine (TCM), the dry rhizome of *P. odoratum* (Miller, 1768) Druce, 1906 is known as Yuzhu (Polygonati Odorati Rhizoma), while the dry rhizomes of *P. sibiricum* Redouté, 1811, *P. kingianum* Collett et Hemsley, 1890, and *P. cyrtonema* Hua, 1892, are known as Huangjing (Polygonati Rhizoma) (Chinese Pharmacopoeia Commission 2020). They are both Yin-nourishing herbs that are associated with delaying senescence and are often used to treat osteoporosis, feebleness, fatigue, diabetes and lung disorders (Zhao et al. 2018; Chinese Pharmacopoeia Commission 2020).

Polygonatum species show a high variation in morphology and a wide overlap in geographical distribution, which makes infrageneric classification and species identification very complicated (Tang 1978; Chen and Tamura 2000; Meng et al. 2014). Since the middle of the last century, much conventional cytogenetic work has been conducted to reveal cytotaxonomic relationships and evolutionary trends of karyotype within the genus (Suomalainen 1947; Therman 1953; Kumar 1959; Mehra and Pathania 1960; Kawano and Iltis 1963; Inoue 1965; Mehra and Sachdeva 1976; Kim and Kim 1979; Fang et al. 1984; Wang et al. 1987, 1991; Yang et al. 1988, 1992; Chen et al. 1989; Hong and Zhu 1990; Tamura 1990, 1993; Wang et al. 1993; Shao et al. 1993, 1994; Han et al. 1998; Wu et al. 2001; Weiss-Schneeweiss and Jang 2003; Chen and Zhou 2005; Deng et al. 2009; Zhao et al. 2014; Zhou et al. 2020). Conventional karyotyping revealed significant variation in basic chromosome number among species in the genus, dysploid variation within species, and bimodality of karyotypes of most populations of *Polygonatum* species studied (Wang et al. 1987, 1991; Yang et al. 1992; Chen and Zhou 2005; Deng et al. 2009; Zhao et al. 2014; Wang et al. 2016a; Zhou et al. 2020).

The classification of *Polygonatum* has long been controversial. Baker (1875) classified *Polygonatum* into three sections based on its leaf arrangement, section *Alternifolia*, section *Verticillata*, and section *Oppositifolia*. Tang (1978) divided *Polygonatum* into eight series based on more detailed morphological characters. Tamura (1993) proposed a new classification on the basis of a combination of cytogenetics and morphology, dividing the genus into two sections, section *Polygonatum* (basic chromosome number: $x = 9, 10, 11$) and section *Verticillata* ($x = 14$ or 15). The most recent and widely accepted classification is that of Meng et al. (2014) who divided the genus into three sections based on molecular phylogenetic and morphological evidence: (i) sect. *Polygonatum* including species with alternate leaves and $x = 9–11$, (ii) sect. Sibirica including species with whorled leaves and $x = 12$, and (iii) sect. *Verticillata* including species with variable phyllotaxy and $x = 13–15$. This infrageneric classification system was confirmed by several subsequent molecular phylogenetic studies (Floden and Schilling 2018; Zhao et al. 2019; Xia et al. 2022; Wang et al. 2022; Qin et al. 2024). However, this classification has not been validated by molecular cytogenetics. To date, more than 50 species of *Polygonatum* have been conventionally karyotyped (Zhao et al. 2014). These karyotype analyses can only provide limited information on species identification and karyotype evolution among *Polygonatum* species due to a lack of effective markers. Although FISH (fluorescence *in situ* hybridization) technology has been widely used in genome analysis of plants (Jiang and Gill 2006), there have not been any report of chromosome characterization of *Polygonatum* species using FISH.

The ribosomal genes, 45S (18S-5.8S-26S) and 5S rDNAs, are organized in tandem arrays with high copy numbers, and then widely utilized as probes for FISH in plants. The rDNA FISH signals can be used as informative markers for a better characterization of the chromosomes of plant species, revealing genome organization at molecular cytogenetic level (e.g. Moscone et al. 1999; Chacón et al. 2012; She et al. 2015; Mitrenina et al. 2023). Furthermore, comparison of rDNA patterns (namely the number and location of 5S and 45S rDNA loci) among species within a genus contributes to the understanding of the mechanism of chromosome evolution and phylogenetic relationships between related species (e.g. Moscone et al. 2007; Chacón et al. 2012; Siljak-Yakovlev and Peruzzi 2012; She et al. 2015, 2017, 2020; Senderowicz et al. 2022; Yucel et al. 2022; Mitrenina et al. 2023). However, to date, there has been no molecular cytogenetic kayotype analysis of *Polygonatum* species except for the report of FISH detection of 45S rDNA in *P. odoratum* and *P. cyrtonema* (Wu et al. 2001).

In the present study, comparative molecular cytogenetic analysis of 9 populations of four *Polygonatum* species, *P. cyrtonema*, *P. kingianum*, *P. sibiricum* and *P. odoratum*, was conducted using dual-color FISH with 5S and 45S rDNA oligonucleotide probes. Detailed molecular cytogenetic karyotypes of these populations were quantitatively established using a combination of chromosome measurements and rDNA FISH signals. Four different karyotype asymmetry indices of each population were calculated for evaluating asymmetry of the karyotypes and karyological relationships among the populations. The combined data of karyotypic parameters and rDNA patterns were assessed to gain insights into the intra- and inter-specific karyotype differentiation as well as the phylogenetic relationships among the four species.

Material and methods

Plant material

Plants of 9 populations including four of *P. cyrtonema*, two of *P. kingianum*, two of *P. odoratum* and one of *P. sibiricum* (Suppl. material 1: table S1) were collected from different regions of China, and cultivated in Huangjing germplasm gardens of Agricultural Environment and Ecology Institute of Hunan Academy of Agricultural Sciences. The plants were identified by Dr. Rong Song of Agricultural Environment and Ecology Institute of Hunan Academy of Agricultural Sciences.

Chromosome preparation

The rhizomes used for cytogenetic experiments were cultivated in pots with mixed planting soil consisting of humus soil and sandy soil, and young new roots grew from the rhizomes in about 10 to 14 days. Chromosome spreads were prepared using a protocol previously published by us (She et al. 2015) with minor modifications. Root tips were harvested and treated with saturated α-bromonaphthalene at 28 °C for 5.0 h, and then fixed in 3:1 (v/v) methanol/glacial acetic acid overnight at 4 °C. The fixed root tips were thoroughly rinsed in deionized water and digested in a mixture of 1% cellulase RS and 1% pectolyase Y23 (Yakult Pharmaceutical Industry Co., Ltd. Tokyo, Japan) in citric buffer (pH 4.5) at 37 °C for 2 h. The enzyme solution was replaced by deionized water. The digested root tips were transferred to a glass slide and mashed by using fine-pointed forceps with the fixative solution. Then, the slides were flame-dried. The slides with well-spread metaphase chromosomes were selected under a Olympus BX51 phase contrast microscope and stored at -20 °C until use.

Probe DNA preparation

The 5S rDNA oligonucleotide probes 5S-1 and 5S-2 and the 45S rDNA oligonucleotide probes 45S-1, 45S-2 and 45S-3, which were described previously by Han et al. (2018), were synthesized by Sangon Bioengineering Co., LTD (Shanghai, China). 5S-1 and 5S-2 were labeled with 6-carboxyl fluorescein (6-FAM) at the 5'-terminus and then mixed together to make the 5S rDNA probe solution. 45S-1, 45S-2, and 45S-3 were labeled with 6-carboxyl-tetramethyl rhodamine (TAMRA) at the 5'-terminus and mixed together to make the 45S rDNA probe solution.

FISH and signal detection

FISH was performed according to the procedure described by Han et al. (2018). The hybridization solution (each slide) was as follows: deionized formamide, 10 μL; 50% dextran sulphate, 4 μL; 20 × SSC, 2 μL; salmon sperm DNA, 2 μL (40 ng); 5S rDNA

probe, 1 μL (40 ng); 45S rDNA probe, 1 μL (40 ng). The slides were baked at 65 °C for 45 min, cooled, and then denatured in 70% deionized formamide at 85 °C for 2.5 min. Further, they were dehydrated in 70%, 90%, and 100% alcohol series each for 5 min at –20 °C, followed by air drying. The hybridization solution was poured onto the denatured chromosome slide and then incubated in a moist box infiltrated by 2 × SSC at 37 °C overnight.

The slides were washed in 2 × SSC twice each for 5 min at room temperature after hybridization. Then, the chromosomes were counterstained with 3 μg ml^{-1} DAPI in 30% (v/v) Vectashield H-1000 and visualized with an Olympus BX60 microscope equipped with a QImaging Retiga R6 CCD camera (Teledyne Photometrics, Canada) which was controlled using Ocular software (Teledyne Photometrics, Canada). Observations were made using UV, blue and green excitation filters for DAPI, 6-FAM, and TAMRA, respectively. Grey-scale images were digitally captured and merged by the Ocular software. The final images were adjusted with Adobe Photoshop CS 8.01.

Karyotype analysis

The methodology of karyotype analysis described recently by us was used (She et al. 2023). For each population, five metaphase cells with high condensation were selected for measurement using Adobe Photoshop CS 8.01. The length of long arm (L) and short arm (S) of each chromosome and the length between the center of FISH signal and centromere were measured. For numerically characterizing the karyotypes, the following parameters were calculated: (i) chromosome relative lengths (RL, % of haploid complement); (ii) arm ratios (AR = L/S); (iii) total chromosome length of the haploid complement (TCL; i.e. the karyotype length); (iv) mean chromosome length (C); (v) percent distance from the centromere to the rDNA locus; (vi) mean centromeric index (CI); (vii) Four karyotype asymmetry indices including coefficient of variation (CV) of centromeric index (CV_{CI}), coefficient of variation (CV) of chromosome lengths (CV_{CL}), mean centromeric asymmetry (M_{CA}) and Stebbins' asymmetry category. The meaning and calculation formulae of these indices refer to Paszko (2006) and Peruzzi and Eroglu (2013). The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t) according to arm ratio (Levan et al. 1964). The chromosomes were arranged in order of decreasing length. Idiograms were drawn based on the dataset of chromosome measurements as well as the location and size of rDNA-FISH signals.

Bidimensional scatter diagram for the 9 populations with M_{CA} vs. CV_{CL} was plotted in order to visualize karyotype asymmetry relationships among them. To determine the karyological relationships among the 9 populations, a principal coordinate analysis (PCoA) using Gower's similarity coefficient were performed based on six quantitative parameters (x, $2n$, TCL, CV_{CI}, M_{CA} and CV_{CL}) according to the proposal by Peruzzi and Altınordu (2014).

Results

General karyotype features

The general karyotype features of the 9 populations of *P. cyrtonema*, *P. kingianum*, *P. odoratum* and *P. sibiricum* are listed in Table 1. The measurement data of the chromosomes of each population are given in Suppl. material 2: table S2. Representative mitotic chromosomes hybridized with the 5S and 45S rDNA probes are shown in Fig. 1. The idiograms displaying the chromosome measurements as well as the location and size of rDNA FISH signals are illustrated in Fig. 2.

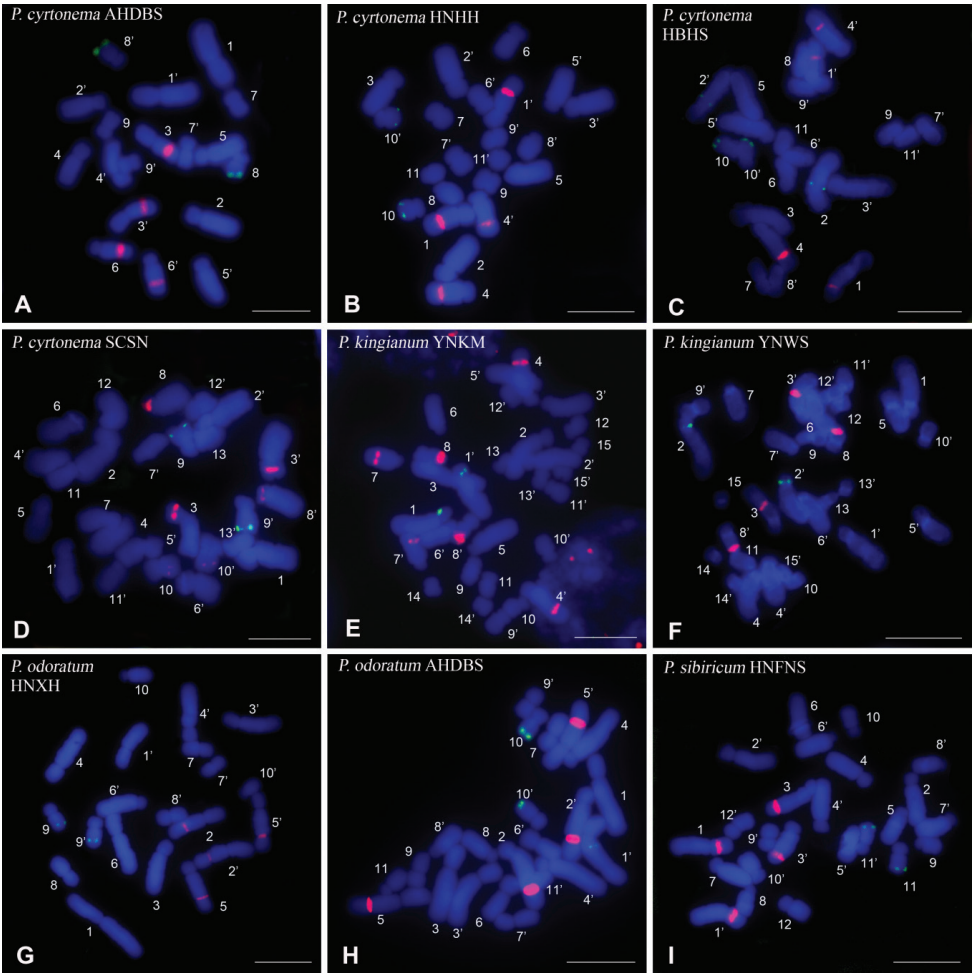

Figure 1. FISH to metaphase chromosomes of 9 populations of four *Polygonatum* species, *P. cyrtonema* (Pc), *P. kingianum* (Pk), *P. odoratum* (Po) and *P. sibiricum* (Ps), using 5S rDNA (green) and 45S rDNA (red) oligonucleotide probes. Chromosomes were counterstained with DAPI (blue). The chromosome numbers were designated by karyotyping **A** Pc AHDBS **B** Pc HNHH **C** Pc HBHS **D** Pc SCSN **E** Pk YNKM **F** Pk YNWS **G** Po HNXH **H** Po AHDBS **I** Ps HNFNS. Scale bars: 10 μm.

Table 1. Karyotype parameters of 9 populations of four *Polygonatum* species.

Populations*	Karyotype formula (KF)	TCL ± SE (μm)	C (μm)	RRL	CI ± SE	CV_{CI}	CV_{CL}	M_{CA}	Stebinns' types
Pc AHDBS	2*n* = 18 = 10m(2SAT) + 6sm + 2st(2SAT)	72.23 ± 8.83	8.03	6.23–17.01	33.45 ± 7.10	21.99	34.38	31.46	2B
Pc HNHH	2*n* = 22 = 4m + 16sm(4SAT) + 2st	80.34 ± 20.91	7.30	5.46–11.95	31.84 ± 7.33	23.02	34.85	36.28	3B
Pc HBHS	2*n* = 22 = 6m + 10sm(2SAT) + 6st(2SAT)	72.39 ± 5.03	6.58	5.01–13.08	32.46 ± 8.32	24.59	38.51	35.07	3B
Pc SCSN	2*n* = 26 = 8m + 14sm(4SAT) + 4st	84.07 ± 6.93	6.47	5.21–10.41	32.22 ± 6.25	19.40	22.94	35.56	3A
Pk YNKM	2*n* = 30 = 6m + 10sm(2SAT) + 14st(4SAT)	93.40 ± 12.14	6.23	2.85–10.13	28.05 ± 9.51	33.92	43.90	43.90	3B
Pk YNWS	2*n* = 30 = 10m + 8sm(2SAT) + 12st(2SAT)	80.38 ± 6.61	5.36	3.34–10.61	31.26 ± 10.62	33.98	42.16	37.48	3B
Po HNXH	2*n* = 20 = 8m(2SAT) + 12sm(2SAT)	86.32 ± 8.18	8.63	5.95–11.61	33.86 ± 7.79	23.00	31.47	32.28	2A
Po AHDBS	2*n* = 22 = 6m + 10sm(2SAT) + 6st(2SAT)	88.61 ± 10.32	8.01	4.90–12.82	30.90 ± 8.85	28.65	36.88	38.20	3B
Ps HNFNS	2*n* = 24 = 12sm(2SAT) + 12st(2SAT)	76.14 ± 5.30	6.35	5.91–11.16	28.30 ± 5.85	20.67	23.61	43.40	3A

* Pc = *P. cyrtonema*, Pk = *P. kingianum*, Po = *P. odoratum*, Ps = *P. sibiricum*.

The four populations of *P. cyrtonema* have three different chromosome numbers: 2*n* = 18 for Pc AHDBS, 2*n* = 22 for Pc HNHH and Pc HBHS, and 2*n* = 26 for Pc SCSN, among which 2*n* = 26 is reported for the first time. Both populations of *P. kingianum* have the same chromosome number 2*n* = 30. The chromosome numbers of the two populations of *P. odoratum* are different: 2*n* = 20 for Po HNXH, 2*n* = 22 for Po AHDBS. The chromosome number of *P. sibiricum* is 2*n* = 24. Among the 9 populations, the total length of the haploid complement (TCL) ranges from 72.23 μm (Pc AHDBS) μm to 93.40 μm (Pk YNKM) with a mean chromosome length between 5.36 μm (Pk YNWS) and 8.63 μm (Po HNXH), showing both inter- and intra-specific variation. According to the classification of Lima-de-Faria (1980), the metaphase chromosomes of the four *Polygonatum* species are of medium size. In regard to range of relative length (RRL), the smallest RRL is observed in Ps HNFNS (5.91–11.16), while the largest RRL is showed in Pk YNKM (2.85–10.13). That is, Ps HNFNS and Pk YNKM exhibit the smallest and the largest variation in chromosome length, respectively. The mean centromeric index (CI) of the chromosome complements varies between 33.86 ± 7.79 (Po HNXH) and 28.05 ± 9.51 (Pk YNKM). That is, Po HNXH and Pk YNKM are characterized by the smallest and the largest level of variation in the centromeric index, respectively.

The karyotypes are composed of m, sm and st chromosomes or consisted of two of these types of chromosomes (Table 1, Suppl. material 2: table S2; Fig. 2). The karyotype formulas are different among populations. This is true even in the populations of the same species with the same number of chromosomes. In Po HNXH, the lengths of the homologous chromosomes of pairs 1 and 6 differ significantly, exhibiting heterozygosity in chromosomal morphology (Fig. 1G). There are clear gaps in chromosome length between the 6th and 7th pair in Pc AHDBS and Po HNXH, between the 5th and 6th pair in Pc HNHH, Pc HBHS and Po AHDBS, exhibiting distinct bimodal

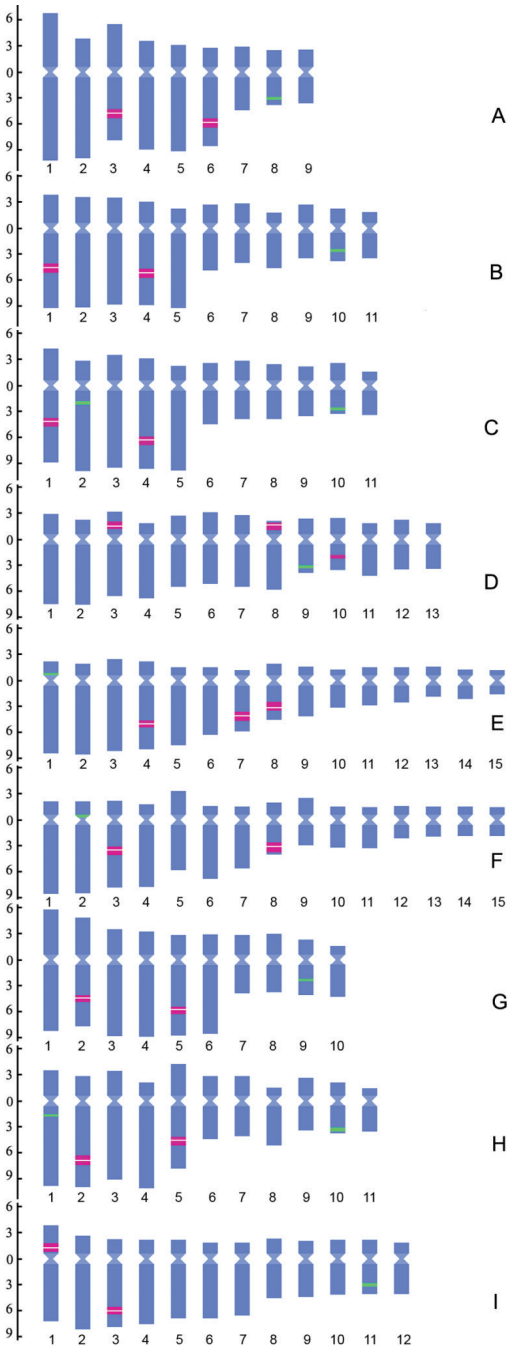

Figure 2. Idiograms of 9 populations of four *Polygonatum* species, *P. cyrtonema* (Pc), *P. kingianum* (Pk), *P. odoratum* (Po) and *P. sibiricum* (Ps), that display the chromosome measurements, and the location and size of the 5S (green) and 45S (red) rDNA FISH signals **A** Pc AHDBS **B** Pc HNHH **C** Pc HBHS **D** Pc SCSN **E** Pk YNKM **F** Pk YNWS **G** Po HNXH **H** Po AHDBS **I** Ps HNFNS. The ordinate scale on the left indicates the relative length of the chromosomes (i.e. % of haploid complement). The numbers at the bottom indicate the the serial number of chromosomes.

karyotypes (Fig. 2A, B, C, G, H; Suppl. material 2: table S2). The difference between the relative lengths of the chromosomes on either side of the gaps is 3.9–5.17 (Suppl. material 2: table S2). The bimodal karyotype can be described as consisting of several pairs of large chromosomes and several pairs of small chromosomes (large + small). If so, the constitutions of the bimodal karyotypes of Pc AHDBS, Pc HNHH, Pc HBHS, Po HNXH and Po AHDBS are 6 + 3, 5 + 6, 5 + 6, 6 + 4, 5 + 6, respectively. Unusually, Pc SCSN has only a small gap in chromosome length between the 8th and 9th pair, showing indistinct bimodality (Fig. 2D; Suppl. material 2: table S2). In Pk YNKM and Pk YNWS, only small gaps in chromosome length between 9th and 10th pair exist, and four pairs of chromosomes are very short and of similar length (pairs 12 to 15), showing indistinct bimodality (Fig. 2E, F; Suppl. material 2: table S2). Ps HNFNS has a small gap between the 7th and 8th pair, also showing indistinct bimodality (Fig. 2I; Suppl. material 2: table S2). Different numbers and locations of secondary constrictions (SCs) are observed in the 9 populations (Fig. 2, Suppl. material 3: fig. S1). All the four populations of *P. cyrtonema* show four SCs, which are located on the long arms of the 3rd and 6th pairs in Pc AHDBS, on the long arms of the 1st and 4th pairs in Pc HNHH and Pc HBHS, and on the short arms of the 3rd and 8th pairs in Pc SCSN (Fig. 2A, B, C, D, Suppl. material 3: fig. S1A, B, C, D). Pk YNKM had six SCs which are located on the long arms of the 4th , 7th and 8th pairs, while Pk YNWS had four SCs which are located on the long arms of the 3rd and 8th pairs (Fig. 2E, F, Suppl. material 3: fig. S1E, F). Both Po HNXH and Po AHDBS have four SCs which are situated on the long arms of the 2nd and 5th pairs (Fig. 2G, H, Suppl. material 3: fig. S1G, H). In Ps HNFNS, two SCs is found on the short arms of the 1st pair, and another two SCs are located on the long arms of the 3rd pair (Fig. 2I, Suppl. material 3: fig. S1I).

The values of the four karyotype asymmetry indices including CV_{CI}, CV_{CL}, M_{CA} and Stebbins' type are presented in Table 1. According to the critical review by Peruzzi and Eroglu 2013, CV_{CI} is the measure of the heterogeneity of centromere position, CV_{CL} is a powerful statistical parameter for estimating the interchromosomal asymmetry, and M_{CA} is the most appropriate parameter for characterizing the intrachromosomal asymmetry. The ranges of CV_{CI}, CV_{CL} and M_{CA} are as follows: CV_{CI} = 19.40 (Pc SCSN) – 33.98 (Pk YNWS), CV_{CL} = 22.94 (Pc SCSN) – 43.90 (Pk YNKM), M_{CA} = 31.46 (Pc AHDBS) – 43.90 (Pk YNKM). The CV_{CL} values reveal that Pc SCSN and Pk YNKM have the least and the most asymmetric karyotype, respectively, among the 9 populations in terms of interchromosomal asymmetry. The M_{CA} values reveal that Pc AHDBS and Pk YNKM have the lowest and the highest intrachromosomal asymmetry, respectively. With respect to the Stebbins' type, these karyotypes fall into 2A, 2B, 3A or 3B categories, possessing moderate degree of asymmetry (Stebbins 1971).

The karyotype asymmetry relationships among the 9 populations that are expressed by means of bidimensional scatter plot of M_{CA} vs. CV_{CL} are illustrated in Fig. 3. It is obvious that the karyotpye structure of these populations can be discriminated by these two parameters. As demonstrated in the scatter plot, Pc AHDBS and Pc SCSN are the most symmetric karyotypes in terms of intra- and inter-chromosomal index, respectively, while Pk YNKM is the most asymmetric karyotype in terms of both intra- and inter-chromosomal asymmetry.

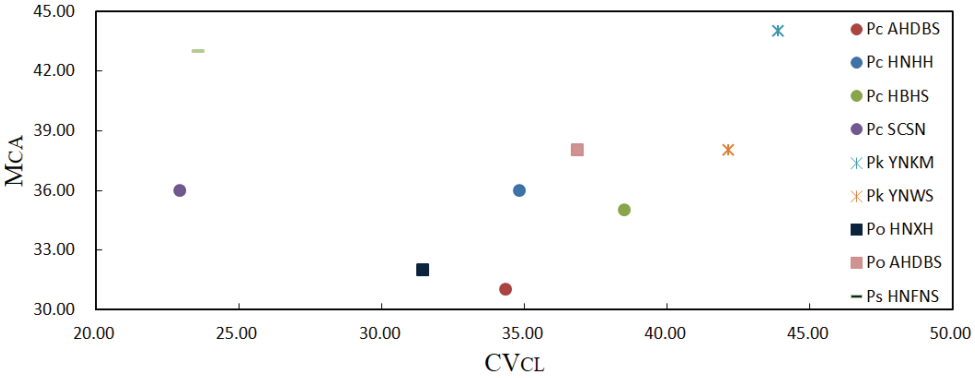

Figure 3. Bidimensional scatter plot of M_{CA} vs. CV_{CL} for the 9 populations of four *Polygonatum* species, *P. cyrtonema* (Pc), *P. kingianum* (Pk), *P. odoratum* (Po) and *P. sibiricum* (Ps).

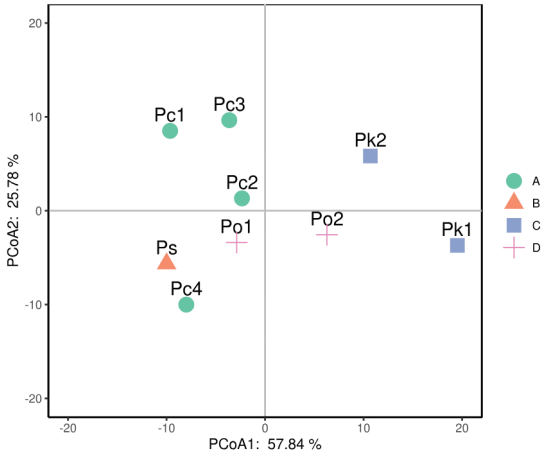

Figure 4. PCoA for the 9 populations of four *Polygonatum* species, *P. cyrtonema* (Pc), *P. kingianum* (Pk), *P. odoratum* (Po) and *P. sibiricum* (Ps), based on *x*, 2*n*, TCL, M_{CA}, CV_{CL} and CV_{CI}. Pc1, Pc2, Pc3 and Pc4 represent Pc AHDBS, Pc HNHH, Pc HBHS and Pc SCSN, respectively. Pk1 and Pk2 represent Pk YNKM and Pk YNWS, respectively. Po1 and Po2 represent Po HNXH and Po AHDBS, respectively. Ps represents Ps HNFNS. PCoA1 reflects the original data characteristics before the dimensionality reduction of 57.84%. PCoA2 reflects the character of the original data before the dimensionality reduction of 25.78%. The sum of the two percentages is 83.62%, indicating that the two-dimensional coordinate system can reflect the characteristics of 83.62% of the original data.

PCoA based on the six quantitative karyological parameters reveals the karyological relationships among the 9 populations (Fig. 4). The PCoA scatter plot shows that the 9 populations are divided into two groups along the direction of PCoA1: Pc AHDBS, Pc HNHH, Pc HBHS, Pc SCSN, Po HNXH and Ps HNFNS in one group with closely clustering together of Pc AHDBS and Pc HBHS , Pc HNHH and Po HNXH, Pc SCSN and Ps HNFNS, respectively; while Po AHDBS, Pk YNKM and Pk YNWS

in another group with Pk YNKM occupying the most isolated position. Po HNXH and Po AHDBS occupy on either side of the middle position and are close to each other along the direction of PCoA2.

FISH mapping of 5S and 45S rDNA sites

The FISH results show inter- and intra-specific variations in number and location of 5S and 45S rDNA loci (Figs 1, 2; Table 2). All but two populations have a single locus of 5S rDNA, which in Pc AHDBS, Pc HNHH, Pc SCSN, Po HNXH and Ps HNFNS is situated in the distal or interstitial regions of the long arms of a small m or sm chromosome pair (Figs 1A, B, D, G, I, 2A, B, D, G, I; Table 2). Pc HBHS and Po AHDBS have one 5S locus in the same position as the five populations described above and another 5S locus that is located in the proximal regions of the long arms of a large st or sm chromosome pair (in Po AHDBS only one member of the chromosome pair showed 5S rDNA signal) (Figs 1C, H, 2C, H; Table 2). The single 5S locus in Pk YNKM and Pk YNWS is located in the proximal regions of the short arms of a large st chromosome pair (Figs 1E, F, 2E, F; Table 2).

As for 45S rDNA site, there exist two loci in Pc AHDBS, Pc HNHH, Pc HBHS, Pk YNWS, Po HNXH, Po AHDBS and Ps HNFNS and three loci in Pc SCSN and Pk YNKM (Figs 1, 2; Table 2). All but one 45S loci in the 9 populations generated SCs (Fig. 2, Suppl. material 3: fig. S1). No SC was observed within the 45S locus that was located on the long arms of pair 10 of Pc SCSN (Fig. 2D, Suppl. material 3: fig. S1D). Among the four populations of *P. cyrtonema*, the locations of the two 45S loci of Pc HNHH and Pc HBHS are almost the same, being located in interstitial regions of the long arms of pairs 1 and 4 (Fig. 2B, C; Table 2); in Pc AHDBS, the lengths and arm ratios of the chromosome pairs with the two 45S loci are changed compared to Pc

Table 2. The number (pair) and location of rDNA loci in 9 populations of four *Polygonatum* species.

Populations[†]	5S rDNA[‡]	45S rDNA[‡]
Pc AHDBS	one[8L-DIS(78.73%)]	two[3L-INT[$](57.57%),6L-INT[$](65.28%)]
Pc HNHH	one[10L-INT(61.65%)]	two[1L-INT(49.19%)[$],4L-INT(57.46%)[$]]
Pc HBHS	two[2L-PRO(19.28%),10L-DIS(79.08%)]	two[1L-INT(45.14%)[$],4L-INT(59.20%)[$]]
Pc SCSN	one[9L-DIS(78.08%)]	three[3S-INT(44.35%)[$], 8S-DIS(82.02%)[$], 10L-INT(46.75%)]
Pk YNKM	one[1S-PRO(23.36%)]	three[4L-INT(61.57%)[$],7L-INT(65.24%)[$], 8L-INT(70.65%)[$]]
Pk YNWS	one[2S-PRO(21.29%)]	two[3L-INT(44.50%)[$], 8L-INT(70.88%)[$]]
Po HNXH	one[9L-INT(58.06%)]	two[2L-INT(57.05%)[$], 5L-INT(65.56%)[$]]
Po AHDBS	one and a half [1L-PRO(18.29%)[|], 10L-DIS(82.99%)]	two[2L-INT(65.41%)[$], 5L-INT(54.92%)[$]]
Ps HNFNS	one[11L-INT(71.79%)]	two[1S-INT(33.76%)[$], 3L-INT(71.86%)[$]]

[†]Pc = P. cyrtonema, Pk = P. kingianum, Po = P. odoratum, Ps = P. sibiricum.

[‡]S and L represent the short and long arms, respectively; CEN, PRO, INT, DIS and TER represent the centromeric (*di* = 0), proximal (0 < *di* < 25%), interstitial (25% ≤ *di* ≤ 75%), distal (75% < *di* < 100%) and terminal (*di* = 100%) positions, respectively; figures ahead of the positions designate the chromosomal pair involved; the percentages in square brackets are the percentage distance from centromere to rDNA locus ($di = d \times 100/a$; *d* = distance of the center of FISH signals from the centromere, *a* = length of the corresponding chromosome arm).

[$]indicates the 45S rDNA loci generating secondary constrictions (SCs).

[|]only a member of the chromosome pair has 5S rDNA site.

HNHH and Pc HBHS, but both loci are still located in interstitial regions of the long arms of two large chromosome pairs (the 3rd and 6th pairs) (Fig. 2A; Table 2; Suppl. material 2: table S2); in Pc SCSN, the two 45S loci which generate SCs are located on the short arms of pairs 4 and 7 and a new minor 45S locus appear in pair 10 (Fig. 2D; Table 2). Among the two populations of *P. kingianum*, the 45S locus that is located on the long arms of pair 8 is conserved, the location of the 45S locus that is located on the long arms of a large st chromosome pair (the 4th or 6th pair) differs somewhat (being closer to the distal region in Pk YNWS than in Pk YNKM), and an additional 45S locus appears on the long arms of pair 7 in Pk YNKM (Fig. 2E, F; Table 2). In *P. odoratum*, the two 45S loci are located on the long arms of pairs 2 and 5 with similar percentage distance between Po HNXH and Po AHDBS, but the arm ratios of the chromosome pairs with the respective loci differ significantly between the two populations (Fig. 2G, H; Table 2; Suppl. material 2: table S2). In *P. sibiricum*, the two 45S loci are located on the short arms of a large chromosome pair (the 1st pair) and the long arms of another large chromosome pair (the 3rd pair), being different from all other populations with two 45S loci (Fig. 2I; Table 2).

Discussion

Karyotype variation

In the present study, a primary molecular cytogenetic characterization of 9 populations of *P. cyrtonema*, *P. kingianum*, *P. odoratum* and *P. sibiricum* is conducted for the first time. The karyotypic parameters and rDNA patterns vary among the populations studied, enabling an accurate distinguishment between individual genomes. The rDNA FISH signals provide new chromosomal markers for investigating the inter- and intraspecific karyotype evolution in the genus *Polygonatum*.

The evolution of chromosome number in *Polygonatum* is mainly dysploidy, and a few species have polyploidy (Deng et al. 2009; Zhao et al. 2014; Wang et al. 2016a). There are two levels of dysploidy in *Polygonatum*. First, there is a significant variation of basic chromosome number among different species, from $x = 8$ to $x = 16$ (Deng et al. 2009; Zhao et al. 2014; Wang et al. 2016a; Zhou et al. 2020). On the other hand, there exists also intraspecific dysploid variation in more than fourteen species in which *P. cyrtonema*, *P. kingianum*, *P. odoratum* and *P. sibiricum* are involved (Zhao et al. 2014; Wang et al. 2016a; Zhou et al. 2020). Moreover, a few species such as *P. cyrtonema* and *P. odoratum* display continuous dysploidy (Wang et al. 2016a). We analyze the basic chromosome numbers of all diploid populations of each of the four species including the populations in our study and those reported in the literature, and calculate the frequency of occurrence of each basic chromosome number in each species (Suppl. material 4: fig. S2). We infer that, in each species, the basic chromosome number that occurs most frequently should be the original character and other basic chromosome numbers should be derived character. Previous studies reported

x = 9, 10, 11 and 12 for *P. cyrtonema* (Fang et al. 1984; Wang et al. 1987, 1991; Chen et al. 1989; Tamura 1990; Shao et al. 1993; Wu et al. 2001; Jin et al. 2002; Chen and Zhou 2005; Zhao et al. 2014; Zhou et al. 2020), a species occurs in south, southeast and southwest China (Chen and Tamura 2000). Our study detected a new basic chromosome number (x = 13) for *P. cyrtonema*, further demonstrating the existence of continuous dysploidy within this species. Among the five basic chromosome numbers of *P. cyrtonema*, x = 11 occurs most frequently (accounting for 60.98%; Suppl. material 4: fig. S2A). For *P. kingianum*, a species occurs in Sichuan, Yunnan and Guizhou provinces, China (Chen and Tamura 2000), x = 13 and 15 have been reported, which were the basic chromosome numbers of the populations from Sichuan and Yunnan, respectively (Yang et al. 1988; Chen et al. 1989; Tamura 1993; Wang et al. 1993; Deng et al. 2009; Zhou et al. 2020). The chromosome numbers ($2n$ = 30) of the two *P. kingianum* populations from Yunnan that we analyzed here are consistent with those of the Yunnan populations reported previously (Tamura 1993; Wang et al. 1993; Zhou et al. 2020). The basic chromosome number of wide-ranging Eurasian species *P. odoratum* has been reported as x = 8, 9, 10 and 11 (Li et al. 1980; Wang et al. 1987, 1988; Chen 1989; Fang 1989; Fu and Hong 1989; Tamura 1990; Wang et al. 1991; Shang et al. 1992; Shao et al. 1993; Han et al. 1998; Wu et al. 2001; Weiss-Schneeweiss and Jang 2003; Chen and Zhou 2005; Zhao et al. 2014; Wang et al. 2016a; Zhou et al. 2020) with x = 10 occurring most frequently (accounting for 75.00%; Suppl. material 4: fig. S2C). Two of the four basic numbers (x = 10 and 11) are detected in the *P. odoratum* populations studied here. It has been showed that most populations of *P. odoratum* from Europe, northeast Asia, northwest and north China had a chromosome number of $2n$ = 20, while populations from east, central and southwest China had a chromosome number fluctuated around $2n$ = 20 (Fang 1989). For *P. sibiricum*, a species occurs in northeastern, northern, central and eastern China, Korea, Mongolia and Russia (Siberia) (Chen and Tamura 2000), x = 12, 15 and 18 has been reported (Mehra and Pathania 1960; Mehra and Sachdeva 1976; Fang et al. 1984; Wang et al. 1987; Chen 1989; Han et al. 1998; Deng et al. 2009; Zhao et al. 2014; Zhou et al. 2020). The *P. sibiricum* population used in this study (Ps HNFNS) shows the basic chromosome number (x = 12) that occurs most frequently in this species (accounting for 85.71%; Suppl. material 4: fig. S2D).

The scatter plot of M_{CA} vs. CV_{CL} reveals that the karyotypic structures vary both among species and among different populations of the same species in terms of both intra- and inter-chromosomal asymmetry (Fig. 3). There are significant variations in the chromosomal organization of the complements between populations with different basic chromosome numbers of the same species. In *P. cyrtonema*, the karyotypes of the populations with x = 9, 10, 11 and 12 are usually of distinct bimodality, whose number of large and small chromosomes are 6 + 3 (Fang et al. 1984; Shao et al. 1993; Zhao et al. 2014; Zhou et al. 2020; this study), 5 + 5 or 6 + 4 (Chen 1989; Wang et al. 1991; Shao et al. 1993; Zhou et al. 2020), 5 + 6 (Wang et al. 1987, 1991; Shao et al. 1993; Jin et al. 2002; Chen and Zhou 2005; Zhou et al. 2020; this study) and 4 + 8 (Jin et al. 2002), respectively. However, the karyotype of the population with

x = 13 becomes indistinctly bimodal (Fig. 2D; Suppl. material 2: table S2). Similarly, the karyotypes of the *P. odoratum* populations with x = 8, 9, 10 and 11 are mainly of distinct bimodality, whose number of large and small chromosomes are 6 + 2 (Shao et al. 1993), 7 + 2 or 6 + 3 (Wang et al. 1988; Shao et al. 1993; Wu et al. 2001; Chen and Zhou 2005; Zhao et al. 2014), 6 + 4 or 7 + 3 (Chen 1989; Tamura 1990; Wu et al. 2001; Weiss-Schneeweiss and Jang 2003; Zhao et al. 2014; Zhou et al. 2020; this study) and 5 + 6 (Fang 1989; Fu and Hong 1989; Zhao et al. 2014; this study), respectively. Some of the *P. odoratum* populations with x = 10 reported previously had unimodal karyotypes (Fang 1989; Hong and Zhu 1990; Tamura 1990, 1993; Wang et al. 1991; Shang et al. 1992; Han et al. 1998). As mentioned above, x = 11 and 10 should be the original basic chromosome numbers of *P. cyrtonema* and *P. odoratum*, respectively, thus there should be a concomitant decrease and increase of basic chromosome number on the basis of x = 11 or 10 in the continuous dysploid variation of the two species. Compared with Pc HNHH and Pc HBHS (x = 11), Pc AHDBS (x = 9) increased by one pair of large chromosomes and decreased by three pairs of small chromosomes with a production of two pairs of large m chromosomes (the 1st and 3rd pairs) and changes of the relative lengths and arm ratios of the chromosomes bearing 45S rDNA loci (Fig. 2A, B, C), and Pc SCSN increased by two pairs of chromosomes, and underwent translocations of the two major 45S loci from long arms to short arms and loss of bimodality (Fig. 2D). Compared with Po HNXH (x = 10), Po AHDBS (x = 11) increased by two pairs of small chromosomes (probably the 6th and 8th pairs), underwent changes of the arm ratios of the chromosomes bearing the two 45S loci as well as the percentage distances of both 45S and 5S loci (Fig. 2G, H). The above analysis of intraspecific increase and decrease of basic chromosome number in *P. cyrtonema* and *P. odoratum* shows that there exists basically a one-to-two or two-to-one relationship between changes in the number of large and small chromosomes in the continuous dysploid variation, but there are not any small st and t chromosomes in their karyotypes (Fig. 2A, B, C, G, H). Thus, the intraspecific dysploidy was not a classic Robertsonian transformation (chromosomal fission or fusion) process (Olson and Gorelick 2011). Considering that changes of the relative lengths and arm ratios of some chromosomes are accompanied, and even the bimodality of karyotypes of some populations has been lost, we suggest that complex chromosomal rearrangements, probably including centromere fission or fusion, unequal translocations, and pericentric inversions, have contributed to the continuous dysploid variation within these species (Moscone et al. 2007).

Chromosome arrangements also occur between populations with the same basic chromosome number. The karyotypes of Pc HNHH and Pc HBHS (both x = 11) show some differences, mainly including significant changes of the arm ratios of pairs 2 and 8 between the two populations, and the occurrence of another 5S locus on pair 2 in Pc HBHS (Fig. 2B, C; Suppl. material 2: table S2). Among the reported populations of *P. cyrtonema* with x = 10, the majority had bimodal karyotypes composed of 5 + 5 (Chen et al. 1989; Shao et al. 1993) or 6+4 (Wang et al. 1991; Zhou et al. 2020), a few had unimodal karyotypes (Wang et al. 1991), indicating

multiple chromosomal arrangements between the populations with $x = 10$. Also, the karyotypes of Pk YNKM and Pk YNWS (both $x = 15$) have some differences, mainly including significant changes in the arm ratios of pairs 5 and 9 between the two populations, changes in the percentage distance of their sharing two pairs of 45S loci, and the presence of an additional 45S locus on pair 7 of Pk YNKM (Fig. 2E, F; Suppl. material 2: table S2). As for *P. odoratum*, previous reports showed that some populations with $x = 10$ had bimodal karyotypes composed of 7 + 3 (Shang et al. 1992; Wu et al. 2001) or even unimodal karyotype (Wang et al. 1987, 1988, 1991; Fang 1989; Hong and Zhu 1990; Tamura 1990, 1993; Han et al. 1998) instead of a bimodal karyotype composed of 6 + 4 as the populations studied by us and other previous authors (Chen 1989; Weiss-Schneeweiss and Jang 2003; Zhao et al. 2014; Zhou et al. 2020), indicating the occurrence of multiple chromosome rearrangements among different populations with $x = 10$.

Although the chromosomal rearrangements inferred from the changes in chromosomal morphology and rDNA pattern may only represent the tip of the iceberg of the dysploidy within species of the genus *Polygonatum*. However, it has been revealed that, in the evolutionary process, geographically diverse populations of *Polygonatum* species are easy to preserve large-scale and multiple chromosomal rearrangements. The reasons for this may be the perennial and clonal nature of *Polygonatum* species (Wang et al. 1987). It is the abundant chromosomal rearrangements and the resulting dysploid variation that leads to the highly morphological variation within widely-distributed *Polygonatum* species such as *P. cyrtonema* and *P. odoratum* (Wang et al. 1991; Shao et al. 1993).

The direction of the basic chromosome number evolution in the interspecific dysploidy of *Polygonatum* has long been an important and challenging issue in the cytogenetic study of the genus. From our comparative molecular cytogenetic karyotype analysis, there are obvious differences in chromosome number, karyotypic structure and rDNA pattern among *P. cyrtonema* and *P. odoratum* (representatives of sect. *Polygonatum*), *P. kingianum* (a representative of sect. *Verticillata*) and *P. sibiricum* (the representative of sect. Sibirica) (Meng et al. 2014). It is generally believed that, in morphology, the section with alternate phyllotaxy is relatively primitive and the section with whorled (verticillate) phyllotaxy is relatively evolved (Wang et al. 1987; Shao et al. 1994). Therefore, it was speculated that ascending dysploidy may be the main evolutionary mode of the karyotype in *Polygonatum* (Deng et al. 2009). Wang et al. (1987) speculated that the ancestral basic chromosome number of *Polygonatum* was most likely $x = 10$, based on which the ascending dysploidy was predominant and the descending dysploidy was secondary. Bayesian analyses of the molecular phylogenetic study based on four regions of chloroplast genomes supported the alternate-leaf arrangement as the ancestral state for *Polygonatum* (Meng et al. 2014). However, a recent comparative analysis of chloroplast genomes of *Polygonatum* species showed that the verticillate leaf might be the ancestral state of this genus (Wang et al. 2022). Therefore, further studies are needed to determine whether the interspecific dysploidy of *Polygonatum* is ascending or descending.

Phylogenetic relationships

According to the infrageneric classification system of Meng et al. (2014) and recent molecular phylogenetic studies of *Polygonatum* (Wang et al. 2016b; Floden and Schilling 2018; Zhao et al. 2019; Wang et al. 2022; Xia et al. 2022), *P. cyrtonema* and *P. odoratum* are placed on different sister branches of the same lineage of sect. *Polygonatum*, *P. sibiricum*, the only species of sect. Sibirica, is sister to sect. *Polygonatum* in one major branch, and *P. kingianum* is placed in another major branch (sect. *Verticillata*). Comparison of the karyotypic structures and rDNA patterns of these four representative species is helpful to reveal the chromosome evolution among three sections of the genus and the phylogenetic relationships among these species. However, this comparison is complicated by the presence of dysploid variation within these species which results in intra-specific variations in both karyotypic structure and rDNA pattern. As mentioned above, the original basic chromosome numbers of *P. cyrtonema*, *P. kingianum*, *P. odoratum* and *P. sibiricum* should be x = 11, 15, 10 and 12, respectively (Suppl. material 4: fig. S2), so it is both reasonable and valid to use the populations with these basic chromosome numbers (Pc HNHH, Pc HBHS, Pk YNKM, Pk YNWS, Po HNXH and Ps HNFNS) for comparison.

The similarities and differences in rDNA patterns among species reflect the closeness of relatedness between species (e.g. Moscone et al. 2007; Chacón et al. 2012; Siljak-Yakovlev and Peruzzi 2012; She et al. 2015, 2020; Senderowicz et al. 2022; Yucel et al. 2022; Mitrenina et al. 2023). Among the 9 populations of the four species investigated here, seven populations have one 5S rDNA locus and two 45S rDNA loci, suggesting one locus of 5S rDNA and two loci of 45S rDNA being the ancestral state of *Polygonatum* species. Another 5S locus in Pc HBHS (localization: 2L-PRO), a half locus of 5S in Po AHDBS (localization: 1L-PRO), another 45S locus in Pc SCSN (localization: 10L-INT) and Pk YNKM (localization: 7L-INT) probably originated from chromosomal arrangements (Table 2; Fig. 2C, D, E, H) (Chacón et al. 2012; She et al. 2015; Senderowicz et al. 2022) or the action of transposable elements which accumulate at the proximity or around rDNA loci (Raskina et al. 2008). As for the distribution of the conserved locus of 5S rDNA, that of *P. cyrtonema* (Pc HNHH and Pc HBHS), *P. odoratum* (Po HNXH) and *P. sibiricum* (Ps HNFNS) is located in the interstitial or distal regions of the long arms of a pair of small m or sm chromosomes, while that of *P. kingianum* (Pk YNKM and Pk YNWS) is located in the proximal regions of the short arms of a pair of large st chromosomes (Fig. 2B, C, E, F, G, I). With regard to the distribution of the two conserved loci of 45S rDNA, those of *P. cyrtonema* (Pc HNHH and Pc HBHS) and *P. odoratum* (Po HNXH) are located in the interstitial regions of the long arms of two pairs of large chromosomes; those of *P. sibiricum* (Ps HNFNS) are also located on two pairs of large chromosomes, but one is located in the interstitial regions of the short arms and the other in the interstitial regions of the long arms; one of those of *P. kingianum* is located in the interstitial regions of the long arms of a pair of large chromosomes, and the other in the interstitial regions of the long arms of a pair of small chromosome (Fig. 2B, C, E, F, G, I). These facts suggest that, among the four species,

P. cyrtonema and *P. odoratum* are most closely related to each other, and *P. cyrtonema* and *P. odoratum* are closely related to *P. sibiricum* and distantly related to *P. kingianum*. This inference is consistent with the phylogenetic relationships among these species revealed by molecular phylogenetic studies (Meng et al. 2014; Wang et al. 2016b; Floden and Schilling 2018; Zhao et al. 2019; Wang et al. 2022; Xia et al. 2022).

PCoA based on *x*, 2*n*, TCL, CV_{CI}, M_{CA}, CV_{CL} is – thus far – the most legitimate approach to use for comparing karyotypes and reconstructing karyological relationships among taxa (Peruzzi and Altınordu 2014; Dehery et al. 2020; Kadluczka and Grzebelus 2021; She et al. 2023). It seems that the karyological relationships between the four species are not clearly delineated by the PCoA scatter plot of the 9 populations since Pc SCSN and Ps HNFNS are closely clustered, and Po AHDBS is distantly separated from Po HNXH and placed in the group that *P. kingianum* is in (Fig. 4). However, when only the populations with the original basic chromosome numbers are considered, the karyological relationships among the four species are basically consistent with the molecular phylogenetic relationships among these species (Meng et al. 2014; Wang et al. 2016b; Floden and Schilling 2018; Zhao et al. 2019; Wang et al. 2022; Xia et al. 2022). As the PCoA scatter plot showed (Fig. 4), along the direction of PCoA1, *P. cyrtonema* (Pc HNHH and Pc HBHS), *P. odoratum* (Po HNXH) and *P. sibiricum* (Ps HNFNS) are in one group with the former two species (Pc HNHH and Po HNXH) closely clustering, while *P. kingianum* (Pk YNKM and Pk YNWS) was in another group and away from the middle position of the two groups. Therefore, it is effective to use populations with the original basic chromosome number of each species for PCoA-based karyological relationship construction among species that possess intraspecific dysploidy.

Conclusions

Detailed molecular cytogenetic karyotypes of 9 populations of four *Polygonatum* species, *P. cyrtonema*, *P. kingianum*, *P. odoratum* and *P. sibiricum*, are established for the first time using the dataset of chromosome measurements and FISH signals of 5S and 45S rDNA. Comparative karyotyping reveals distinct variations in the karyotypic parameters and rDNA patterns among and within species, and intraspecific dysploidy of *P. cyrtonema* and *P. odoratum*. The evolutionary relationships among the four species revealed by rDNA pattern comparison and PCoA based on *x*, 2*n*, TCL, CV_{CI}, M_{CA} and CV_{CL} are basically accordant with the phylogenetic relationships revealed by molecular phylogenetic studies.

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ORCID

Chao-Wen She https://orcid.org/0000-0003-1935-5509

Supplementary material 1

The plant materials

Authors: Yan-Fang Wei, Xiang-Hui Jiang, Rong Song, Chao-Wen She

Data type: doc

Link: https://doi.org/10.3897/compcytogen.17.122399.suppl1

Supplementary material 2

Chromosome measurements of 9 populations of four *Polygonatum* species

Authors: Yan-Fang Wei, Xiang-Hui Jiang, Rong Song, Chao-Wen She

Data type: docx

Link: https://doi.org/10.3897/compcytogen.17.122399.suppl2

Supplementary material 3

Metaphase chromosomes of 9 populations of four *Polygonatum* species, *P. cyrtonema* (Pc), *P. kingianum* (Pk), *P. odoratum* (Po) and *P. sibiricum* (Ps)

Authors: Yan-Fang Wei, Xiang-Hui Jiang, Rong Song, Chao-Wen She

Data type: docx

Link: https://doi.org/10.3897/compcytogen.17.122399.suppl3

Supplementary material 4

Diagrams of the distribution of basic chromosome numbers within four *Polygonatum* species, *P. cyrtonema*, *P. kingianum*, *P. odoratum* and *P. sibiricum*

Authors: Yan-Fang Wei, Xiang-Hui Jiang, Rong Song, Chao-Wen She

Data type: docx

Link: https://doi.org/10.3897/compcytogen.17.122399.suppl4

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Karyotype analysis of *Quasipaa spinosa* David, 1875 (Anura, Dicroglossidae) with conventional cytogenetic techniques

Liaoruilin Zhang[1], Jianguo Xiang[1], Juan Li[1], Jie Zhou[1], Jinliang Hou[1], Yanfei Huang[1], Hong Li[1]

1 *College of Fisheries, Hunan Agricultural University, No.1 Nongda Road, Furong District, Changsha, Hunan Province,410128, China*

Corresponding author: Jianguo Xiang (xiangjianguo055x@hunau.edu.cn)

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Abstract

The current study analyzed the chromosomal karyotype of *Quasipaa spinosa* David, 1875 from Hunan Province, China. The karyotype, C-banding, BrdU-banding pattern were characterized using direct preparation of bone-marrow cells and hemocyte cultures. The findings indicated that *Q. spinosa* was a diploid species (2n = 26) that lacked heteromorphic chromosomes and secondary constrictions. C-banding analysis revealed an abundance of positive signals in the centromere regions, while the BrdU-banding pattern showed three phases in both male and female, occurring consistently and in chronological sequence during S-phase. Notably, there was no asynchronous replication in the late phase. This study enhanced our understanding of the karyotypic structure of *Q. spinosa* by conventional cytogenetic techniques, thus providing essential scientific insights into the cytogenetics of *Q. spinosa*.

Keywords

BrdU-banding, C-banding, karyotype, *Quasipaa spinosa*

Introduction

Quasipaa spinosa David, 1875 (Anura, Dicroglossidae) is an amphibian native to and widely distributed in southern and southeastern China, where it thrives in forests and hilly areas at altitudes of 500–1500 meters (Zhao 1998). *Quasipaa spinosa* holds considerable economic significance in China's frog-breeding industry (Yu et al. 2008), as it is prized for its therapeutic and medicinal properties, together with its high reproductive rate and large populations (Lau et al. 2008). This frog species has significant nutritional and medicinal value, and has been described as the "King of a Hundred Frogs" (Mei et al. 2018). However, over the past decade, its population has declined significantly, by over 30% due to overhunting and habitat destruction. This decline has led to its being classified as endangered by the International Union for Conservation of Nature (IUCN) and the China Species Red List (Shen et al. 2015). Efforts in artificial breeding were initiated in the 1980s to meet the market demand (Chan et al. 2014).

Chromosome karyotype studies are valuable in elucidating the phylogeny of the species studied as well as assisting in its classification (Gokhman 2023) and providing an understanding of species alterations in *Q. spinosa* from a cytogenetic perspective. Regarding karyotypic research on *Q. spinosa*, Li and Wang (1983), Zheng and Hong (1984), as well as Long et al. (2021) had reported Giemsa-stained karyotypes, which consistently demonstrate a chromosomal count of 2n=26 but differing karyotypic formulas across different geographical regions.

This study presents an analysis of the karyotype of *Q. spinosa* using conventional cytogenetic techniques, aiming to contribute novel and valuable karyotypic information while enhancing the existing cytogenetic database for *Q. spinosa*.

Material and methods

The study utilized 15 healthy adult male and 15 female *Q. spinosa*, each weighing between 90–100 g, sourced from farm located in Taiping Town (29°58'42"N, 111°05'25"E), Shimen County of Hunan Province, China. Animal treatment and the study protocol strictly adhered to ethical guidelines formulated by the Animal Protection Committee (APC) of Hunan Agricultural University (201903297) (ethics license: No. LSK 202-3-D106). Specimens were transported to the laboratory via a specialized vehicle designed to maintain breeding environment temperatures thereby reducing mortality due to temperature-induced stress.

Bone marrow-cell suspensions were prepared as previously described by Baldissera et al. (1993), with minor modifications. The animals were injected intraperitoneally with 10 μg/g of colchicine solution and allowed to rest for 4 h. They were then anesthetized with 0.05% MS-222 and bone marrow was harvested from the thighs. The harvested bone marrow was treated with 0.34% KCL solution at low osmolality for 1 h and then fixed in two changes of Carnoy's solution, after which the cell suspension was aspirated onto the center of a slide. The slides underwent standard Giemsa staining

(Howell and Black 1980), and C-banding (Sumner 1972). 20 clear images were selected from a total of 180 images captured using an Olympus BX5 digital camera. The chromosomes were classified as metacentric or submetacentric according to the criteria of Green and Sessions (1991).

The animals were anesthetized and blood was collected from the heart. The blood was injected into human peripheral blood lymphoid medium (0.2 ml of blood for every 5 ml of culture medium) which was then incubated for 90 hours in a thermostatic incubator at 28 °C in the dark. The cells were treated with 100 μg/ml bromodeoxyuridine (BrdU) 9 h before the end of the culture period, followed by 40 μg/mL fluorodeoxyuridine (FdU) for 6 h and the addition of colchicine to a final concentration of 0.03 μg/ml for 2 h. Acridine orange was added at a final concentration of 10 μg/mL 1 h before harvesting (Matsubara and Nakagome 1983). After drying, the chromosome slides were soaked in 2×SSC solution in a thermostatic water bath at 40 °C and exposed to UV light from a distance of 8 cm for 45 min. The slides were then stained with 10% Giemsa (pH = 6.8) for 15 min.

Results

Q. spinosa exhibited a diploid number of 2n = 26 lacking heteromorphic chromosomes and secondary constrictions (Fig. 1), The chromosome pairs 2, 4, 8, and 10 were submetacentric, while the remaining chromosomes were metacentric. Table 1 shows the relative length (Guzman et al. 2022), and arm ratio, and chromosomal classification. Most chromosomes had a C-banding pattern restricted to centromere regions (Fig. 2A, B).

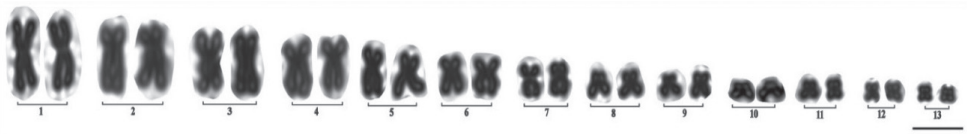

Figure 1. *Q. spinosa* karyotype. Scale bar: 10 μm.

Table 1. Chromosome number (CN), relative length (RL), arm ratio (AR), and chromosomal classification (CC) of mitotic chromosome. M = metacentric chromosome; SM = submetacentric chromosome. LHV= lower and higher values for each chromosome.

CN	1	2	3	4	5	6	7	8	9	10	11	12	13
RL	15.38± 0.84	12.46± 0.81	11.65± 0.82	10.75± 0.62	10.04± 0.56	7.45± 0.58	6.98± 0.55	5.93± 0.55	4.97± 0.61	4.17± 0.13	3.88± 0.21	3.48± 0.11	2.96± 0.85
LHV	13.88- 16.51	11.11- 13.95	10.31- 12.69	9.83- 11.68	9.16- 10.65	6.64- 8.41	6.15- 7.92	5.06- 6.93	4.15- 5.95	3.97- 4.37	3.54- 4.17	3.32- 3.67	2.81- 3.07
AR	1.25± 0.05	1.85± 0.12	1.50± 0.05	2.39± 0.10	1.25± 0.06	1.34± 0.05	1.34± 0.05	1.88± 0.08	1.15± 0.02	2.23± 0.07	1.28± 0.05	1.06± 0.03	1.41± 0.04
LHV	1.20- 1.34	1.70- 2.03	1.40- 1.58	2.18- 2.54	1.15- 1.33	1.24- 1.42	1.26- 1.43	1.76- 2.04	1.11- 1.19	2.12- 2.34	1.19- 1.36	1.02- 1.12	1.33- 1.5
CC	M	SM	M	SM	M	M	M	SM	M	SM	M	M	M

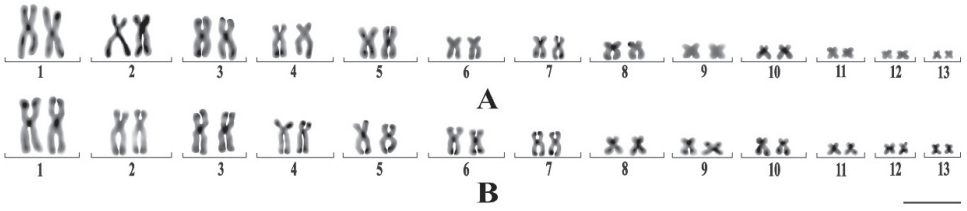

Figure 2. C-banding karyotypes. *Q. spinosa* male (**A**) and *Q. spinosa* female (**B**). Scale bar: 15 μm.

After BrdU infiltration, abundant chromosomal division phases and replicative banding patterns were observed as the cell culture progressed. During the S-phase, chromosomes that had completed DNA synthesis were stained dark purplish-red or dark, while segments that were still undergoing synthesis post-BrdU infiltration appeared lavender-colored or light purplish-blue. Consequently, the replication bandings were classified into three periods based on the proportion of dark and light staining during the intermediate stage. First, in the early-replication stage at the time of BrdU treatment, the bands showed a roughly 1:1 ratio between early-replicated dark-stained bands and late-replicated light-stained bands. Second, in the mid-replication stage, dark-stained areas predominated while the chromosomes nevertheless remained distinguishable, and third, in the late replication phase, the chromosomes were almost entirely dark-stained following the completion of replication.

The replication timing of each of the 13 *Q. spinosa* chromosomes ranged from the very early to the late stages (Fig. 3A, B). As seen in the figure, their sequential arrangement from left to right indicated a decreasing BrdU substitution throughout the remaining S-phase. Male *Q. spinosa* displayed stronger replication bands in the early S-phase compared to the late-replication phase, while females exhibited weaker but more uniformly distributed, replication bands. Over time, these faint bands intensified, forming larger blocks. Intriguingly, the late-replication banding patterns in *Q. spinosa* suggested that neither sex exhibited heterozygosity. However, males showed more pronounced early replication banding in chromosomes 1–10 compared to females.

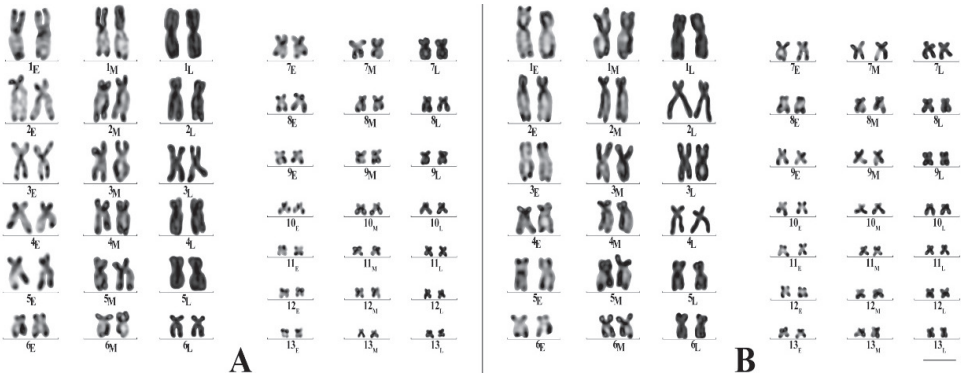

Figure 3. BrdU karyotypes. *Q. spinosa* male (**A**) and *Q. spinosa* female (**B**). E: early- replication phase; M: middle-replication phase; L: late-replication phase (**A, B**). Scale bar: 10 μm.

Discussion

Q. spinosa in Hunan exhibited a karyotype comprising nine pairs of metacentric and four pairs of submetacentric chromosomes (4sm+9m), consistent with the finding reported by Long et al. (2021). However, Zheng and Hong (1984) reported a distinct karyotype for *Q. spinosa* in Fujian, characterized by seven pairs of metacentric and six pairs of submetacentric chromosomes (6sm+7m), while Li and Wang (1983) identified ten pairs of metacentric and three pairs of submetacentric chromosomes (3sm+10m) in Anhui. Furthermore, Qing et al. (2012) discovered that all 33 populations of *Q. boulengeri* Günther, 1889 were diploid with a consistent chromosome count of 2n=26 across different regions in Sichuan, China. Nevertheless, at least five distinct karyotypes were primarily observed, with notable variations occurring on chromosomes 1 and 6. Specifically, chromosome 1 was composed of either two large metacentric chromosomes (Ms) or one large M and one large telocentric chromosome (T), while chromosome 6 consisted of either two small Ms, one small M and one large subtelocentric chromosome (ST), or two large STs. These findings suggested that geographic differentiation contributed to the observed karyotype variations within the same species.

The C-banding positive region was predominantly localized in the centromere region of *Q. spinosa*, with chromosomes 1–5 exhibiting the most pronounced positivity. Moreover, C-banding positivity was also observed within the respective centromere regions of *Limnonectes taylori* Matsui, Panha, Khonsue et Kuraishi, 2010 (Phimphan and Aiumsumang 2021), *Hypsiboas pulchellus* Faivovich, Haddad, Garcia, Frost, Campbell et Wheeler, 2005 (Baraquet et al. 2013), *Rana ridibunda* Pallas, 1771 (Arslan et al. 2010). These findings collectively indicate a prevalent occurrence of C-banding across species (Kakampuy et al. 2013). The BrdU-banding findings revealed that the BrdU-banding patterns of chromosomes 1–5 in both males and females was consistent with those reported by Richard et al. (2016), with the degree change in the BrdU-banding clearly observed in all of them. Additionally, the chromosomal BrdU-banding patterns for the three stages of the S-phase resembled those described by Schempp (1981), categorized as early, middle, and late. In the present study of *Q. spinosa*, three replication stages were observed chromosomes in the S-phase for both males and females, occurring in chronological order but asynchronous replication phenomena were not observed in the late phase.

In conclusion, *Q. spinosa* was a diploid species (2n=26) with the absence of heteromorphic chromosomes and secondary constrictions. Notably, heterochromatin in the centromere region and patterns of change in the BrdU-banding were observed. In this study, the karyotypic structure of *Q. spinosa* was analyzed, providing further genetic information on *Q. spinosa*.

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RESEARCH ARTICLE

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Physical chromosomal mapping of major ribosomal genes in 15 ant species with a review of hypotheses regarding evolution of the number and position of NORs in ants

Monique Telcia dos Santos Damasceno[1,2], Gisele Amaro Teixeira[1], Paulo Castro Ferreira[1,2], Rodrigo Batista Lod[1,2], Luísa Antônia Campos Barros[1], Hilton Jeferson Alves Cardoso de Aguiar[1,2]

1 *Universidade Federal do Amapá, Campus Binacional – Oiapoque, n°3051, Bairro Universidade, Oiapoque, Amapá, 68980-000, Brazil* **2** *Programa de Pós-graduação em Biodiversidade Tropical, Universidade Federal do Amapá, Campus Marco Zero do Equador, Macapá, Amapá, 68.903-419, Brazil*

Corresponding author: Hilton Jeferson Alves Cardoso de Aguiar (hilton@unifap.br)

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Abstract

Recently, hypotheses regarding the evolutionary patterns of ribosomal genes in ant chromosomes have been under discussion. One of these hypotheses proposes a relationship between chromosomal location and the number of rDNA sites, suggesting that terminal locations facilitate the dispersion of rDNA clusters through ectopic recombination during meiosis, while intrachromosomal locations restrict them to a single chromosome pair. Another hypothesis suggests that the multiplication of rDNA sites could be associated with an increase in the chromosome number in Hymenoptera due to chromosomal fissions. In this study, we physically mapped rDNA sites in 15 new ant species and also reviewed data on rDNA available since the revision by Teixeira et al. (2021a). Our objectives were to investigate whether the new data confirm the relationship between chromosomal location and the number of rDNA sites, and whether the increase in the chromosome number is significant in the dispersion of rDNA clusters in ant karyotypes. Combining our new data with all information on ant cytogenetics published after 2021, 40 new species and nine new genera were assembled. Most species exhibited intrachromosomal rDNA sites on a single chromosome pair, while three species showed these genes in terminal regions of multiple chromosome pairs. On one hand, the hypothesis that the chromosomal location of rDNA clusters may facilitate the

dispersion of rDNA sites in the ant genome, as previously discussed, was strengthened, but, on the other hand, the hypothesis of chromosomal fission as the main mechanism for dispersion of ribosomal genes in ants is likely to be refuted. Furthermore, in certain genera, the location of rDNA sites remained similar among the species studied, whereas in others, the distribution of these genes showed significant variation between species, suggesting a more dynamic chromosomal evolution.

Keywords
Formicidae, FISH, karyotype, molecular cytogenetics, rDNA sites

Introduction

In Formicidae, molecular cytogenetic studies involving fluorescence *in situ* hybridization (FISH) for physical mapping of major ribosomal genes, 45S ribosomal DNA (rDNA), here referred to as rDNA clusters, were first conducted in Australian ants of the genus *Myrmecia* Fabricius, 1804 (Imai et al. 1992; Hirai et al. 1994, 1996). In recent years, FISH has been widely employed in several ant species, particularly in the Neotropical region (Santos et al. 2016; Aguiar et al. 2017; Micolino et al. 2019, 2022; Barros et al. 2021a, b, 2022a, b; Murakami et al. 2021; Silveira 2022; Teixeira et al. 2022, 2023; Jacintho et al. 2023). These molecular cytogenetic studies have provided valuable insights into various biological aspects of these insects, including evolution, taxonomy, and reproduction.

For instance, the physical mapping of ribosomal genes in certain ant genera has enabled the proposal of chromosomal rearrangements during their karyotypic evolution, such as the occurrence of inversions in *Myrmecia* (Hirai et al. 1996), *Dolichoderus* Lund, 1831 (Santos et al. 2016), *Mycetophylax* Emery, 1913 (Micolino et al. 2019), and *Acromyrmex* Mayr, 1865 (Barros et al. 2016; Teixeira et al. 2021a). Additionally, chromosomal polymorphisms involving the rDNA clusters, with homozygous and heterozygous karyotypes, which may arise from duplications/deletions due to unequal crossing-over or the formation of extrachromosomal circular DNA (eccDNA) have been observed in *Gnamptogenys regularis* Mayr, 1870 (Teixeira et al. 2020a) and *Odontomachus bauri* Emery, 1892 (Teixeira et al. 2021a). These may arise from duplications/deletions due to unequal crossing-over or the formation of extrachromosomal circular DNA (eccDNA) in these species. EccDNA can replicate via a rolling circle mechanism and then either get reintegrated into the genome or deleted from it, respectively causing duplications or deletions of these sequences (Teixeira et al. 2020a, 2021a).

Regarding ant taxonomy, mapping the chromosomal distribution of rDNA clusters has been important in helping to delimit specific boundaries between taxa, as is the case of the ants *Camponotus renggeri* Emery, 1894 and *Camponotus rufipes* (Fabricius, 1775) (Aguiar et al. 2017). These two species were subjects of discussion regarding taxonomic synonymization. However, the number of chromosomes bearing the rDNA clusters differs between them, with *C. rufipes* possessing one pair and *C. renggeri* possessing two pairs, a hereditary characteristic capable of distinguishing these two *Camponotus*

Mayr, 1861 species (Aguiar et al. 2017). Additionally, *C. rufipes* and *C. renggeri* differ in ecological, molecular, and behavioral traits, and this further confirms their status as valid species (Ronque et al. 2015).

Furthermore, cytogenetic data, including the chromosome location of rDNA sites, in the fungus-growing ant *Mycocepurus smithii* (Forel, 1893), have contributed to enhancing the understanding of cytological mechanisms associated with thelytokous parthenogenesis in this species (Barros et al. 2022a). Karyotypic variations were observed in the asexual population (2n=9, 10, and 11) with a decay of the diploid structure in the absence of meiosis and genetic recombination, whereas in the sexual population, the karyotype remained stable (2n=14) with appropriate homologous pairing. The data mapping of rDNA sites in *M. smithii* shows a single chromosome pair bearing these genes in the sexual population and in the karyomorphs 2n=9 and 2n=11 of the asexual population, supporting the idea that asexual individuals are indeed diploids. However, these data demonstrate the decay of the diploid structure, particularly in the 2n=11 karyomorph, in which there is a variation in size between the homologs of the pair bearing rDNA sites (Barros et al. 2022a).

Recently, based on new and previously published data regarding the chromosomal mapping of ribosomal genes from 63 species, 19 genera and six subfamilies of ants, Teixeira et al. (2021a) proposed important insights into the general patterns of these genes in ant chromosomes. These authors showed that rDNA clusters have a non-random distribution within the ant genome in which there is a relationship between chromosomal location and the number of rDNA sites. Most ant species have a single intrachromosomal (pericentromeric/interstitial) rDNA site, whereas species with multiple rDNA sites have these genes located in the terminal regions. Based on Hirai's model (2020), Teixeira et al. (2021a) argued that the terminal location of rDNA sites in ants would facilitate association with other non-homologous chromosome terminal sequences during the meiotic bouquet, forming affinity systems, which would lead to the occurrence of ectopic recombination and dispersion of rDNA clusters to other chromosomes. However, the intrachromosomal location of rDNA sites would hinder interaction with other chromosomes, restricting these genes to a single chromosome pair.

Alternatively, Menezes et al. (2021) proposed that the multiplication of rDNA sites could be linked to an increase of the chromosome number in most groups of Hymenoptera (ants, wasps and bees), suggesting that chromosomal fissions play a pivotal role in the dispersal of rDNA clusters in the karyotypes of these insects.

Despite notable advances in molecular cytogenetic data in ants, entire genera and even subfamilies have not yet been studied in this respect. Thus, in this study, we performed chromosomal mapping of ribosomal genes through FISH in 15 new ant species belonging to 9 genera, and also reviewed molecular cytogenetic data involving rDNA sites available since the paper by Teixeira et al. (2021a) was published. Our goal was to verify whether the chromosomal distribution of ribosomal genes in these ant species follows a relationship between the chromosomal location and the number of rDNA sites, and whether the increase in the chromosome number is significant in the dispersion of these genes in ant karyotypes.

Materials and methods

Field campaigns to collect ant colonies were performed in French Guiana and Brazil in regions of Amazonian and Atlantic rainforests (Table 1) from the following locations: Campus Agronomique, Kourou (5.17312°N, 52.65480°W) and Petit Saut route (5.13051°N, 52.94385°W), both in French Guiana; Oiapoque, Amapá State (3.84151°N, 51.84112°W) and Viçosa, Minas Gerais State (20.75696°S, 42.87314°W), both in Brazil. Sampling license in Brazil was provided by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (SISBIO accession numbers 87049-1). Adult specimens were deposited in the Coleção Entomológica do Laboratório de Coleoptera (CELC), at the Universidade Federal de Viçosa (UFV), Viçosa, Brazil.

In addition, for comparative purposes, we performed a survey of molecular cytogenetic data involving chromosomal mapping of ribosomal genes through FISH in ants since the last review by Teixeira et al. (2021a). The cytogenetic data are shown in Table 2 and the following traits were considered for each species: general number of chromosomes, number of chromosomes bearing rDNA clusters, and location of rDNA clusters on chromosomes.

For cytogenetic analysis, mitotic metaphase chromosomes were obtained from the cerebral ganglia of larvae after meconium elimination according to the methods described by Imai et al. (1988). The 18S rDNA probes were amplified via polymerase chain reaction (PCR) using primers 18SF1 (5`-TCATATGCTTGTCTCAAAG-3`) and 18SR1.1 (3`-TCTAATTTTTTCAAAGTAAACG-5`) designed for *Melipona quinquefasciata* Lepeletier, 1836 (Pereira 2006) in the genomic DNA from the ant *Camponotus rufipes*. Gene amplification was performed following Pereira (2006). The probes were labeled with digoxigenin-11-dUTP using Dig-Nick-Translation Mix (Roche Applied Science, Mannheim, Germany), and the FISH signals were detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol.

The rDNA sites were mapped on the chromosomes of Neotropical ant species using FISH according to Pinkel et al. (1986) with modifications described in Teixeira et al. (2021a): the slides were treated with RNase A (100 µg/ml) and kept in a moist chamber at 37 °C for 1 h. After that, they were washed in 2×SSC for 5 min, incubated in 5 µg/ml pepsin in 0.01 N HCl for 10 min, washed in 1× PBS for 5 min, and dehydrated in 50%, 70% and 100% alcohol series for 2 min each. After this pretreatment, metaphase chromosomes were denatured in 70% formamide/2×SSC at 75 °C for 5 min, and 20 µl of hybridization mix including 200 ng of labeled probe, 2×SSC, 50% formamide, and 10% dextran sulfate was denatured for 10 min at 85 °C and added on preparations. The slides were kept in a moist chamber up to 37 °C overnight. Then, the slides were washed in 2×SSC for 5 minutes; the detection solution including anti-digoxigenin-rhodamine was added on slides that were kept in a moist chamber at 37 °C for 1 h. The slides were washed three times in 4×SSC/Tween 20 (4×SSC, 0.05% [v/v] Tween 20) and dehydrated in an alcohol series. Finally, counterstaining with DAPI (DAPI Fluoroshield, Sigma Aldrich) was performed.

Slides subjected to FISH with the 18S rDNA probes were photographed using an epifluorescence microscope Olympus BX60 attached to an Olympus DP23M camera, and CellSens image capture software, using the filters WG (510–550 nm) for the rhodamine, and WU (330–385 nm) for DAPI. Images of the chromosomes were arranged using Adobe Photoshop® CS6. At least 20 metaphases for each species were analyzed to determine the FISH patterns.

Results

In this study, we physically mapped rDNA genes in the karyotypes of 15 species from 9 genera and 4 subfamilies (data for six genera have not been previously published) (Table 1). Among these species, 13 exhibit a single chromosomal pair bearing rDNA clusters, which are located in the pericentromeric region: in *Brachymyrmex admotus* Mayr, 1887 (Fig. 2A), *B. heeri* Forel, 1874 (Fig. 2B), *Nylanderia* sp. (Fig. 2D), *Cephalotes cordatus* (Smith, 1853) (Fig. 3A), *Cyphomyrmex laevigatus* Weber, 1938 (Fig. 3B), *Megalomyrmex* aff. *incisus* Smith, 1947 (Fig. 3D), *Pheidole jelskii* Mayr, 1884 (Fig. 3E), *P. vorax* (Fabricius, 1804) (Fig. 3F), *Strumigenys schulzi* Emery, 1894 (Fig. 3G), *Neoponera unidentata* (Mayr, 1862) (Fig. 4A), and *Pseudoponera stigma* (Fabricius, 1804) (Fig. 4B). In *Azteca andreae* Guerrero, Delabie et Dejean, 2010 (Fig. 1) and *Cephalotes minutus* (Fabricius, 1804) (Fig. 3C), the rDNA sites are located on the short arms. Furthermore, two species showed rDNA clusters on the short arms of more than one chromosome pair: *Camponotus cameroni* Forel, 1892 in two submetacentric and two subtelocentric pairs (Fig. 2C) and *Solenopsis saevissima* (Smith, 1855) in two submetacentric pairs (Fig. 3H).

Table 1. Ant species in which chromosomal mapping of rRNA genes was performed in this study, their respective localities and Brazilian biomes, and diploid chromosome numbers.

Ant species	Localities	Brazilian biomes	Chromosome numbers
Dolichoderinae			
Azteca andreae	Petit Saut route, French Guiana	Amazonian rainforest	2n=28
Formicinae			
Brachymyrmex admotus	Viçosa, MG, Brazil	Atlantic rainforest	2n=18
Brachymyrmex heeri	Oiapoque, AP, Brazil	Amazonian rainforest	2n=18
Camponotus cameroni	Viçosa, MG, Brazil	Atlantic rainforest	2n=36
Nylanderia sp.	Viçosa, MG, Brazil	Atlantic rainforest	2n=30
Myrmicinae			
Cephalotes cordatus	Oiapoque, AP, Brazil	Amazonian rainforest	2n=24
Cephalotes minutus	Kourou, French Guiana	Amazonian rainforest	2n=44
Cyphomyrmex laevigatus	Oiapoque, AP, Brazil	Amazonian rainforest	2n=14
Megalomyrmex aff. *incisus*	Oiapoque, AP, Brazil	Amazonian rainforest	2n=46
Pheidole jelskii	Oiapoque, AP, Brazil	Amazonian rainforest	2n=20
Pheidole vorax	Oiapoque, AP, Brazil	Amazonian rainforest	2n=42
Solenopsis saevissima	Viçosa, MG, Brazil	Atlantic rainforest	2n=32
Strumigenys schulzi	Oiapoque, AP, Brazil	Amazonian rainforest	2n=18
Ponerinae			
Neoponera unidentata	Oiapoque, AP, Brazil	Amazonian rainforest	2n=12
Pseudoponera stigma	Oiapoque, AP, Brazil	Amazonian rainforest	2n=14

Brazilian states: MG- Minas Gerais; AP - Amapá.

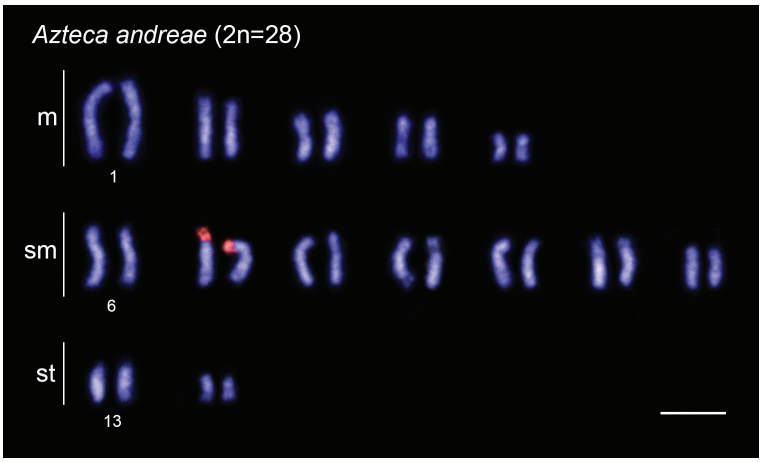

Figure 1. FISH with 18S rDNA probe (red signals) performed in ant *Azteca andreae* (Dolichoderinae). Scale bar: 5 μm.

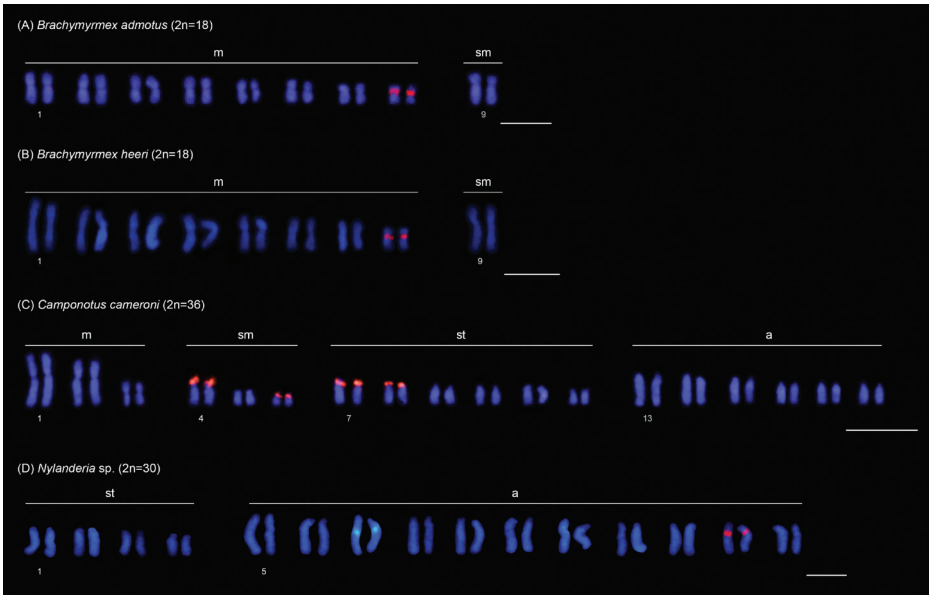

Figure 2. FISH with 18S rDNA probe (red signals) performed in different ant species of the subfamily Formicinae. Scale bars: 5 μm.

Chromosome mapping data from rDNA sites, which have been available since the review conducted by Teixeira et al. (2021a), encompassing 25 species across 10 genera (including three new genera), were reviewed (Table 2). This new information focused on ants from the Neotropics. However, some invasive populations of *Solenopsis invicta* Buren, 1972 from eastern Asia were also investigated. In most taxa, a single chromosomal pair bearing rDNA sites, located in the pericentromeric or interstitial regions, was observed. However,

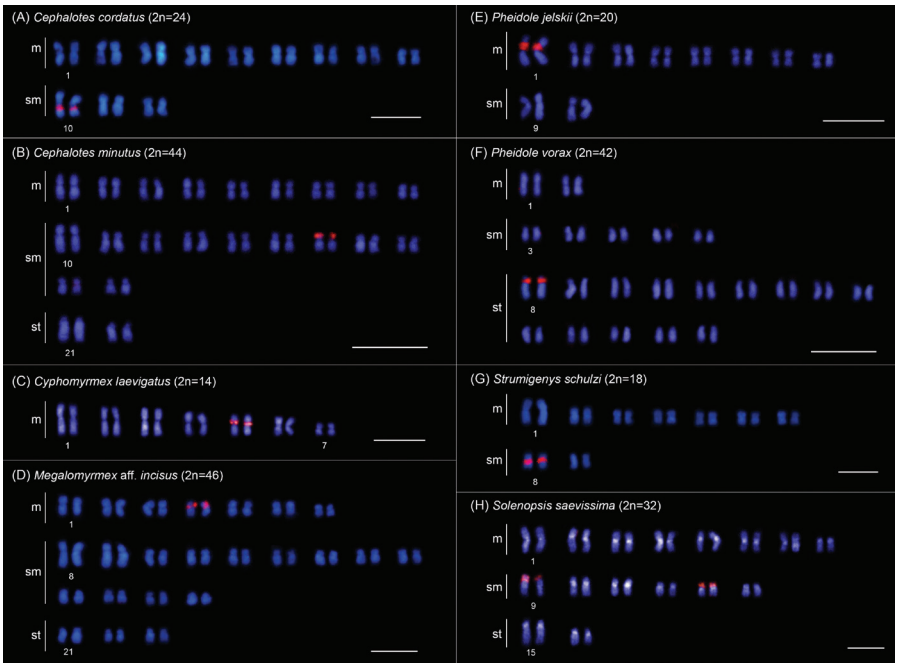

Figure 3. FISH with 18S rDNA probe (red signals) performed in different ant species of the subfamily Myrmicinae. Scale bars: 5 μm.

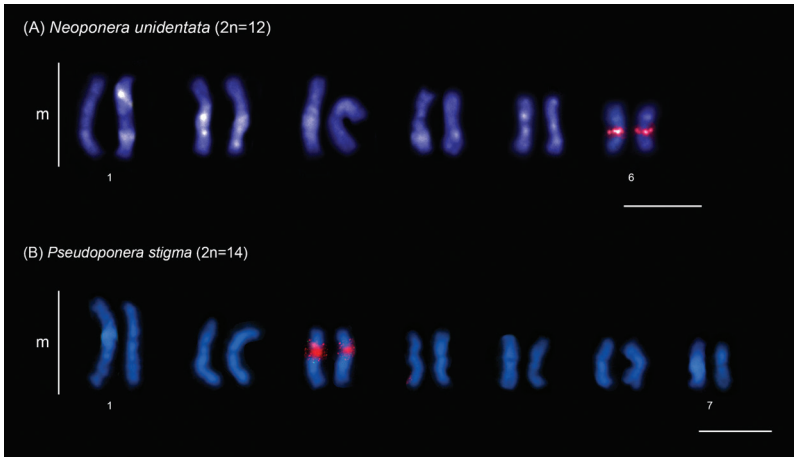

Figure 4. FISH with 18S rDNA probe (red signals) performed in different ant species of the subfamily Ponerinae. Scale bars: 5 μm.

populations of *S. invicta* exhibited multiple rDNA terminal sites: the native population from Argentina had two chromosome pairs carrying rDNA clusters, while invasive populations from Argentina, United States, and Taiwan showed large variation on this pattern, from 1 to 11 chromosomes carrying rDNA sites depending on the ploidy (Table 2).

Table 2. Summary of the available molecular cytogenetic data, including this study and published data after the revision by Teixeira et al. (2021a), concerning major rDNA clusters detected by FISH in ants.

Species	2n	rDNA cluster location		References
		Chromosome pair	Chromosome region	
Dolichoderinae				
Azteca andreae	28	2nd sm	short arm	**This study**
Technomyrmex vitiensis	16	m	pericentromeric	Barros et al. (2022b)
Formicinae				
Brachymyrmex admotus	18	8th m	pericentromeric	**This study**
Brachymyrmex heeri	18	8th m	pericentromeric	**This study**
Camponotus cameroni	32	4th sm, 6th sm, 7th st and 8th st	short arm	**This study**
Nylanderia sp.	30	10th a	pericentromeric	**This study**
Myrmicinae				
Acromyrmex ameliae	36	1st st	terminal	Barros et al. (2021b)
Acromyrmex balzani	38	1st st	short arm	Barros et al. (in press)
Acromyrmex brunneus	38	1st st	terminal	Barros et al. (in press)
Acromyrmex laticeps	38	1st st	terminal	Barros et al. (in press)
Acromyrmex subterraneus	38	1st st	terminal	Barros et al. (in press)
Amoimyrmex bruchi	22	2nd m	pericentromeric	Micolino et al. (2022)
Amoimyrmex silvestrii	22	2nd m	pericentromeric	Micolino et al. (2022)
Atta cephalotes	22	4th m	interstitial	Teixeira et al. (2022)
Cephalotes cordatus	24	1st sm	pericentromeric	**This study**
Cephalotes minutus	44	7th sm	short arm	**This study**
Crematogaster aff. *erecta*	28	3rd m	pericentromeric	Silveira (2022)
Crematogaster erecta cytotype I	22	2nd sm	interstitial	Silveira (2022)
Crematogaster erecta cytotype II	22	3rd m	pericentromeric	Silveira (2022)
Crematogaster limata	38	1st m	pericentromeric	Silveira (2022)
Crematogaster sp.	38	5th m	interstitial	Silveira (2022)
Crematogaster tenuicula	38	5th m	interstitial	Silveira (2022)
Cyphomyrmex laevigatus	14	5th m	pericentromeric	**This study**
Cyphomyrmex rimosus	22	3rd m	pericentromeric	Teixeira et al. (2023)
Cyphomyrmex transversus	18	2nd m	pericentromeric	Teixeira et al. (2021b)
Eurhopalothrix reichenspergeri	16	2nd m	terminal	Jacintho et al. (2023)
Megalomyrmex aff. *incisus*	46	4th m	pericentromeric	**This study**
Mycetomoellerius relictus	20	5th m	interstitial	Teixeira et al. (2021b)
Mycocepurus smithii	9	1st sm	interstitial	Barros et al. (2022a)
	11	1st sm	interstitial	
	14	1st sm	interstitial	
Pheidole jelskii	20	1st m	pericentromeric	**This study**
Pheidole vorax	42	1st st	pericentromeric	**This study**
Solenopsis invicta (native population from Argentina)	32	two chromosome pairs	short arm	Murakami et al. (2021)
Solenopsis saevissima	32	1st sm and 5th sm	short arm	**This study**
Strumigenys crassicornis	26	3rd m	interstitial	Jacintho (2023)
Strumigenys denticulata	18	2nd m	pericentromeric	Jacintho (2023)
Strumigenys louisianae	4	1st m	interstitial	Jacintho (2023)
	20	2nd m	pericentromeric	Barros et al. (2021b)
	26	4th m	interstitial	Jacintho (2023)
Strumigenys schulzi	18	3rd m	pericentromeric	**This study**
Strumigenys aff. *stenotes*	16	2nd m	interstitial	Jacintho (2023)
Strumigenys subedentata	18	3rd m	pericentromeric	Jacintho (2023)
Ponerinae				
Neoponera unidentata	12	6th m	pericentromeric	**This study**
Pseudoponera stigma	14	3rd m	pericentromeric	**This study**

Chromosomal classification: m – metacentric; sm – submetacentric; st – subtelocentric.

Discussion

Molecular cytogenetic data, which involve chromosomal mapping of rDNA clusters in ants, are now available for 103 species/subspecies, 28 genera and 6 subfamilies (this study, Table 2; reviewed in Teixeira et al. 2021a). Considering the number of chromosome pairs bearing rDNA clusters observed among the ant taxa analyzed since the review by Teixeira et al. (2021a) was published, 36 of them showed only a single pair bearing these genes while three species, namely *S. saevissima* and *S. invicta*, had two pairs, and *C. cameroni* had four pairs (Table 2). These patterns indicate that a diploid genome with a single chromosome pair carrying ribosomal genes should be considered an ancestral feature in Formicidae, as previously discussed by Teixeira et al. (2021a), and later suggested for the Hymenoptera in general (Menezes et al. 2021).

The rDNA physical mapping also strongly reinforces the relationship between the number of rDNA sites and their location discussed by Teixeira et al. (2021a) since the species *C. cameroni*, *S. saevissima*, and *S. invicta* have multiple rDNA sites located on short arms (including terminal regions) of the chromosomes while most other species studied show a single intrachromosomal rDNA site (Table 2). Based on the Hirai's model (2020), terminal location of rDNA sites in ants would facilitate association with other non-homologous chromosome terminal sequences during the meiotic bouquet, forming affinity systems, which would lead to the occurrence of ectopic recombination and dispersion of rDNA clusters to other chromosomes. Hirai's (2020) model for the dispersion of rDNA clusters in karyotypes does not address the presence of haploid males (haplodiploid reproductive system), such as those found in ants. We hypothesize that males could inherit rearrangements involved in the dispersal of rDNA clusters that occurred in the queens' genomes. Due to the haploid nature of hymenopteran males, the occurrence of ectopic recombination is restricted to females which are diploid. Therefore, it is possible that the evolution of repetitive DNA sequences (such as rDNA clusters), through the mechanisms described by Hirai (2020) may be slower in Hymenoptera when compared to other insect orders that reproduce in diplodiploid fashion.

Furthermore, our results seem to refute the hypothesis of chromosomal fission as the main mechanism for dispersion of ribosomal genes in ants proposed by Menezes et al. (2021) since: (i) species with the same chromosome number show differences in the number of rDNA sites, such as in *Solenopsis geminata* (Fabricius, 1804) (2n=32; one pair with rDNA), *S. saevissima* (2n=32; two pairs with rDNA) and the native population of *S. invicta* from Argentina (2n=32; two pairs with rDNA), in addition to *C. rufipes* (2n=40; one pair with rDNA) and *C. renggeri* (2n=40; two pairs with rDNA); (ii) in *Camponotus* Mayr, 1861, *C. cameroni* has 2n=36 chromosomes and four pairs bearing rDNA sites compared to other *Camponotus* species with 2n=40 chromosomes and only one or two pairs with rDNA clusters; (iii) in the fungus-growing ant *M. smithii*, asexual populations had a certain degree of relaxed chromosome stability (2n=9 and 11) when compared to sexual populations (2n=14) as discussed by Barros et al. (2022a), and still both populations had a single chromosome pair bearing the intrachromosomal rDNA sites; and (iv) in several ant genera studied, a wide variation in chromosome number is observed between species that present only a single pair with

rDNA sites, for example, *Cephalotes* Latreille, 1802 (2n=24 to 44), *Pheidole* Westwood, 1839 (2n=20 to 42), and *Strumigenys* Smith, 1860 (2n=4 to 40) (this study, Table 2, reviewed in Teixeira et al. 2021a). As discussed by Hirai (2020), centric fissions could generate chromosomes with very short heterochromatic arms (acrocentrics) and rDNA clusters in terminal/subterminal positions, which could facilitate affinity associations between these genes and other terminal chromosomal sequences promoting dispersion of rDNA sites in the karyotype of ant species.

For several ant genera studied, only single species have any kind of molecular cytogenetic data on rDNA clusters available, as in *Nylanderia* Emery, 1906, *Megalomyrmex* Forel, 1885, and *Neoponera* Emery, 1901 (this study, Table 2, reviewed in Teixeira et al. 2021a), which limits comparisons and in-depth discussion, however, these data are important to start understanding the chromosomal evolution of ribosomal genes in these genera. For other genera, such as *Brachymyrmex* Mayr, 1868, *Camponotus*, *Solenopsis* Westwood, 1840, *Pseudoponera* Emery, 1900, *Strumigenys*, *Cyphomyrmex* Mayr, 1862, *Pheidole*, and *Cephalotes* ribosomal gene mapping data is available for some species (this study, Table 2, reviewed in Teixeira et al. 2021a), which allowed interspecific comparisons and the observation of some karyotypic patterns, as discussed below.

rDNA cluster distribution patterns in the subfamily Dolichoderinae

The subfamily Dolichoderinae includes 22 genera and more than 900 species, grouped into four monophyletic tribes: Tapinomini, which is sister to the clade encompassing Bothriomyrmecini, Dolichoderini, and Leptomyrmecini (Ward et al. 2010). Data on the distribution of rDNA clusters in this subfamily are available for two species in Tapinomini and some species in Dolichoderini (reviewed in Teixeira et al. 2021a; Barros et al. 2022b). All these species had only one pair of chromosomes bearing rDNA sites.

In this study, we provide the first data for the arboreal ant genus *Azteca* Forel, 1878 (comprising 84 valid species, Bolton 2024), which is included in the tribe Leptomyrmecini. We performed the chromosomal mapping of rDNA sites through FISH in *A. andreae* (2n=28). However, location data of Nucleolar Organizer Regions (NORs), which include the major ribosomal genes (45S), obtained through Ag-NOR banding, are available for *A. trigona* Emery, 1893 (2n=28) (Cardoso et al. 2012). Although the karyotypes of these two *Azteca* species were organized according to different chromosomal classification systems, it is possible to observe that the data from FISH with rDNA probe in *A. andreae* and the Ag-NOR banding in *A. trigona* were similar with rDNA clusters located in the terminal region of the short arm of a medium-sized chromosome pair (this study, Cardoso et al. 2012). Regarding classical cytogenetic data, six *Azteca* species, including *A. andreae* in this study, had 2n=28 chromosomes, and only *A. alfari* Emery, 1893 had 2n=26 (Mariano et al. 2019; Barros et al. 2021c). Increasing the efforts to physically map rDNA clusters in other *Azteca* species may unveil variations in the location of rDNA sites, similarly to the observed chromosome number in *A. alfari*, thereby enhancing our understanding of karyotypic evolution in this genus.

rDNA cluster distribution patterns in the subfamily Formicinae

The subfamily Formicinae encompasses 52 genera and more than 3000 species, grouped into 11 monophyletic tribes, in which Myrmelachistini is sister to the clade that includes all other tribes (Ward et al. 2016). Chromosomal mapping data of rDNA clusters in the subfamily are available for some *Camponotus* species (Camponotini) and *Gigantiops destructor* (Fabricius, 1804) (Gigantiopini) (reviewed in Teixeira et al. 2021a). In this study, we present previously unknown data for the basal tribe Myrmelachistini, which includes *Brachymyrmex*, as well as for the tribe Lasiini, which contains *Nylanderia*.

A similar chromosomal distribution pattern of rDNA clusters has been observed in the two *Brachymyrmex* species, which showed these genes located in the pericentromeric region of the smaller metacentric pair. *Brachymyrmex* is composed of 40 species and has a challenging taxonomic history due to some morphological traits such as small body size (3 mm) and superficially similar external morphology among species (Ortiz-Sepulveda et al. 2019). To date, all the three studied taxa had the same karyotype with 2n=18 chromosomes, with rDNA sites mapped to the same regions and chromosomes (this study, Teixeira et al. 2020b). However, these cytogenetic data are limited, and an increase in the number of species studied using classical and molecular cytogenetic methods may reveal the putative presence of any derived lineage with chromosomal distinctions within *Brachymyrmex*.

In contrast, distinct patterns in the number and chromosomal location of rDNA sites were observed among *Camponotus* species included in the subgenus *Myrmobrachys* Forel, 1912, varying numbers of pairs bearing rDNA clusters were observed among the studied species: one pair in *C. rufipes* (2n=40), *C. atriceps* (Smith, 1858) (2n=40), and *C. cingulatus* Mayr, 1862 (2n=40), two pairs in *C. renggeri* (2n=40) (Aguiar et al. 2017; Teixeira et al. 2021a), and four pairs in *C. cameroni* (2n=36) (this study). The presence of multiple rDNA sites located in terminal regions in *C. renggeri* and *C. cameroni* may be associated with ectopic recombination, as discussed earlier.

rDNA cluster distribution pattern of the subfamily Myrmicinae

The subfamily Myrmicinae comprises 147 genera and over 7000 species, grouped into six monophyletic tribes, with Myrmicini being sister to the clade that includes other five tribes (Ward et al. 2015). This subfamily concentrates the largest number of cytogenetic data concerning the physical location of rDNA clusters, which are available for Attini, Crematogastrini, and Solenopsidini. Nearly all species exhibit only one chromosome pair carrying rDNA sites (reviewed in Teixeira et al. 2021a), except for populations of *S. invicta*, which have multiple terminal rDNA sites (Murakami et al. 2021). In this study, we present the first results for *Cephalotes* (Attini) and *Megalomyrmex* (Solenopsidini).

Within the fire ant genus *Solenopsis* (comprising more than 190 species, Bolton 2024), variations in the number of chromosome pairs carrying rDNA sites were observed. For instance, *S. geminata* (2n=32) exhibits one pair, while *S. saevissima* (2n=32) and the native population of *S. invicta* from Argentina (2n=32) possess two pairs (this study; Teixeira et al. 2021a; Murakami et al. 2021). Additionally, in invasive/established

populations of *S. invicta*, notable intraindividual chromosomal variations were observed concerning the number of chromosomes carrying rDNA sites and the ploidy in females and males. For example, females exhibit karyotypes with 1 to 11 chromosomes carrying rDNA sites, depending on their ploidy, while males show haploid to tetraploid karyotypes with 1 to 9 chromosomes carrying rDNA sites (Murakami et al. 2021). These authors suggest hybridization between invasive populations and closely related species, or between genetically distant populations, and/or the use of insecticides to control these ants and other insects as potential causes of these chromosomal variations observed in *S. invicta*. Studies conducted on populations of *S. saevissima* also reveal the presence of polyploid cells in immature stages, but reversal occurs in the final stages of development, suggesting some fitness advantage from the presence of polyploidy in immature stages, necessitating further investigation (Alves-Silva 2016; Andrade et al. 2023).

With many taxonomic issues, the speciose genus *Strumigenys* (with more than 850 species) is subdivided into several groups of species according to morphological traits (Bolton 2000). Chromosomal mapping data of rDNA sites are available for *S. schulzi* (2n=18) of the *schulz*i-group (this study), *S. diabola* Bolton, 2000 (2n=40) of the *mandibularis*-group (Teixeira et al. 2021a), *S. denticulata* Mayr, 1887 (2n=18), *S. subedentata* Mayr, 1887 (2n=18), *S. crassicornis* Mayr, 1887 (2n=26) and *S.* aff. *stenotes* (Bolton, 2000) (2n=16) from the *gundlachi*-group, and *S. louisianae* Roger, 1863 (2n=4, 20, 26) from the *louisianae*-group (Barros et al. 2021b; Jacintho 2023). These data show a notable variation in the karyotypic distribution pattern of these genes, even in closely related species. For example, *S. denticulata* and *S. subedentata* share the same chromosome number (2n=18); but differ in the chromosome pair bearing these genes, which is the second and third metacentric pair, respectively (Jacintho 2023). Furthermore, in *S. louisianae* three distinct karyotypes, differing in chromosome number and distribution of rDNA clusters, are observed in three different populations, reinforcing the existence of a species complex in this taxon (Barros et al. 2021b; Jacintho 2023). The variations in the location of rDNA sites among species indicate the intensity of the karyotype evolutionary dynamics, encompassing the rDNA regions. A deeper understanding of the evolutionary patterns of these genes in *Strumigenys* could be achieved in a species group context.

The occurrence of chromosomal rearrangements involving the rDNA region during karyotypic evolution in the fungus-growing ant genus *Cyphomyrmex* (comprising 23 valid species, Bolton 2024) has also been suggested (Teixeira et al. 2023). The data obtained in this study reinforce this hypothesis, since *C. laevigatus* Weber, 1938 (2n=14) showed rDNA sites on the short arm of the 4th metacentric pair, differing from the other two *Cyphomyrmex* species previously studied, *C. transversus* Emery, 1894 (2n=18), and *C. rimosus* (Spinola, 1851) (2n=24), where rDNA clusters are located on the short arm of the 2nd pair and the long arm of the 3rd pair, respectively (Teixeira et al. 2021b, 2023).

Pheidole is the most speciose ant genus (with more than 1100 species with worldwide distribution), which is subdivided into several species groups based on external morphology (Wilson 2003), and chromosomal mapping data of rDNA sites also show variations among the studied species. Within the *fallax* group, only *P. jelskii* (2n=20) of

this study has chromosomal distribution data for rDNA sites, which were located in the pericentromeric region of the largest metacentric pair. Conventional cytogenetics was performed on other species of this group, namely *Pheidole fallax* Mayr, 1870, *P. dentata* Mayr, 1886, *P. desertorum* Wheeler, 1906, *P. hyatti* Emery, 1895, and *P. nitidula* Emery, 1888, and all of them presented 2n=20 chromosomes which is the modal chromosome number among over 70 taxa studied within the genus, including representatives from the Old and New Worlds (reviewed in Lorite and Palomeque 2010 and Cardoso et al. 2018). Classic cytogenetic studies highlight size heteromorphism between the homologs of the largest chromosomal pair in *P. fallax*, *P. nitidula*, and *P. hyatti* (Goñi et al. 1983; Taber and Cokendolpher 1988). Heteromorphisms in the size of rDNA clusters are common in ants, and they can alter the size between homologs of the same chromosome pair (reviewed in Teixeira et al. 2021a). Thus, it is possible to hypothesize that the heteromorphism observed in *P. fallax*, *P. nitidula*, and *P. hyatti* may be related to the difference in size of rDNA clusters. If this is true, the location of the rDNA clusters in the largest chromosome pair in these three species would be similar to that observed in *P. jelskii* and could be the ancestral character in this group.

Furthermore, considering the *tristis* group of *Pheidole*, *P. vorax* of this study had 2n=42 chromosomes, with rDNA clusters located in the pericentromeric region of the short arm of the largest subtelocentric chromosome pair. Another species previously studied and included in the *tristis* group, namely *P. germaini* Emery, 1896, presented 2n=22 chromosomes, with rDNA sites located in the pericentromeric region of the only subtelocentric pair (Teixeira et al. 2021a). Conventional cytogenetics performed on *P. subarmata* Mayr, 1884 (cited as *P. cornutula* Emery, 1890) showed that this species has 2n=20 chromosomes and size heteromorphism between homologs of the largest chromosome pair (Goñi et al. 1983). Based on the discussion above, it is possible that the largest metacentric chromosome pair in *P. subarmata* (2n=20) may carry the rDNA clusters. Considering that this pattern may be the ancestral character in *Pheidole*, since the majority of studied species have 2n=20 chromosomes (reviewed in Lorite and Palomeque 2010 and Cardoso et al. 2018), we hypothesize that the occurrence of chromosomal fissions involving the chromosomal pair carrying rDNA clusters could give rise to the karyotypes in *P. germaini* (2n=22) and *P. vorax* with 2n=42 chromosomes, respectively, and rDNA clusters located in a subtelocentric pair (this study; Teixeira et al. 2021a).

The arboreal ant genus *Cephalotes* comprises 118 species (Bolton 2024) and according to the most recent molecular phylogeny of the genus, which includes 60% of its species, *C. cordatus* occupies a basal position, whereas *C. minutus* has a derived position (Price et al. 2022). The former species showed 2n=24 and rDNA sites located in the pericentromeric region of a larger metacentric pair, while the latter species presented 2n=44 and rDNA sites located in the short arm of a medium-sized submetacentric pair. The occurrence of fissions followed by tandem growth of heterochromatin apparently enhances telomeric stability, and therefore it could explain the increase in the chromosome number from 2n=24 to 2n=44, and inversions could change the chromosomal location of rDNA clusters from the pericentromeric region to the entire short arm.

rDNA cluster distribution patterns in the subfamily Ponerinae

The subfamily Ponerinae comprises 50 genera and over 1200 species, divided into two monophyletic tribes: Platythyreini, represented solely by *Platythyrea* Roger, 1863, and Ponerini, which includes all other genera (Schmidt 2013). Chromosomal mapping of rDNA clusters has been performed in some Ponerini species. Most of them exhibit only one chromosomal pair carrying rDNA sites, except for *Dinoponera gigantea* (Perty, 1833), which has multiple terminal rDNA sites (reviewed in Teixeira et al. 2021a). In this study, we provide new data for *Pseudoponera* and the first results on *Neoponera*.

Pseudoponera has six valid species (Bolton 2024), and *P. stigma* and *P. gilberti* (Kempf, 1960) are sympatric and share several morphological similarities. There are important examples of the usefulness of molecular cytogenetic data solving taxonomic challenges in ants (Aguiar et al. 2017). *P. stigma* (2n=14) and *P. gilberti* (2n=12) have different karyotypes (Correia et al. 2016), and the patterns of rDNA genes help to distinguish these two *Pseudoponera* species, because *P. stigma* have rDNA sites located in the pericentromeric region of the third metacentric pair (this study), while *P. gilberti* shows these genes located in the interstitial region of the largest metacentric pair (Teixeira et al. 2021a).

Conclusions

In summary, the molecular cytogenetic data from this study, as well as those available after the publication of the revision by Teixeira et al. (2021a), describe chromosome patterns for 40 new species and nine new genera. These new data strengthen the hypothesis suggesting that a single rDNA site per haploid genome represents the ancestral condition in ants. Furthermore, the data reinforce the observed non-random chromosomal distribution of ribosomal genes in Formicidae karyotypes, in which the chromosomal location (terminal or intrachromosomal) of these genes possibly influences the dispersion of rDNA sites in the ant genome. In certain genera, the location of rDNA sites in relation to which chromosomal pair carries the rDNA sites and whether it is located on the short or long arm, remained similar among the species studied, however in others, the distribution of these genes exhibited significant variation between species, suggesting a more dynamic chromosomal evolution. The expansion of molecular cytogenetic studies encompassing other ant subfamilies will continue to enhance our understanding of the chromosomal evolution of ribosomal genes in the genomes of these insects.

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ORCID

Monique Telcia dos Santos Damasceno https://orcid.org/0000-0003-0373-1389
Gisele Amaro Teixeira https://orcid.org/0000-0002-7106-5798
Paulo Castro Ferreira https://orcid.org/0009-0000-1640-9757
Rodrigo Batista Lod https://orcid.org/0000-0002-3710-5354
Luísa Antônia Campos Barros https://orcid.org/0000-0002-1501-4734
Hilton Jeferson Alves Cardoso de Aguiar https://orcid.org/0000-0001-7738-1460

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RESEARCH ARTICLE

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The first record of *Chironomus nuditarsis* Keyl, 1961 from Sevan Lake (Armenia) confirmed by morphology, karyotype and *COI* gene sequence

Viktor Bolshakov[1], Alexander Prokin[1], Elena Ivanova[2], Ekaterina Movergoz[1]

1 *Papanin Institute for Biology of Inland Waters Russian Academy of Sciences, Yaroslavl reg., Nekouz dist., Borok, 152742, Russia* **2** *Cherepovets State University, Lunacharski 5, Cherepovets 162600, Russia*

Corresponding author: Viktor Bolshakov (victorb@ibiw.ru)

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Abstract

Chironomus nuditarsis Keyl, 1961 is recorded from Sevan Lake for the first time. This species is widespread in Europe, the Caucasus, and Siberia. For species identification, we used a comprehensive approach that included morphological, cytogenetic and molecular genetic analyses. Morphological analysis showed a high similarity with the description. Nine chromosome banding sequences ndtA1, ndtA2, ndtB2, ndtC1, ndtD1, ndtE1, ndtF1, ndtG1, and ndtG2 were found. The banding sequences ndtA1, ndtA2, ndtG1, and ndtG2 are species-specific for *C. nuditarsis* and allow us to accurately distinguish it from the sibling species *Ch. curabilis* Belyanina, Sigareva et Loginova, 1990. Molecular-genetic analysis of the *COI* gene sequences has shown low genetic distances of 0.38–0.95% in the sibling species *Ch. nuditarsis* and *Ch. curabilis* complex and the insufficiency of using a single *COI* as a molecular marker for their separation.

Keywords

Barcode, *Chironomus nuditarsis*, *COI*, Diptera, karyotype, Lake Sevan

Introduction

The study of chironomids from Sevan Lake began in 1936 – 1938, when more than 1,500 midges and 220 larvae and pupae were collected (Pankratova et al. 1980). Initially, only three species from the genus *Chironomus* Meigen, 1803 were recorded from Sevan

Lake: *Ch. plumosus* (Linnaeus, 1758), *Ch. tentans* (Fabricius, 1805), and *Ch. thummi* Kief. (now *Ch. riparius* Meigen, 1804) (Fridman 1950; Sharonov 1951; Pankratova et al. 1980). Later, the list was expanded with new species: *Ch. markosjani* Shilova, 1983 (Shilova 1983), which is probably a sibling species to *Ch. annularius* Meigen, 1818 (Bolshakov et al. 2023), inhabiting depths of 10–20 m; *Ch. piger* Strenzke, 1959 and *Ch. dorsalis* Meigen, 1818 (Shilova and Zelentsov 1988); *Ch. bonus* Shilova et Dzhvarsheishvili, 1974 (Kiknadze et al. 2016); *Ch. pilicornis* Fabricius, 1787 (Shcherbina 2016).

In one of the samples from Sevan Lake, we found one *Ch. nuditarsis* Keyl, 1961 larva among other aquatic organisms. Unfortunately, we did not have the opportunity to collect more samples of this species. We regularly suggest to use a comprehensive approach to the species identification of the genus *Chironomus*, which includes morphology, cytogenetics and molecular genetics (Bolshakov and Movergoz 2022; Bolshakov et al. 2022a, 2022b). In this study, we have decided to use a single *Chironomus* larva to obtain a complex of scientific data.

Despite the long history of Sevan Lake *Chironomus* investigations, the list of species is still incomplete, and possibly the fauna is enriched with new species due to changing environmental conditions in the lake, as a result of climate change and human activity, such as water level regulation connected with electricity production in the Hrazdan River cascade of power plants, irrigation of agricultural lands, etc. (Hakobyan and Jenderedjian 2016). In this article, we have added the 8th species *Chironomus nuditarsis* Keyl, 1961 to the list of *Chironomus* of Sevan Lake. Previously, this species has already been recorded for Armenia from the Hrazdan River, Aghrlich (the Artashar Canal) (Petrova et al. 2011) without the karyotype data.

Dr. Fischer has done excellent work on the biology, physiology and genetics of *Ch. nuditarsis* (Rosin and Fischer 1966; Fischer 1969, 1974; Fischer and Rosin 1969; Fischer and Tichy 1980; Adamek 2020); thanks to the study of the biology of *Ch. nuditarsis*, it became possible to maintain a laboratory culture of this species (Fischer 1969). A detailed morphological description of the imago was later performed by Klotzli (Klotzli 1974).

The studied species *Ch. nuditarsis* has a wide Palearctic range; therefore it is a convenient object for studying the chromosomal divergence of populations and species (Polukonova et al. 2005a, 2005b; Kiknadze et al. 2006; Jabłońska-Barna and Michailova 2009; Kiknadze et al. 2016; Karmokov 2020). Chromosome maps of *Ch. nuditarsis* have been described for larvae collected in Germany by Keyl (Keyl 1961). In Caucasian populations, inversion polymorphism was observed for the majority of chromosomal arms, except for C and E (Karmokov 2020).

P. Michailova (Michailova 1989) found two karyological races in Bulgarian populations of *Ch. nuditarsis*, one with small centromeric bands and the other with large centromeric bands (Michailova 1989). Later it was found that the second karyological race, with large centromeric bands, corresponds to the species *Ch. curabilis* Belyanina, Sigareva et Loginova, 1990, which was originally described by morphological characteristics and the karyotype was mapped later (Beljanina et al. 1990) according to the Maximova system (Maximova 1976). Some authors argued that *Ch. nuditarsis* did not belong to any group according to morphological characters, although its position

on the tree constructed according to cytogenetic data indicated its high affinity to the *Ch. plumosus* group (Kiknadze et al. 2004). Thus, *Ch. nuditarsis* and *Ch. curabilis* were placed in the *Ch. nuditarsis* group of sibling species (Polukonova et al. 2005b). However, the results of molecular genetic analysis further supported the suggestion that both species belong to the *Ch. plumosus* group (Djomin 2011; Karmokov 2019; Bolshakov and Movergoz 2022; Bolshakov et al. 2022a) so both species are now considered members of the *Ch. plumosus* group.

The *COI* gene sequences for *Ch. nuditarsis* are known for only two regions, Germany and the United Kingdom; the species was identified by larval morphology and polytene chromosomes (Pfenninger et al. 2007), and by using only eDNA (Bista et al. 2017), respectively. Unfortunately, the length of the *COI* gene sequences of *Ch. nuditarsis* corresponding to Germany is only 416 nucleotides.

Traditionally, many samples of the *COI* gene sequence used for phylogenetic analysis were obtained using only one identification method based on imago or larva morphology, which can often lead to a misidentification, thus working with *COI* gene of chironomids requires an integrated approach (DeSalle et al. 2005; Ekrem et al. 2007; Kondo et al. 2016; Bolshakov and Movergoz 2022).

Occasionally, samples are received from locations that are difficult to access or infrequently visited, and there is no opportunity to return to collect the material. Consequently, every sample is of significant value, and we endeavor to obtain as much information as possible about each *Chironomus* larva. In this study, we demonstrate that even a single individual of *Ch. nuditarsis* from Lake Sevan can be studied using a comprehensive approach that includes morphological, cytogenetic, and molecular genetic methods.

Material and methods

One IV instar larva was found in Sevan Lake (Artanish Bay), Gegharkunik Province, Armenia (among other macroinvertebrates in the macrozoobenthos sample) on October 10, 2019. Coordinates – 40.462450, 45.355983. The depth at the sampling site was 1.3 m, and the bottom sediments were black silted gravel.

The larva was fixed in 96% ethanol. For morphological analysis, the head capsule of larva was mounted on a slide in the Fora-Berlese solution, the morphological terminology proposed by Sæther (Sæther 1980) was used. The age was determined by the standard method (Ilyinskaya 1983). The salivary glands were extracted through an incision (1–3 body segments) with thin preparation needles. Karyotype analysis was performed using the ethanol-orcein method (Dyomin 1989). The specimens were analyzed with a light microscope (Micromed-6 LOMO, St-. Petersburg, Russia) with an objective of ×100 and a digital camera (ToupCam 5.1., ToupTek Photonics, Hangzhou, China), and with a light stereomicroscope (Olympus CX43 with digital camera DP23, Olympus, Tokyo, Japan). For identification of chromosome banding sequences previously published cytomaps were used (Kiknadze et al. 2006, 2016; Karmokov 2020). Arms A, E and F were mapped in the system of Keyl (Keyl 1962), and arms C and D – in the system of

Dévai et al. (Dévai et al. 1989). The morphological preparation of the head capsule and cytological preparation are deposited in the collection of Papanin Institute for Biology of Inland Waters Russian Academy of Sciences, Russia, Borok (IBIW).

DNA extraction was performed by the "M-sorb-OOM" (Sintol, Moscow) kit with magnet particles according to manufacturer's protocol. For amplification of *COI* (cytochrome oxidase subunit I) primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA -3') were used (Eurogen, Moscow) (Folmer et al. 1994). Amplification reaction was carried out in 25 µl reaction mixture (1× buffer, 1.5 µM MgCl2, 0.5 mM of each primer, 0.2 µM dNTP of each nucleotide, 17.55 µL deionized water, 1 µL template DNA, 1 unit Taq-polymerase (Evrogen, Moscow). PCR was performed at 94 °C (3 min), followed by 30 cycles at 94 °C (15 s), 50 °C (45 s), 72 °C (60 s) and a final at 72 °C (8 min). PCR products were visualized in1% agarose gel and purified with ethanol and ammonium acetate (3 M). Both strands were sequenced on an Applied Biosystems 3500 DNA sequencer (Thermo Scientific, USA) following the manufacturer's instructions.

For alignment of *COI* nucleotide sequences we used MUSCLE in the MEGA6 software (Tamura et al. 2013). Pairwise genetic distances were calculated in the MEGA6 software using Kimura 2-parameter (K2P) with codon position preferences: 1st, 2nd, 3rd and noncoding sites. The program MrBayes v.3.2.6 was used for the Bayesian analysis (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) with previously suggested settings (Karmokov 2019; Bolshakov et al. 2022a), for 1 000 000 iterations and 1000 iterations of burn-in, nst = 6 (GTR + I + G). The phylogenetic trees resulting from Bayesian inference analyses were visualized and edited using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). For the estimation of the number of haplotypes we used DNA SP v.6 software (Librado and Rozas 2009), and to create a network of haplotypes we used PopArt 1.7 software, with the Median Joining algorithm (Leigh and Bryant 2015).

In addition, thirty one *COI* gene sequences of the genus *Chironomus* from GenBank were analyzed. Accession numbers of used sequences in GenBank: *Ch. acutiventris* Wülker, Ryser et Scholl, 1983 (AF192200), *Ch. annularius* Meigen, 1818 (AF192189), *Ch. anthracinus* Zetterstedt, 1860 (KF278222), *Ch. balatonicus* Dévai, Wülker et Scholl, 1983 (JN016826), *Ch. bernensis* Klötzli, 1973 (AF192188), *Ch. borokensis* Kerkis, Filippova, Shobanov, Gunderina et Kiknadze, 1988 (AB740261), *Ch. cingulatus* Meigen, 1830 (AF192191), *Ch. commutatus* Keyl, 1960 (AF192187), *Ch. curabilis* Belyanina, Sigareva et Loginova, 1990 (MT535221, JN016811, KX118693), *Ch. entis* Shobanov, 1989 (AF192195), *Ch. heteropilicornis* Wülker, 1996 (MK795770), *Ch. luridus* Strenzke, 1959 (AF192203), *Ch. maturus* Johannsen, 1908 (DQ648204), *Ch. melanescens* Keyl, 1961 (MG145351), *Ch. melanotus* Keyl, 1961 (OL546775), *Ch. novosibiricus* Kiknadze, Siirin et Kerkis, 1993 (AF192197), *Ch. nuditarsis* Keyl, 1961 (KY225345), *Ch. pallidivittatus* Malloch, 1915 (AF110164), *Ch. piger* Strenzke, 1959 (AF192202), *Ch. pilicornis* Fabricius, 1787 (HM860166), *Ch. plumosus* Linnaeus, 1758 (AB740263), *Ch. riparius* Meigen, 1804 (KR756187), *Ch. sokolovae* Istomina, Kiknadze et Siirin, 1999 (MW471100), *Ch. sororius* Wülker, 1973 (MZ324811),

Ch. tentans Fabricius, 1805 (AF110157), *Ch. tuvanicus* Kiknadze, Siirin et Wülker, 1993 (AF192196), *Ch. usenicus* Loginova et Belyanina, 1994 (JN016806), *Ch. whitseli* Sublette et Sublette, 1974 (KR683438). Species *Drosophila melanogaster* Meigen, 1830, Drosophilidae (HQ551913) was used as an out-group in phylogenetic analysis.

In order not to miss the details, we used all 25 available *COI* gene sequences of *Ch. nuditarsis* and *Ch. curabilis* from the GenBank database, including short ones (416 bp). *Ch. nuditarsis*: Germany (DQ910577, DQ910575, DQ910573, DQ910568, DQ910574, DQ910576, DQ910569, DQ910570, DQ910567, DQ910579, DQ910578, DQ910572, DQ910571). *Ch. curabilis*: Montenegro (MT535377, MT535005, MT534682, MT534976), Germany (OP927609, OP927503, OP927448, OP927684, OP927470, OP927434) and Russia, Saratov reg. (JN016810, JN016812).

Study area

Sevan Lake is the largest freshwater lake in Armenia and the Caucasus region; it is located in the northern part of the Armenian Volcanic Highland at an altitude of approximately 1,900 meters above sea level, with a surface of 1278.04 km^2 (Hakobyan and Jenderedjian 2016; Krylov 2016; Jenderedjian et al. 2019).

We suggest that in the future, knowledge of the vegetation at chironomid larvae collection sites may help to reveal the relationship with species richness. The study of the aquatic vegetation of Sevan Lake is conducted regularly; *Cladophora glomerata* (L.) Kütz., *Myriophyllum spicatum* L. were registered in sampling site. The composition of the aquatic core of the flora also includes: *Vaucheria dichotoma* (L.) Mart., *Drepanocladus aduncus* (Hedw.) Warnst., *Butomus umbellatus* L., *Potamogeton pectinatus* L., *P. perfoliatus* L., *Ceratophyllum demersum* L., *Lemna gibba* L., *L. trisulca* L., Salix elbursensis Boiss, *Schoenoplectus tabernaemontani* (C.C.Gmel.) Palla, *Typha angustifolia* L., *Butomus umbellatus* L., *Phragmites australis* (Cav.) Trin. ex Steud. (Arnoldi 1929; Barsegyan 1975; Tsaturyan et al. 1985; Bobrov 2016).

Results

Morphological characters of *Ch. nuditarsis* from Sevan Lake

The morphological characteristics of the 4th instar larva are shown in Fig. 1. The body length was about 15 mm. The length of the ventral tubules exceeds the length of the posterior parapods. The head capsule was yellow or light brown (Fig. 1a). The gular spot has blurred borders. The exterior tooth of the premandible is significantly narrower (3 times) than the inner tooth (Fig. 1b). All four teeth of the mandible are dark brown (Fig. 1c). The mentum is black-brown; the fourth tooth of the mentum is not lower than the neighboring teeth (Fig. 1d). The basal segment of the antenna is cone-shaped; the antenna blade is extended above the fifth segment (Fig. 1e). Ventromental plates have a flat front edge and without a wrinkly surface (Fig. 1f).

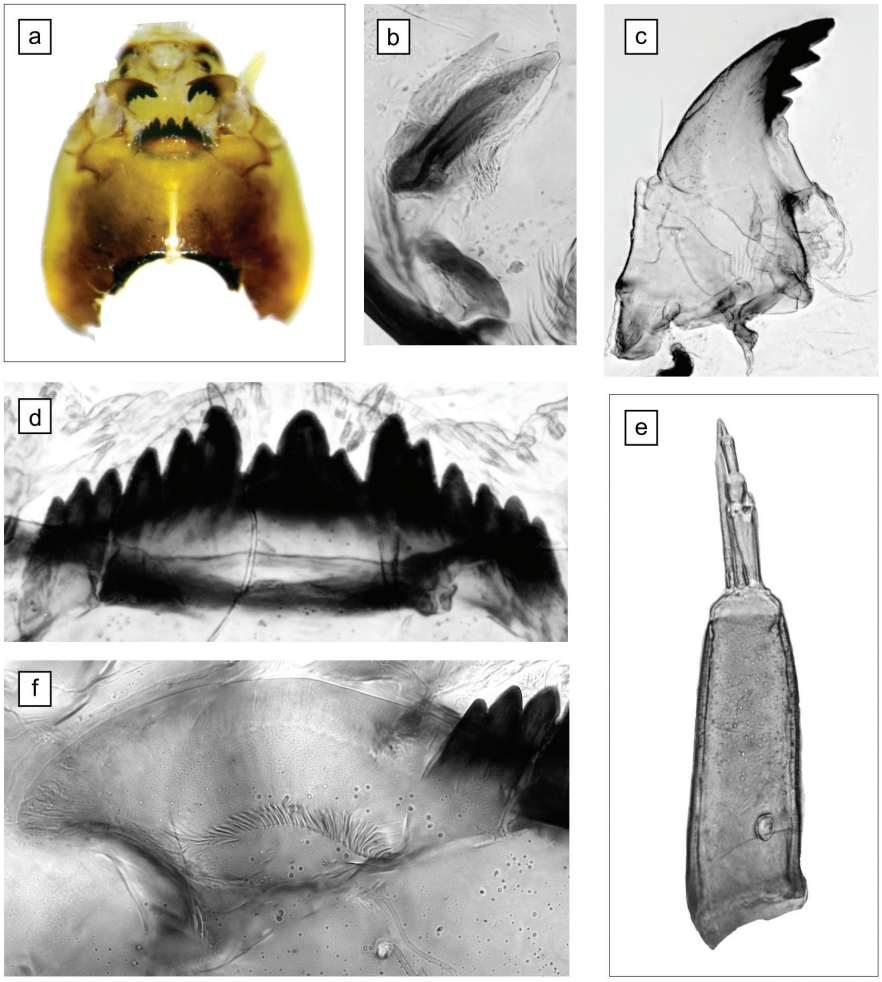

Figure 1. Larva morphology of *Ch. nuditarsis* from Sevan Lake, Armenia **a** head capsule, ventrally **b** premandible **c** mandible **d** mentum **e** antenna **f** ventromental plate. Scale bar: 100 μm.

The measurement results: length of the first antennal segment (L1) - 141 μm, length of the second segment (L2) - 37 μm, width of the first segment (W1) - 49 μm; the distance of the ring organ from the base of the first antenna segment - 43 μm; mental size (MS), the distance between the first lateral teeth - 91 μm; number of epipharyngeal teeth – 14.

Karyotype of *Chironomus nuditarsis* from Sevan Lake

The species has a 2n = 8 set of chromosomes. The chromosome arm combination is AB CD EF G ("thummi" cytocomplex). The chromosomes AB and CD are metacentric, EF is submetacentric, short G is telocentric. Balbiani rings are located in arms B and G, nucleolus is located in arm G (Fig. 2).

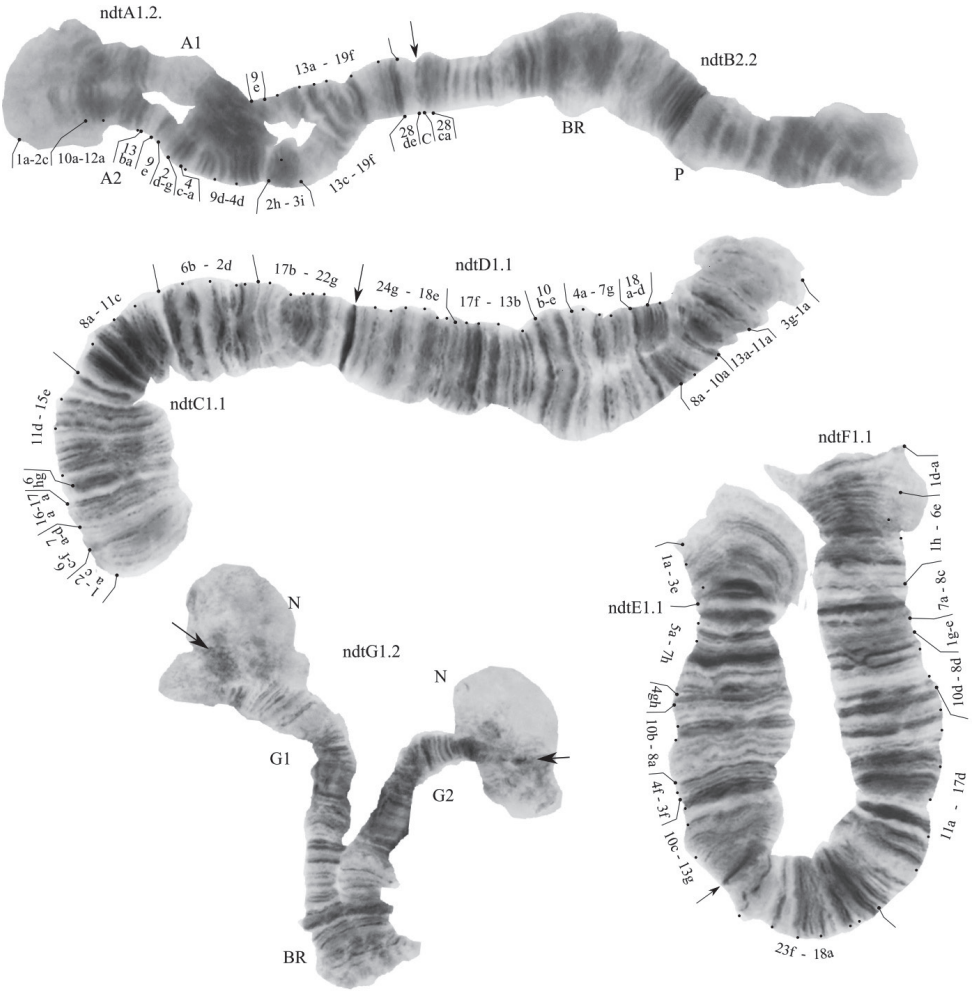

Figure 2. Karyotype of *Ch. nuditarsis* from Sevan Lake, Armenia. Arrows indicate centromeric bands; ndtA1.2, ndtB2.2., etc., genotypic combinations of banding sequences of chromosome arms; BR, Balbiani rings; N, nucleolus.

One zygotic combination was found: ndtA1.2.B2.2.C1.1.D1.1.E1.1.F1.1.G1.2. All nine banding sequences in our study were previously described from different populations (Michailova et al. 2002; Polukonova 2005b; Kiknadze et al. 2006, 2016; Karmokov 2020).

Arm A. Two banding sequences: ndtA1 1a-2c 10a-12c 3i-2h 4d-9d 4a-c 2g-d 9e 13a-19f [28de] C. in heterozygous state with ndtA2 1a-2c 10a-12a 13ba 9e 2d-g 4c-a 9d-4d 2h-3i 12cb 13c-19f [28de] C.

Arm B. One banding sequence: ndtB2, not mapped.

Arm C One banding sequence: ndtC1 1a-2c 6c-f 7a-d 16a-17a 6hg 11d-15e 8a-11c 6b-2d 17b-22g C.

Arm D. One banding sequence: ndtD1 1a-3g 11a-13a 10a-8a 18d-a 7g-4a 10e-b 13b-17f 18e-24g C.

Arm E. One banding sequence: ndtE1 1a-3e 5a-7h 4gh 10b-8a 4f-3f 10c-13g C.

Arm F One banding sequence: ndtF1 1a-d 6e-1h 8c-7a 1e-g 8d-10d 17d-11a 18a-23f C.

Arm G. Two banding sequences: ndtG1, not mapped and ndtG2,not mapped.

Ch. nuditarsis has many banding sequences similar to *Ch. curabilis*; in *Ch. nuditarsis* from Sevan Lake we have found four banding sequences which are considered specific for *Ch. nuditarsis*: ndtA1, ndtA2, ndtG1 and ndtG2 (Polukonova et al. 2005b).

COI gene sequences and phylogenetic analysis of *Ch. nuditarsis* from Sevan Lake

The obtained *COI* gene sequence of *Ch. nuditarsis* from Sevan Lake was deposited in GenBank with the accession number - OR652398, length - 658 bp. Percentage of nucleotides A: 26; T: 37; G: 17; C: 20.

We found one *COI* gene sequence length of 608 bp in the GenBank that belongs to *Ch. nuditarsis* (KY225345), other sequences had a length of 416 bp, and this decreased the accuracy of the analysis. The minimum genetic distances were between *COI* gene sequences *Ch. nuditarsis* (OR652398) from Sevan Lake and *Ch. curabilis* (KX118693) from Iran – 0.49%, and *Ch. curabilis* (JN016811) from Saratov reg. (Russia) – 0.73% (Table 1). The genetic distance between *COI* gene sequences of *Ch. nuditarsis* (OR652398) from Sevan Lake and *Ch. nuditarsis* (KY225345) from the United Kingdom was 0.98%. Another low genetic distance of 1.23% was found between *Ch. nuditarsis* (OR652398) from Sevan Lake and *Ch. curabilis* (MT535221) from Skadar Lake in Montenegro. Almost all the estimated distances between sequences of *Ch. nuditarsis* and *Ch. curabilis* were less than the accepted interspecific threshold value of 3% (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016), the only exception is the distance of 3.75% between *Ch. nuditarsis* (DQ910574) from Germany and *Ch. curabilis* (JN016812) from Saratov region (Russia). The distances between sequences of *Ch. nuditarsis* and other species from the *Ch. plumosus* group varied from 4.01 to 8.54% (Table 1), exceeding the interspecific threshold value of 3% (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016).

COI gene sequences of *Ch. nuditarsis* (OR652398) from Sevan Lake form a single cluster with *Ch. curabilis* from Iran (KX118693), Russia (JN016811) and Montenegro (MT535221), and *Ch. nuditarsis* (KY225345) from the United Kingdom (Fig. 3). It can be assumed that only the samples of *Ch. curabilis* (JN016811, JN016812) from Saratov region (Russia) were identified by cytogenetics (Polukonova et al. 2009), the others by morphology (Chimeno et al. 2023), or only by *COI* gene sequence (Bista et al. 2017). All short sequences of *Ch. nuditarsis* and *Ch. curabilis* were combined into one large cluster; we do not consider it fitting to give this figure here. All short sequences of *Ch. nuditarsis* and *Ch. curabilis* were used to create a haplotype network.

Table 1. The pairwise genetic distances (Kimura-2p) between *COI* gene sequences of *Chironomus* species.

No.	1	2	3	4	5	6	7	8	9	10	11
	Ch. nuditarsis **(OR652398) Sevan Lake**	***Ch. nuditarsis*** **(KY225345) United Kingdom**	***Ch. nuditarsis*** **(DQ910574) Germany**	***Ch. curabilis*** **(KX118693) Iran**	***Ch. curabilis*** **(JN016811) Saratov reg. Russia**	***Ch. curabilis*** **(MT535221) Montenegro**	***Ch. curabilis*** **(JN016812) Saratov reg. Russia**	***Ch. usenicus*** **(JN016806) Russia**	***Ch. plumosus*** **(AB740263) Russia**	***Ch. entis*** **(AF192195) Russia**	***Ch. borokensis*** **(AB740261) Russia**
1											
2	0.98										
3	2.23	2.23									
4	0.49	0.98	2.22								
5	0.73	1.23	2.99	1.23							
6	1.23	0.73	2.48	1.23	1.48						
7	1.47	1.97	3.75	1.97	0.73	2.22					
8	4.27	4.01	5.84	4.53	4.26	3.75	5.04				
9	4,53	4,26	6,10	4,78	4,52	4,52	5,30	0,73			
10	6,37	6,10	8,54	6,36	6,09	6,36	6,89	5,06	5,85		
11	5,86	5,59	7,47	6,12	5,85	5,85	6,65	3,00	3,25	7,50	

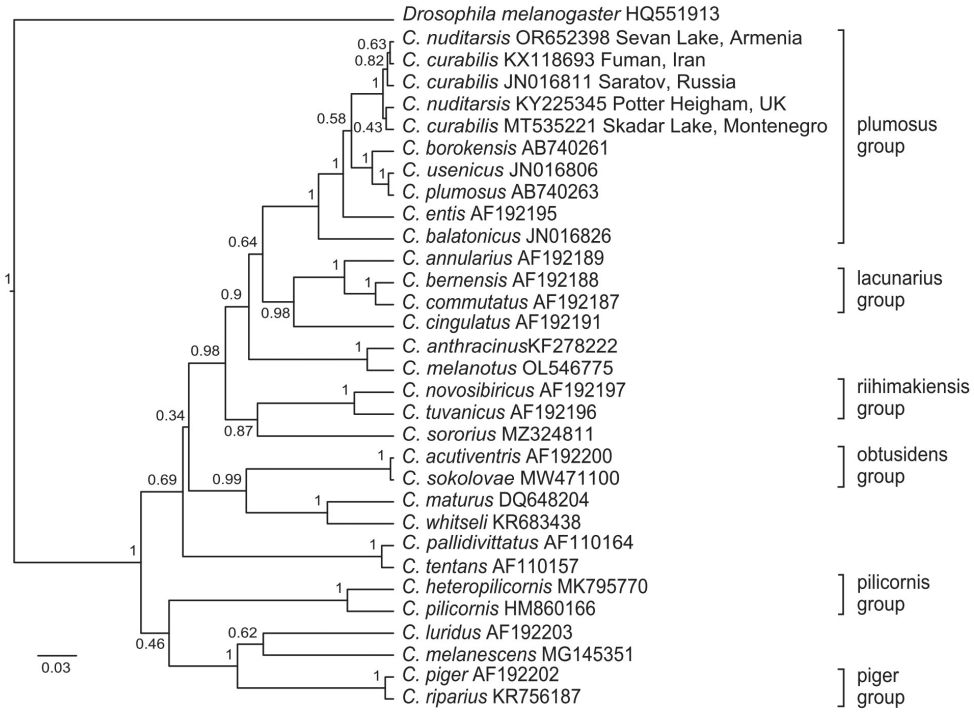

Figure 3. Bayesian tree of the analyzed samples of *Chironomus* spp., inferred from *COI* gene sequences. Species name, GenBank accession numbers and group name are shown to the right of the branches. Support p-values are given if they exceed 0.3.

The resulting haplotype network of *Ch. nuditarsis* and *Ch. curabilis* has a quite complex structure and consists of 21 haplotypes (Fig. 4). *COI* gene sequences of both species have the same haplotypes. Haplotype 2 consists of *Ch. nuditarsis* (KY225345) from the United Kingdom and *Ch. curabilis* (OP927684) from Germany. Haplotype 6 includes *COI* gene sequences of *Ch. nuditarsis* (DQ910568, DQ910570) and *Ch. curabilis* (OP927609, OP927448, OP927470, OP927434) from Germany. Haplotype 8 includes sequences of *Ch. nuditarsis* (DQ910576) and *Ch. curabilis* (OP927503) from Germany, and *Ch. curabilis* (MT535377) from Montenegro. The maximum number of mutation steps (20) was found between *Ch. curabilis* (JN016812) from Saratov region, Russia, and *Ch. nuditarsis* (DQ910574) from Germany. The haplotype of *Ch. nuditarsis* (OR652398) from Sevan Lake is separated from *Ch. nuditarsis* (DQ910569) from Germany and *Ch. curabilis* (KX118693) from Iran by three mutation steps.

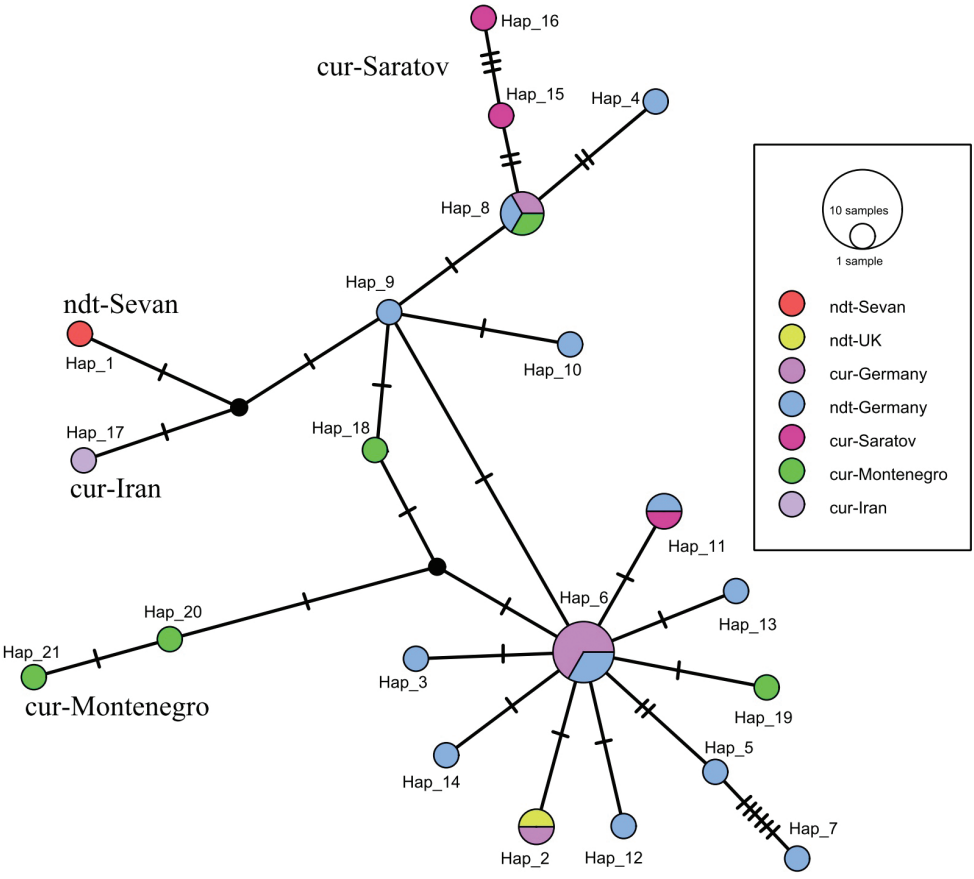

Figure 4. Median Joining Network showing phylogenetic relationships within *Ch. nuditarsis* and *Ch. curabilis* species. Each bar represents a single mutational change. The diameter of the circles is proportional to the number of individuals in each haplotype sampled (see open circles with numbers). Black dots represent hypothetical intermediate haplotypes. Sevan, Saratov, etc. – names of localities.

Discussion

Hydrobiological and entomological investigations, including studies of chironomids, are performed regularly in Lake Sevan (Pankratova et al. 1980; Shilova 1983; Shilova and Zelentsov 1988; Shcherbina and Zelentzov 2011; Hakobyan and Jenderedjian 2016; Shcherbina 2016). As a result of that long-term study, a total of 8 species of the genus *Chironomus* are known today from the lake, but some of them still need confirmation using a comprehensive approach.

In Artanish Bay, the locality of our material of *Ch. nuditarsis*, previously only *Ch. tentans* was recorded (Shilova and Zelentsov 1988). We assume that species *Ch. nuditarsis* has not been found for a long time previously due to the high diversity of habitat conditions in Sevan Lake (Pavlov et al. 2010; Hakobyan and Jenderedjian 2016; Krylov 2016; Jenderedjian et al. 2019), and the possible changing of the fauna. Incredibly, chironomids were found at a depth of up to 63 meters in 2015, because of the relatively high concentration of dissolved oxygen in this period in the profundal zone (Hakobyan and Jenderedjian 2016).

During the course of preparing this article, we encountered some confusion regarding the author of the name for the species in question. The author of the species, at the first mention (Keyl 1961), attributes name authorship to Strenzke (Strenzke 1959), who in reality did not use such a name in the cited publication. In addition, Dr. Fischer's articles indicate the species name as *Ch. nuditarsis* Str., yet the year is not specified (Fischer 1969, 1974; Fischer and Rosin 1969). The publication that provides a detailed description of the imago indicates *Ch. nuditarsis* Keyl, 1961 (Klotzli 1974). In a comprehensive article on the morphological characteristics of *Chironomus* larvae, this species is listed as *Ch. nuditarsis* Keyl, 1961 (Webb and Scholl 1985). In Polukonova (Polukonova 2005a) two alternative names are used, where different years of description are indicated *Ch. nuditarsis* Keyl, 1962 and *Ch. nuditarsis* Str. (Keyl, 1961) (Polukonova 2005a). Later, in an article by Jabłońska-Barna and Michailova, the species was named *Ch. nuditarsis* Strenzke, 1959 (Jabłońska-Barna and Michailova 2009). In recent works by Kiknadze et al. (Kiknadze et al. 2016) and Karmokov (Karmokov 2020) the species is named *Ch. nuditarsis* Keyl, 1961. To sum up, the work of Keyl (Keyl 1961) should be accepted as the first mention and original description of the species.

The morphological characteristics of the larva we found correspond to the description of *Ch. nuditarsis* (Webb and Scholl 1985; Polukonova 2005a) and differs from the sibling species of *Ch. curabilis*. The head capsule of *Ch. nuditarsis* is darker (dark yellow or brown), the gular spot has blurred borders, in *Ch. curabilis* the head capsule is yellow and has a clear gular spot (Webb and Scholl 1985; Polukonova 2005a). In *Ch. nuditarsis*, the basal segment of the antenna is cone-shaped; the antenna blade is extended beyond the fifth segment; this is well illustrated in Fig. 1e. In *Ch. curabilis*, the basal segment of the antenna is cylindrical; the antenna blade reaches the base of the fifth segment (Webb and Scholl 1985; Polukonova 2005a). The measurements of the most significant morphological characteristics also demonstrated a match with the description of *Ch. nuditarsis* (Webb and Scholl 1985; Polukonova 2005a).

The cytogenetic analysis of *Ch. nuditarsis* in Caucasian populations demonstrated a high diversity of chromosome banding sequences (Kiknadze et al. 2006; Karmokov 2020), explained by the authors as a result of high diversity of habitat conditions. Some chromosome banding sequences registered in Sevan Lake are rare among Caucasian populations. Heterozygote ndtA1.2 in the populations of the Central Caucasus was found only in two localities of Kabardino-Balkaria, with the maximum frequency of occurrence of 1.1–1.8%, but it is common in the populations of the Northwestern Caucasus and Europe, with the frequency of occurrence of about 30–40% (Karmokov 2020). In former studies of *Ch. nuditarsis* in Caucasian populations the ndtA2 banding sequence was not observed at all (Polukonova and Karmokov 2013). Homozygote ndtB2.2 is common for Siberian populations and occurs in all individuals; it is rare in Central Caucasus and European populations and more common in the northwest and east Caucasus (Kiknadze et al. 2006; Karmokov 2020). Heterozygote ndtG1.2 is common in European and Caucasian populations, but not in Siberian populations (Kiknadze et al. 2006; Polukonova and Karmokov 2013; Karmokov 2020). It is noted that the frequency of occurrence of zygotic combination ndtG1.2 may depend on the altitude of the habitat, it is more common in the plains, but rare in the highlands (Polukonova and Karmokov 2013). In addition, the zygotic combinations ndtB2.2 and ndtG1.2 do not follow the Hardy-Weinberg expectation in some Caucasian populations (Karmokov 2020). Based on the obtained data, we can assume that the combination of chromosome banding sequences of the Sevan Lake larva might be more similar to the northwest Caucasian population.

Previously, the presence of two karyological races was noted in Bulgarian populations of *Ch. nuditarsis*, differing in the size of the centromeric region (Michailova 1989). Later, the karyoform with large centromeric bands was associated with the species *Ch. curabilis* (Polukonova et al. 2005b). As noted earlier, karyoforms with large centromeric bands are not found in the Caucasian populations (Karmokov 2020), and the larvae we found in Sevan Lake also has thin centromeric bands. These two species, *Ch. nuditarsis* and *Ch. curabilis*, have many similar chromosome banding sequences (Polukonova et al. 2005b), which is also characteristic of many species of the genus *Chironomus* (Kiknadze et al. 2006, 2008, 2016). It is known that banding sequences ndtA1, ndtA2, ndtG1, and ndtG2 are specific for *Ch. nuditarsis* (Polukonova et al. 2005b), which confirms the accuracy of the identification of the species in this publication.

The estimated genetic distances between *COI* gene sequences of *Ch. nuditarsis* and *Ch. curabilis* were less than the accepted 3% threshold (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016), only in one case, *Ch. nuditarsis* (DQ910574) from Germany and *Ch. curabilis* (JN016812) from Saratov region (Russia), the distance was 3.75% (Table 1).

To make sure that the species is correctly identified, we studied the publications with available data on the locality and methods for species identification. In the case of *Ch. curabilis* (KX118693) from sediments of Anzali Wetland, Iran, the species was probably identified by the morphology of the larva as the authors usually indicate that they used genus identification without the need for routine mounting of larvae (Salehzadeh et al. 2019). Samples of *Ch. nuditarsis* (DQ910569 – DQ910579) collected on the Rhine River plain in southwestern Germany were identified cytogenetically

(Pfenninger et al. 2007). One sample of *Ch. nuditarsis* (KY225345) was collected as part of eDNA, from the water bodies of Potter Heigham, Great Yarmouth, United Kingdom, and was identified only by *COI* gene sequence (Bista et al. 2017). Samples of *Ch. curabilis* (with prefix OP927…) that were collected in Dark-Sky Reserve within the Westhavelland Nature Park in the Berlin-Brandenburg Metropolitan Region, Germany, were identified by morphology of imago (Chimeno et al. 2023). Samples of *Ch. curabilis* (with prefix MT535…) that were collected from the Dark-Sky Reserve within the Westhavelland Nature Park in the Berlin-Brandenburg Metropolitan Region, Germany, were identified by imago morphology (Gadawski et al. 2022). Samples of *Ch. curabilis* (with prefix JN016…) from the Saratov region, Russia, were identified by cytogenetics (Polukonova et al. 2009). As we can see, the species identification in most samples was performed by imago morphology and cytogenetically, and we can consider this data relatively reliable, therefore, the accuracy of the results may be affected by the length of the obtained nucleotide sequences.

On the Bayesian tree, the *COI* gene sequences of *Ch. nuditarsis* and *Ch. curabilis* combined into one cluster (Fig. 3), which combines with the *Ch. plumosus* group, and it matches the previous data (Kiknadze et al. 2004; Pfenninger et al. 2007; Djomin 2011). Some authors suggest to include the species in the *Ch. plumosus* group (Djomin 2011). The group of sibling-species has an artificial character, where species with similar cytogenetic characteristics and morphological features are combined, often without clear criteria (Shobanov 2000). Due to the development of techniques, we believe that it is necessary to add molecular genetic criteria for sibling species separation together with morphology and cytogenetics.

Further calculations only with additional sequences of *Ch. nuditarsis* and *Ch. curabilis* showed that there was no association within the cluster either by species name or by locality. Within a network of haplotypes based on the *COI* gene sequences of both species *Ch. nuditarsis* and *Ch. curabilis* (Fig. 4), we can see the same situation as in the Bayesian tree: sequences are combined into common haplotypes. This is visible in haplotype 6, which contains sequences from Germany of both species. The haplotype of *Ch. nuditarsis* from Sevan Lake differs from the *Ch. curabilis* from Iran and *Ch. nuditarsis* from Germany by three mutational steps. The highest number of mutation steps, 20, was found between *Ch. nuditarsis* (DQ910574) from Germany and *Ch. curabilis* (JN016812) from Saratov reg. (Russia), with the genetic distance of 3.75%, and only in this case species distinguished as separate. At this stage of the study, it is clear that populations from Europe have the greatest diversity, and other haplotypes diverge from them. Unfortunately, we do not have the *COI* gene sequences of samples from Siberia, and we cannot follow the changes from West to East like with chromosomal polymorphism (Kiknadze et al. 2006; Petrova et al. 2013). Although such trends are already being observed, populations from Saratov (Russia), Iran, Sevan Lake (Armenia), and Skadar Lake (Montenegro) are beginning to form separate haplotypes.

This situation indicates a close relationship between two sibling species and the insufficiency of using single *COI* gene as a molecular marker for their separation in the case studied of *Ch. nuditarsis* and *Ch. curabilis*. Previously performed investigations

of the diversity of *COI*, *gb2b* gene sequences, and the possibility of their use in species delimitation indicate that the calculated threshold cannot be used to separate all *Chironomus* species (Proulx et al. 2013). Another explanation is interspecific hybridization and horizontal transfer of mitochondrial genes with fixation in one of the initial species in a population (Guryev and Blinov 2002; Polukonova et al. 2009; Karmokov 2019; Bolshakov et al. 2021). Previously, we confirmed the existence of hybrids even between species from different cytocomplexes (Bolshakov et al. 2022b).

Chironomid larvae are an important component of aquatic ecosystems and a model object for ecological and hydrobiological studies, as well as a convenient object for cytogenetics (Keyl 1962; Kiknadze et al. 2016). Recently, the study of chironomids phylogenetic relationships has increasingly used the analysis of mitochondrial genes (Li et al. 2022). New knowledge allows for comprehensive monitoring of the ecological state of the environment. According to the changes in environmental conditions caused by global warming and human activities, it is necessary to continue a long-term study of such unique and regionally important reservoirs as Sevan Lake.

Using the case of *Ch. nuditarsis*, we have shown that even a single larva can be subjected to a comprehensive examination, including morphological, cytogenetic and molecular genetic analysis, and a lot of interesting information can be obtained. Do not ignore even one larva.

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ORCID

Viktor Bolshakov https://orcid.org/0000-0002-8028-3818
Alexander Prokin https://orcid.org/0000-0002-9345-5607
Elena Ivanova https://orcid.org/0000-0002-6976-1452
Ekaterina Movergoz https://orcid.org/0000-0002-3677-6631

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RESEARCH ARTICLE

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The complete chloroplast genome of *Rhododendron ambiguum* and comparative genomics of related species

Wen Bao Ma[1], Yafei Ou[1], Buddhi Dayananda[2], Hui Juan Ji[1], Tao Yu[3]

1 *Ecological Restoration and Conservation of Forests and Wetlands Key Laboratory of Sichuan Province, Sichuan, Academy of Forestry, Chengdu 610081, China* **2** *School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD 4072, Australia* **3** *Guizhou Provincial Key Laboratory for Rare Animal and Economic Insect of the Mountainous Region, Guiyang University, Guiyang 550005, China*

Corresponding authors: HuiJuan Ji (ence:ybsc02@163.com); Tao Yu (yutao123@bjfu.edu.cn)

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Abstract

Rhododendron Linnaeus, 1753, the largest genus of woody plants in the Northern Hemisphere, includes some of the most significant species in horticulture. *Rhododendron ambiguum* Hemsl, 1911, a member of subsection *Triflora* Sleumer 1947, exemplifies typical alpine *Rhododendron* species. The analysis of the complete chloroplast genome of *R. ambiguum* offers new insights into the evolution of *Rhododendron* species and enhances the resolution of phylogenetic relationships. This genome is composed of 207,478 base pairs, including a pair of inverted repeats (IRs) of 47,249 bp each, separated by a large single-copy (LSC) region of 110,367 bp and a small single-copy (SSC) region of 2,613 bp. It contains 110 genes: 77 protein-coding genes, 29 tRNAs, four unique rRNAs (4.5S, 5S, 16S, and 23S), with 16 genes duplicated in the IRs. Comparative analyses reveal substantial diversity in the *Rhododendron* chloroplast genome structures, identifying a fourth variant pattern. Specifically, four highly divergent regions (*trnI-rpoB*, *ndhE-psaC*, *rpl32-ndhF*, *rrn16S-trnI*) were noted in the intergenic spacers. Additionally, 76 simple sequence repeats were identified. Positive selection signals were detected in four genes (*cemA*, *rps4*, *rpl16*, and *rpl14*), evidenced by high Ka/Ks ratios. Phylogenetic reconstruction based on two datasets (shared protein-coding genes and complete chloroplast genomes) suggests that *R. ambiguum* is closely related to *R. concinnum* Hemsley, 1889. However, the phylogenetic positions of subsection *Tsutsusi* Pojarkova, 1952 species remain unresolved, indicating that the use of complete chloroplast genomes for phylogenetic research in *Rhododendron* requires careful consideration. Overall, our findings provide valuable genetic information that will enhance understanding of the evolution, molecular biology, and genetic improvement of *Rhododendron* spieces.

Keywords
Chloroplast genome, molecular evolution, phylogenetic analysis, *Rhododendron*, *Rhododendron ambiguum*

Introduction

The genus *Rhododendron* Linnaeus, 1753, comprising approximately 1000 species, stands as the largest genus within the family Ericaceae. It holds significant importance due to its high species diversity and broad distribution across the temperate regions of Europe, Asia, and North America (Chamberlain et al. 1996). These plants are renowned for their attractive flowers and foliage, and are extensively cultivated for their ornamental value, occupying a significant position in the realms of gardening and landscaping (Srivastava 2012). Moreover, *Rhododendron* species play a crucial role in montane ecosystems, hosting numerous dominant and ecologically significant species that contribute to the stability of plant communities in alpine or subalpine regions (Yu et al. 2017; Zhang et al. 2021). However, due to excessive deforestation and ongoing habitat deterioration, multiple *Rhododendron* species have been categorized as vulnerable on the IUCN Red List, including *R. protistum* var. *Giganteum* Chamb. 1979, *R. redowskianum* Maximovich, 1870, and *R. aureum* Georgi, 1775 (Lu et al. 2021). Nonetheless, understanding of interspecific relationships and the timeline of diversification within the genus *Rhododendron* remains unresolved, largely due to the influence of natural hybridization and introgression (Ma et al. 2022b; Mo et al. 2022).

The molecular basis of *Rhododendron* species has been extensively explored in recent years, with nine *Rhododendron* genomes and various chloroplast genomes, including those of *R. calophytum* Franchet, 1886 and *R. delavayi* Franche, 1886, being published in recent studies (Li et al. 2020b; Shirasawa et al. 2021; Ma et al. 2022a; Shen et al. 2022; Li et al. 2023). The genetic information obtained has provided a range of genetic markers that have the potential to aid in reconstructing the phylogenetic connections among *Rhododendron* species. A comprehensive phylogenomic study of *Rhododendron* has been published recently, utilizing a partial chloroplast genome that encompasses 161 *Rhododendron* species (Mo et al. 2022). The advantages of utilizing the chloroplast genome include its moderate nucleotide substitution rates and the absence of homologous recombination, which makes it a valuable tool for elucidating relationships among *Rhododendron* species. This approach has provided a dependable taxonomic framework for the genus *Rhododendron*. However, only a limited number of studies have delved into the examination of chloroplast genome structure and variations within the genus *Rhododendron* (Li et al. 2020b; Shen et al. 2022). Since the comparison of *Rhododendron* chloroplast genomes not only establishes a crucial foundation for evolutionary investigations but also facilitates the exploration of fundamental chloroplast genome variability, there is a need for more comprehensive comparative studies utilizing the entirety of the chloroplast genome (Yu et al. 2021; Shen et al. 2022).

R. ambiguum Hemsley, 1911, belonging to subsection *Triflora*, is native to central and western Sichuan (China), thriving in thicket or woodland environments at

elevations ranging from 2,300 to 3,300 meters, and occasionally reaching up to 4,500 meters (Shrestha et al. 2017). Functioning as an alpine species, *R. ambiguum* can be artificially cultivated and holds substantial horticultural significance. Studies have demonstrated the presence of abundant glycosides, terpenoids, and essential oils within *R. ambiguum*, indicating its promising potential for future commercial development (Shrestha et al. 2017). Currently, the genomic information available for *R. ambiguum* is rather limited. Furthermore, there is no complete nuclear genome data for subsection *Triflora*, and only a single chloroplast genome has been published (Shirasawa et al. 2021). The frequent occurrence of natural hybridization between subsection *Triflora* species and natural hybridization with other subgroups in the subgenus make it difficult to confirm and identify the phylogenetic location of species (Mo et al. 2022). The complete chloroplast genome will provide genetic data resources for the subsection *Triflora* and the whole genus *Rhododendron* phylogenetic analysis. In the current work, we report the characterization of the chloroplast genome of *R. ambiguum* and provide a comparative assessment alongside other relatives within the genus *Rhododendron*. This research focuses on (1) analyzing the chloroplast genome structural characteristics of *Rhododendron*, (2) identifying hypervariable regions and SSR loci that could serve as DNA barcodes in future, investigating the relative synonymous codon usage (RSCU), (3) gene Ka/Ks ratios in *Rhododendron* chloroplast genomes, (4) inferring the phylogenetic position of *R. ambiguum* within the genus *Rhododendron* using complete cp genome alignments.

Material and methods

Sample collection and DNA sequencing.

Fresh leaves of *R. ambiguum* were collected from Gongga Mountain National Nature Reserve in Sichuan, China (Longitude: 102.01791, Latitude: 29.77135, Altitude: 3291 meters). The leaves were immediately desiccated using silica gel for further analysis. The corresponding voucher specimens were deposited at the West China Subalpine Botanical Garden in Dujiangyan, Sichuan Province (Voucher No. 2019-GG-015). DNA was extracted from the leaves using a modified CTAB method (Doyle 1987). The purified genomic DNA was sequenced on an Illumina HiSeq X Ten platform by Biomarker Technologies, Beijing, China. Overall, we obtained a total of 8G paired-end reads after sequencing, according to previous studies, most *Rhododendron* genomes are about 650M, the sequencing depth in this research is about 18X (Shirasawa et al. 2021; Shen et al. 2022). The sequences were then trimmed using NGSQC (Dai et al. 2010) with default parameters.

Assembly and annotation.

We assembled the sequences using MIRA software (http://sourceforge.net/apps/mediawiki/mira-assembler), with default settings, and NOVOplasty (Dierckxsens et al. 2016) using the reference genomes of *R.* × *pulchrum* (accession number: MN182619.1)

and *R. calophytum* (accession number: OM373082.1) (Shen et al. 2020; Ma et al. 2022a). Genome annotation was performed using Cpgavas (Yong and Zheng 2012), complemented by manual curation and BLAST for gene verification. Circular chloroplast genome maps for *R. ambiguum* were generated using OGDRAW. The complete genome was uploaded to the National Center for Biotechnology Information with the GenBank accession number OR455462.

Chloroplast genome boundaries comparison, divergence hotspot and SSRs analysis

The junctions between the chloroplast genomes of 18 Rhododendron species were examined using IRscope (Amiryousefi et al. 2018), which visualizes the expansion and contraction of inverted repeats (IRs) and the positioning of genes. Nucleotide diversity (Pi) was assessed through sliding window analysis using DnaSP v5 (Librado and Rozas 2009), with a step size of 200 bp and a window length of 600 bp. Simple sequence repeats (SSRs) were identified using MISA v1.0 with parameters set for various repeat units (Beier et al. 2017).

Analysis of codon usage characteristics and selection pressure

Coding sequences from *Rhododendron* chloroplast genomes were extracted, and codon usage was analyzed using CodonW v1.4.2 (http://codonw.sourceforge.net). A clustered heatmap of Relative Synonymous Codon Usage (RSCU) values was generated at cloud.genepioneer.com. Selection pressures were calculated by determining nonsynonymous (Ka) and synonymous (Ks) substitution rates and their ratios using KaKs_Calculator v2.0 (Zhang et al. 2006), with non-applicable or infinite values defined as zero.

Phylogenetic relationship reconstruction

The phylogenetic tree was constructed using Bayesian Inference (BI) methods, based on datasets of shared protein-coding genes (PCGs) and complete chloroplast genomes. Multiple sequence alignments were conducted using PhyloSuite (Zhang et al. 2020), with default settings. BI trees were inferred using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) under a N/A model with 3,000,000 generations, discarding the initial 25% of sampled data as burn-in. The substitution models used were GTR+G+I for the PCGs dataset and GTR+G for the complete chloroplast genome dataset. *Vaccinium bracteatum* Thunberg, 1784, *V. macrocarpon* Aiton, 1789 and *V. uliginosum* Linnaeus, 1753 were selected as outgroups

Availability of data and materials

The datasets generated or analyzed during the current study are available in the NCBI Bioproject repository OR455462.

Results

Characteristics of *R. ambiguum* chloroplast genome

The *R. ambiguum* chloroplast genome was typically circular quadripartite (Fig. 1). It spans a total length of 207,478 bp, comprising a pair of inverted repeats (IRs) of 47,249 bp each, separated by the large single-copy (LSC) region of 110,367 bp and the small single-copy (SSC) region of 2,613 bp. This genome encodes 110 unique genes, including 77 protein-coding genes, 29 tRNAs, and four unique rRNAs (4.5S, 5S,

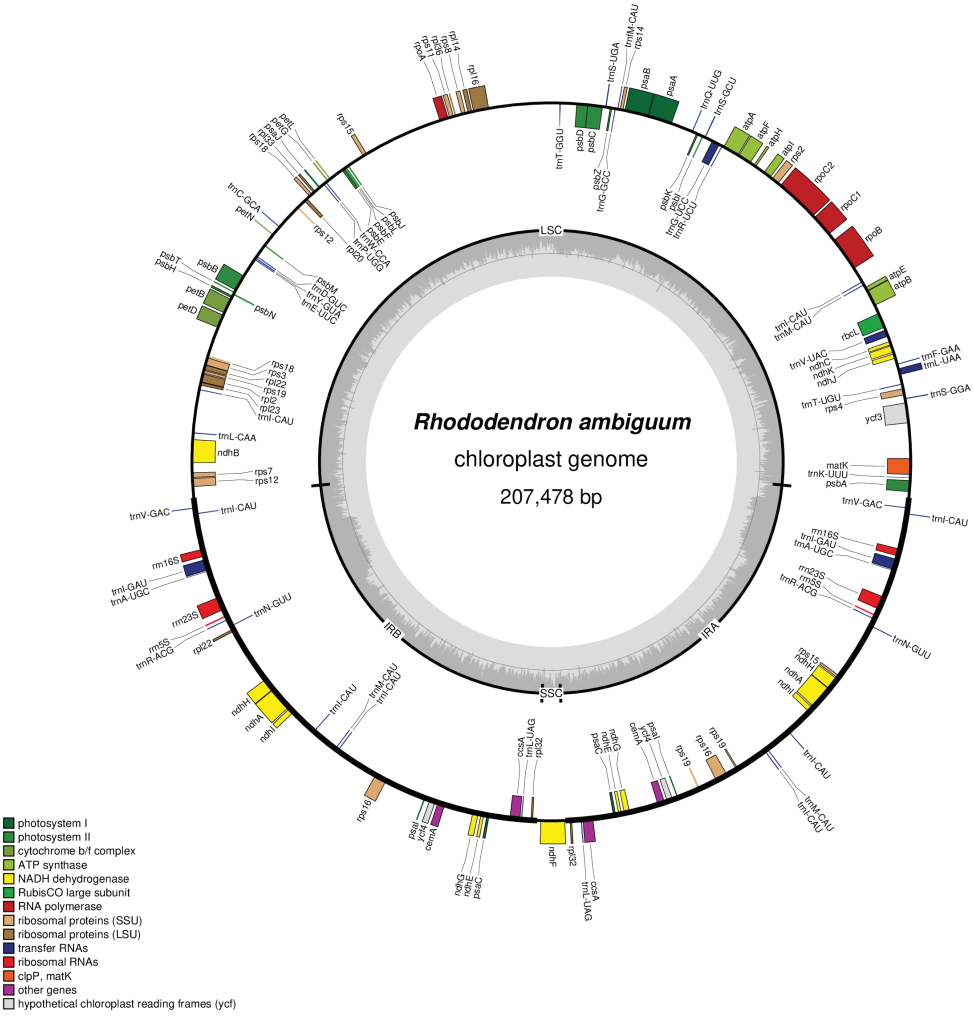

Figure 1. Chloroplast genome map of *Rhododendron ambiguum*. Genes located outside of the circle were transcribed counter-clockwise, while genes shown inside were transcribed clockwise. The thick lines indicate the inverted repeat regions (IRa and IRb) that separate the genome into small (SSC) and large (LSC) single copy regions. Different genes were color coded.

Table 1. Genes present in the *Rhododendron ambiguum* chloroplast genome.

Group of gene	Genes name
Photostsyem I	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*, *ycf3***, *ycf4*
Photostsyem II	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*
Cytochrome b/f complex	*petB**, *petD**, *petG*, *petL*, *petN*
ATP synthase	*atpA*, *atpB*, *atpE*, *atpF**, *atpH*, *atpI*
NADH dehydrogenase	*ndhA**, *ndhB**, *ndhC*, *ndhE*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *ndhJ*, *ndhK*
RubisCO large subunit	*rbcL*
RNA polymerase	*rpoA*, *rpoC1*, *rpoC*2
Ribosomal proteins (SSU)	*rps2*, *rps3*, *rps4*, *rps7*, *rps11*, *rps12***^{T}, *rps14*, *rps15*, *rps16*, *rps18*, *rps19*
Ribosomal proteins (LSU)	*rpl2**, *rpl16**, *rpl20*, *rpl22*, *rpl23*, *rpl32*, *rpl33*
Other gene	*matK*, *ccsA*, *cemA*
Transfer RNAs	29 tRNAs (two contain a single intron)
Ribosomal RNAs	*rrn4.5*, *rrn5*, *rrn16*, *rrn23*

Note: a single asterisk (*) preceding gene names indicate intron-containing genes, and double asterisks (**) preceding gene names indicate two introns in the gene; ^{T} , trans-splicing of the related gene.

16S, and 23S rRNA); 16 of these genes are duplicated in the IRs (Table 1). There are 14 genes with introns: ten protein-coding genes (*atpF*, *ndhA*, *ndhB*, *rpl2*, *rpl16*, *rps12*, *rps16*, *petB*, *petD*, and *ycf3*) and four tRNAs (*trnL-UAA*, *trnG-UCC*, *trnI-GAU*, and *trnV-UAC*). Notably, the *ycf3* gene contains two introns. The overall GC content of the *R. ambiguum* chloroplast DNA is 35.8%.

Boundaries comparison and divergence hotspot analysis

We compared the complete chloroplast genomes of 18 *Rhododendron* species (Fig. 2), revealing a rich diversity in gene order within these genomes. Despite the overall retention of the quadripartite structure, the SSC regions exhibited large variations in length, displaying four main forms. The first category included 11 *Rhododendron* species with chloroplast genome lengths around 200,000 bp, where the SSC regions ranged from 2,582 bp to 2,748 bp, with the *ndhF* gene occupying more than 81% of this region. The second category consisted of three species (*R. simsii* Planchon, 1853, *R. latoucheae* Franchet, 1899, and *R.* × *pulchrum* Sweet, 1831) with shorter chloroplast genomes ranging from 146,941 bp to 156,355 bp. In *R. simsii*, the SSC region was notably large at 69,783 bp, only 9,214 bp shorter than the large single-copy (LSC) region. The third category included two species (*R. molle* Don, 1834 and *R. huadingense* Ding et Fang, 2005), which had the longest LSC regions and the shortest IRs, with the IRs comprising only 1% of the LSC region in *R. molle*. The fourth category, consisting of *R. kawakamii* Hayata, 1911 and *R. datiandingense* Tang et Zhuang, 2009, differed significantly from other species in terms of region length and gene arrangement.

In terms of nucleotide variability (Pi), the first category exhibited ranges from 0 to 0.299, indicating high divergence (Fig. 3). We identified four highly variable regions (*trnI-rpoB*, *ndhE-psaC*, *rpl32-ndhF*, *rrn16S-trnI*) with Pi values ≥0.095. The *trnI-rpoB* and *ndhE-psaC* regions were located in the LSC region, *rpl32-ndhF* in the SSC region,

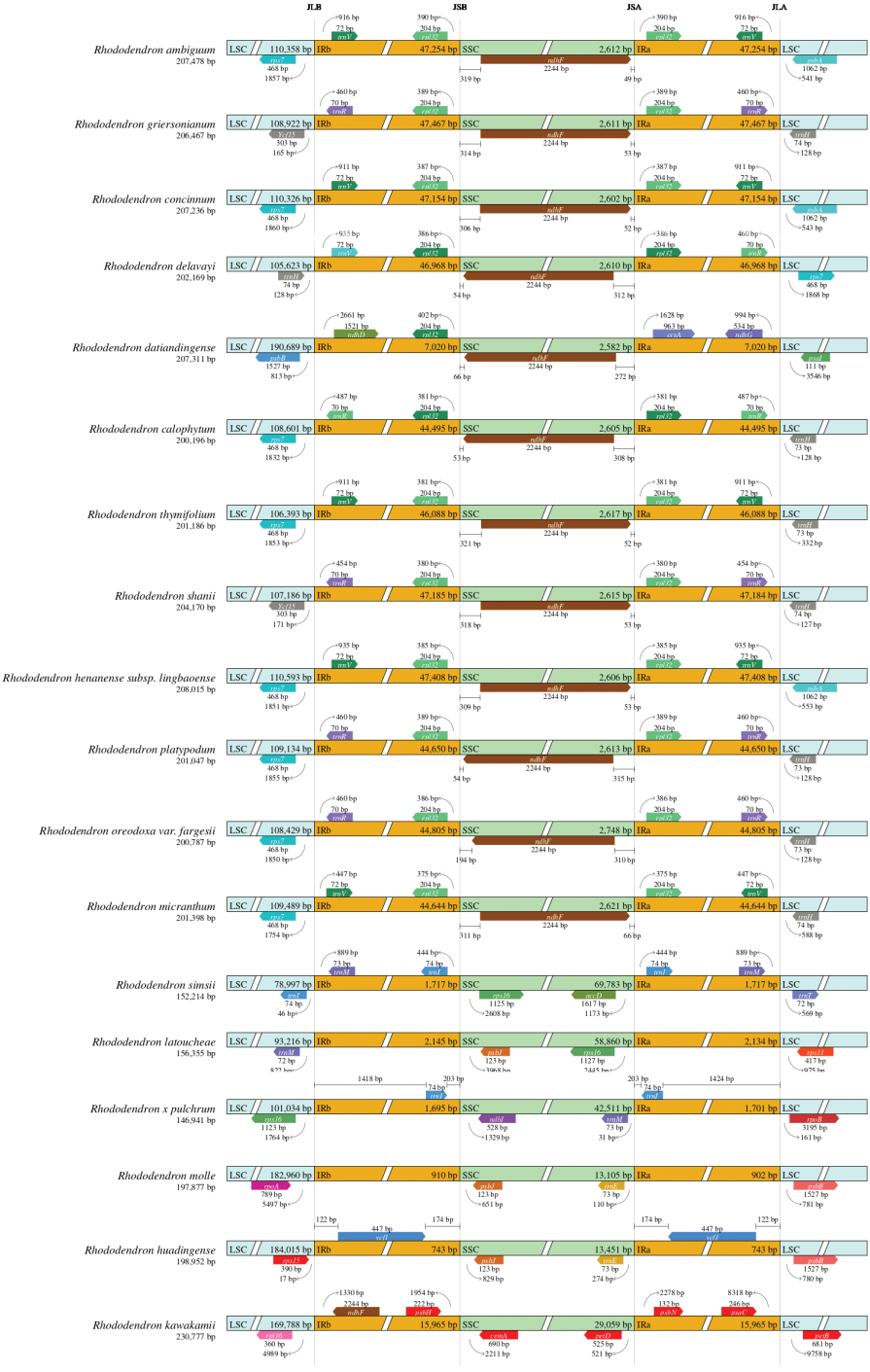

Figure 2. Comparison of the borders of the LSC, SSC and IR regions in 18 complete *Rhododendron* cp genomes. Genes transcribed forward were shown above the lines whereas genes transcribed reversely were shown below the lines. Gene lengths in the corresponding regions were displayed above the boxes of gene names.

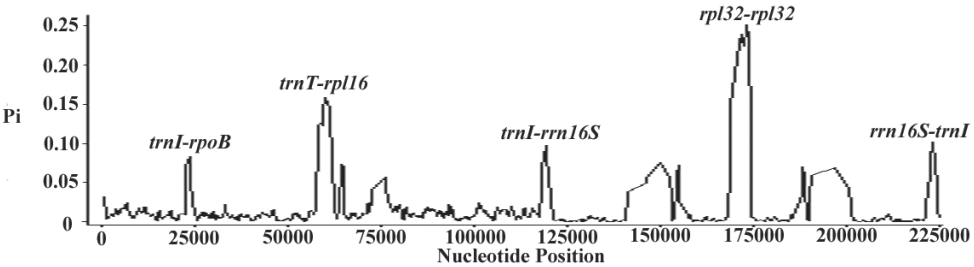

Figure 3. Sliding window analysis of *Rhododendron* chloroplast genome for nucleotide diversity (Pi) compared among 11 species similar structure, with window length 600 bp and step size 200 bp . Peak regions with a Pi value of > 0.08 were labeled with loci tags of intergenic spacers regions. X-axis: position of the midpoint of a window;Y-axis: nucleotide diversity of each window.

and *trnI-rrn16S* in the IR region, highlighting that the LSC and SSC regions are more divergent than the IR regions. Overall, the non-coding regions were more divergent than the coding regions. These results underscore the potential of these regions as valuable phylogenetic markers for *Rhododendron* species.

SSR analysis

In this study, a total of 76 SSRs with a repeat length of one to three bp were detected in *R. ambiguum* chloroplast genome. Among these SSRs, there were 69, six and one for mononucleotides (mono-), dinucleotides (di-), trinucleotides (tri-) nucleotide repeats, respectively, and tetranucleotides (tetra-), pentanucleotide (penta-) and hexanucleotide (hex) repeats were not found (Fig. 4A). The majority of the mononucleotides were composed of A/T, only one SSR was G/C, and total of the dinucleotides were AT/AT. Statistical analysis showed these SSRs were identified mainly in the intergenic regions (59), compared to six and 11 SSRs in the introns and coding region, respectively (Fig. 4B). In terms of distribution, 44 SSRs in LSC regions and 32 SSRs in IRs regions, no SSR was detected in the SSC region (Fig. 4C).

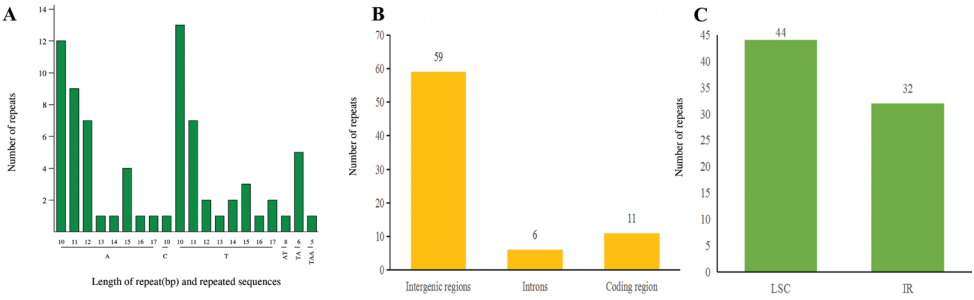

Figure 4. Distribution of SSRs in *Rhododendron ambiguum* chloroplast genomes **A** length of repeat and repeated sequences **B** frequency of SSRs in the coding region, intergenic and intron region **C** frequency of SSRs in the LSC, IR and SSC regions.

Codon usage bias and gene selective pressure analysis

The 18 *Rhododendron* chloroplast genomes were analyzed to investigate the relative synonymous codon usage (RSCU) (Fig. 5A). In this study, 64 codons are encoded 20 amino acids and one stop codon, The three most abundant amino acids were Leu (40,985), Ile (33,752) and Gly (27,610). The uncommon encoded amino acids were Ter (1,615), Cys (4,353) and His (8,417), respectively. Among these codons, RSCU value ranged from 0.317 (CGC) to 2.17 (UUA), and UUA had RSCU value between 2.06 to 2.17, indicating that Leu is preferentially coded by UUA in *Rhododendron* chloroplast genomes (Fig. 5B). Thirty codons showed average codon usage bias with RSCU > 1.00 in the chloroplast genes of *Rhododendron*, there were 13 codons ending in A (GCA,UCA,CCA,ACA,GUA,UAA,GAA,GGA,CGA,AAA,CAA.AGA and UUA), 16 codons ending in U (CUU, AGU, GGU, UUU, GUU, CAU, AUU, AAU, UGU, CGU, GAU, UAU, ACU, CCU, GCU and UCU), and 1 codon ending in G (UUG). This indicates that *R. ambiguum* prefers to end with A/U-ending codons.

The Ka/Ks ratio was calculated for 53 shared protein-coding genes between *Rhododendron* species cp genomes (Fig. 6). In all protein-coding genes of *Rhododendron*, *atpH*, *petG*, *psbD*, *psbF*, *psbL*, *psbJ*, *psbL*, *psbM*, *psbT*, *psbZ* and *rpl32* had no nonsynonymous rate, and four genes (*petG*, *psbL*, *psbT* and *rpl32*) had no synonymous substitution rates. The *rps15* gene had the highest synonymous rate (0.067) and highest nonsynonymous rate (0.073). The average Ka/Ks ratio analyzed in the 18 genomes was 0.496 for 42 protein-coding genes with Ka/Ks ratio, which were not region-specific. Most protein-coding genes showed purifying selection, 38 coding genes showed Ka/Ks ratios < 1, moreover, Ka/Ks ratios in the range of 0.5–1 has 15 protein-coding genes. The genes inferred to be undergoing positive selection were *cemA*, *rps4*, *rpl16* and *rpl14* (Ka/Ks ratios >1). The *rps4*, *rpl16* and *rpl14* were located in the LSC region, except the *cemA* in the IR region.

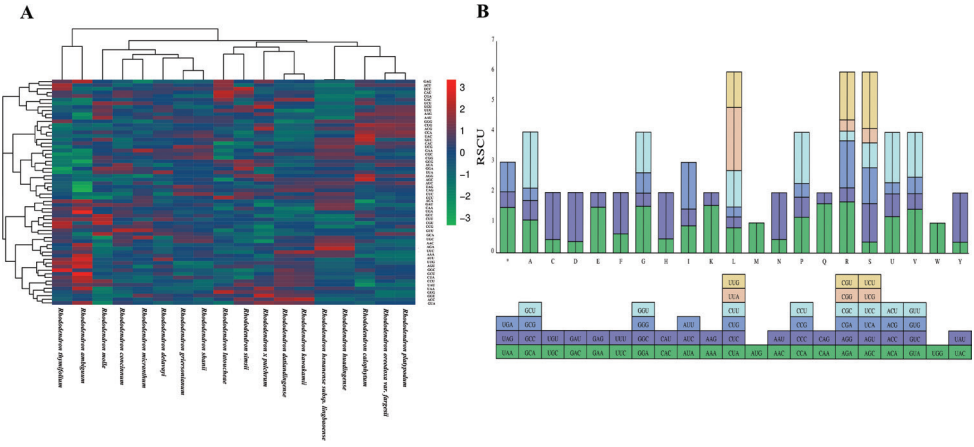

Figure 5. A Heat map analysis for codon distribution of all protein coding genes in 18 sequenced *Rhododendron* chloroplast genomes **B** codon content and codon usage of 20 amino acids and stop codons of all protein coding genes of *Rhododendron ambiguum*.

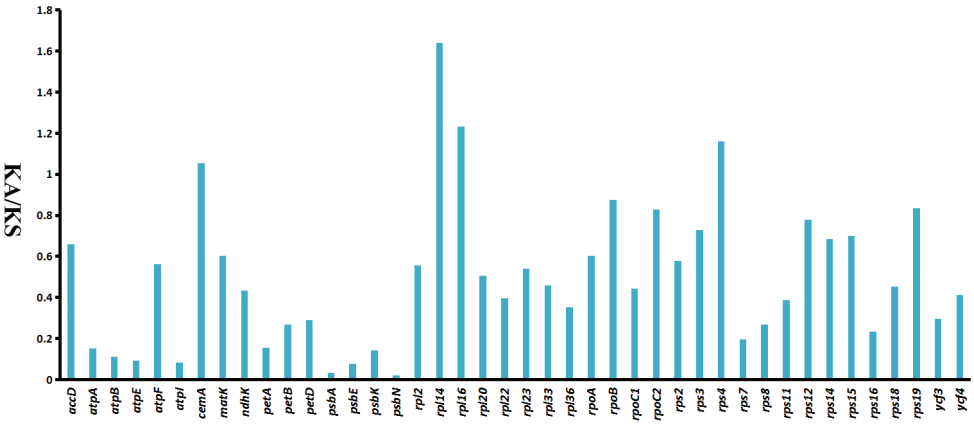

Figure 6. The Ka/Ks values of shared protein-coding genes of 18 *Rhododendron* chloroplast genomes.

Phylogenetic relationship reconstruction

Phylogenetic relationships among *Rhododendron* were estimated with two datasets using Bayesian inference (BI) methods. The BI tree inferred from shared PCGs confirms that *Rhododendron* species clustered three clades, and the boot-strap values of almost nodes were equal to 1 (Fig. 7). The shared genes and the whole chloroplast genome generated trees showed *R. ambiguum* close to *R. concinnum*, but the phylogenetic relationships of *Rhododendron* genus species were unresolved. The first clades showed that part of subgenus *Tsutsusi*, subgenus *Hymenanthes* Koch, 1872 and subgenus *Pentanthera* Don, 1834, species were clustered together. The second clades included species of subgenus *Rhododendron* and *R. datiandingense* Feng, 1996. belong to subgenus *Tsutsusi* in the middle position branch. The third clades composed of *R. latoucheae* Franchet, 1899 belong to subgenus *Azaleastrum* Maximovich, 1900 and *R.huadingense* Ding et Fang, 2005 belong to subgenus *Tsutsusi*. The results showed that confusion about phylogenetic position of subgenus *Tsutsusi* species was the main cause of the disorder phylogenetic tree. Different analyses based on the two datasets generated inconsistent topologies (Fig. 7, Suppl. material 1). In the datasets of chloroplast genome, different groups are not effectively divided indicating that PCGs were more efficient in determining phylogenetic relatedness rather than whole genomes, since the PCGs data exclude the influence of chloroplast genome structural variation.

Discussion

The chloroplast genome of *R. ambiguum* exhibits a typical quadripartite structure, similar to most terrestrial plants, and spans 207,478 bp—slightly larger than the typical angiosperm chloroplast genome, which ranges from 120,000 to 160,000 bp (Fu et al. 2016). However, this size is average compared to the chloroplast genomes of closely

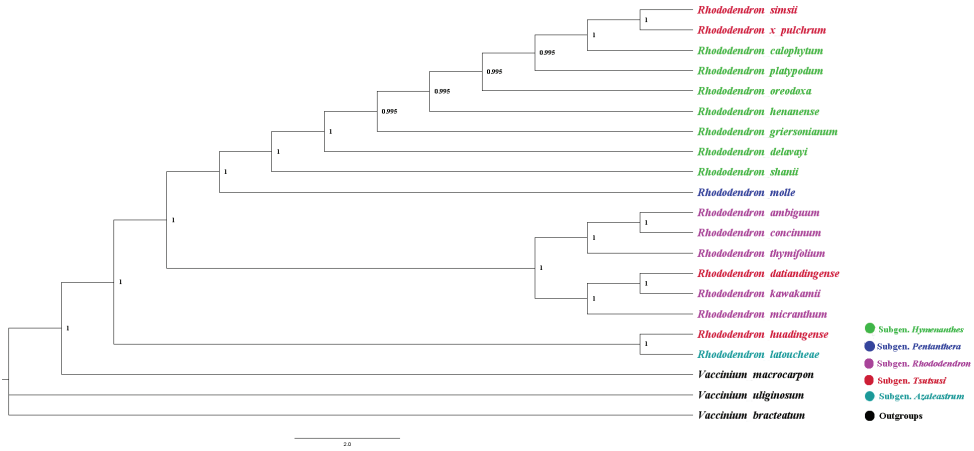

Figure 7. Phylogenetic relationships inferred by Bayesian Inference. This phylogenetic tree is based on shared protein-coding genes (PCGs) of 18 *Rhododendron* species and three outgroup species. Numbers at the nodes represent bootstrap support values.

related *Rhododendron* species. In this study, gene losses and large inversions were detected across *Rhododendron* chloroplast genomes, resulting in variations in genome length from 146,941 bp (*R.* × *pulchrum*) to 230,777 bp (*R. kawakamii*). Structural rearrangements within these genomes highlight extensive variations and significant expansions or contractions of inverted repeats (IRs) among *Rhododendron* species. Typically, the genes at the LSC/IRa/SSC/IRb boundaries are highly conserved within species of the same genus, with only slight expansions or contractions of IR regions observed (Turmel et al. 1999; Wang et al. 2021). Fig. 2 illustrates structural differences in the *Rhododendron* chloroplast genomes that exhibit a unique fourth variation pattern, underscoring the distinctive specificity of *Rhododendron* species, similar to findings in other studies (Li et al. 2020b; Shen et al. 2022). This unusual pattern in the *Rhododendron* chloroplast genomes may be influenced by factors such as genome mapping or annotation, in addition to natural factors.

Previous research has demonstrated that comparative chloroplast genomics is crucial for developing barcoding methods for species identification and advancing population genetics studies (Yuan et al. 2017; Li et al. 2020b). In our study, we identified variable regions within the chloroplast genomes of 11 *Rhododendron* species that exhibit similar structures. Notable differences were observed in regions such as *trnI-rpoB*, *ndhE-psaC*, *rpl32-ndhF*, and *rrn16S-trnI*. Notably, the *rpl32-ndhF* region has been previously utilized to test closely related species within the tribe Hydrangeeae (Granados Mendoza et al. 2013), and the *rrn16S-trnI* region has been extensively employed as a recombination site in chloroplast genome transformation research (Cui et al. 2014; Kaushal et al. 2020). Interestingly, the noncoding regions *ndhE-psaC* and *trnI-rpoB*, though never used in phylogenetic inference, have been recognized as hypervariable regions in monocots (de Santana Lopes et al. 2018).

Codon usage in the chloroplast genome protein-coding sequences of *Rhododendron* species is consistent, showing a preference for A/U-ending codons, similar to other plant genera (Silva et al. 2018; Shen et al. 2022). The amino acids Leu, Ile, and Gly are most frequently coded, which is a common pattern observed across various plant families. Notably, genes such as *cemA*, *rps4*, *rpl16*, and *rpl14* exhibit a relatively high Ka/Ks ratio (>1), suggesting positive selection. The *cemA* gene, encoding an envelope membrane protein, has shown signs of positive selection in the *Dalbergia* species (Wu et al. 2022). The ribosomal proteins *rps4*, *rpl16*, and *rpl14*, crucial for self-replication, have also been detected under positive selection in other taxa like Rosaceae and *Vicia* L., 1753 (Cheng et al. 2017; Li et al. 2020a). Given the diverse environmental conditions of *Rhododendron* habitats, from varying elevation gradients to light-related stress, this positive selection may indicate adaptive evolutionary responses.

Taxonomic determination within *Rhododendron* is challenging due to intermediate morphologies and hybrid origins between different species (Chamberlain et al. 1996; Ma et al. 2022a). Historically, phylogenetic analyses in *Rhododendron* have utilized markers such as ITS, retinol-binding proteins (RPB2I-1, RPB2I-2, RPB2I-3, RPB2I-4, RPB2I-5, RPB2I-6), and cpDNA loci (*atpB-rbcL*, *rbcL*, *matK*, *ndhF*, *psbA-trnH*, *trnL-trnF*, *trnL*, *trnT-trnL*, t*rnS-trnG*) (Kurashige et al. 2001; Shrestha et al. 2018; Mo et al. 2022), while elucidating some phylogenetic relationships, have also highlighted unresolved key nodes such as section *Pentanthera* to section *Rhodora* (60% bootstrap value) was indicated subgenus *Mumeazalea* Makino, 1914 and subgenus *Azaleastrum* Koch, 1872 section *Choniastrum* (59% bootstrap value)(Kurashige et al. 2001; Shrestha et al. 2018). The recent study by Mo et al. (2022) provides strong support for a stable and reliable taxonomic framework for *Rhododendron*, based on extensive chloroplast genome sampling. In Mo et al. (Mo et al. 2022) research revealed that *Rhododendron* species chloroplast genome failed to provide a complete circular structure, which the result file is several scaffold forms. As sequencing technologies advance, particularly with the introduction of third-generation sequencing, the accuracy of *Rhododendron* chloroplast genomes is expected to enhance their phylogenetic resolution significantly.

Conclusions

This study assembled the complete chloroplast genome of *R. ambiguum*, revealing significant structural variations compared to other plant species. Key divergences were identified in four non-coding IGS regions, suggesting their potential as molecular markers for phylogenetic studies. The codon usage analysis showed a preference for A/U-endings, common across *Rhododendron* species. Notably, while most protein-coding genes exhibited purifying selection, genes such as *cemA*, *rps4*, *rpl16*, and *rpl14* showed signs of positive selection, indicating potential adaptation mechanisms to diverse environmental conditions. Phylogenetic analysis confirmed the close relationship between *R. ambiguum* and *R. concinnum* but also highlighted the complexity of relationships within the genus *Rhododendron*, underscoring the need for further research

using complete chloroplast genomes. The insights gained from this study enhance our understanding of *Rhododendron*'s evolutionary biology and support the continued development of genomic resources for ecological and evolutionary studies.

Author Contributions

HuiJuan Ji – methodology, Tao Yu – software, WenBao Ma and Yafei Ou – validation, WenBao Ma and Tao Yu – investigation, Tao Yu – writing and original draft preparation, Buddhi Dayananda – writing – review and editing, WenBao Ma – funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Declarations

Dr. Wenbao Ma has obtained the permission by Sichuan Academy of Forestry to collect plant species from Gongga Mountain national nature reserve, Sichuan, China. Because of the important protective value of *Rhododendron ambiguum*, Dr. Ma only collected a few leaves from the plants for further molecular study without damaging the plant growth. The plant material collection and experimental research were conducted according to the Plant Protection and Regulation of Sichuan Academy of Forestry.

The authors declare no conflict of interest.

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ORCID

WenBao Ma https://orcid.org/0009-0008-4583-0135
Yafei Ou https://orcid.org/0009-0006-2364-9605
Buddhi Dayananda https://orcid.org/0000-0002-7607-0596
HuiJuan Ji https://orcid.org/0009-0008-5172-3600
Tao Yu https://orcid.org/0009-0003-8140-9296

Supplementary material 1

Phylogenetic relationship was inferred used the Bayesian inference method based on whole chloroplast genome of 18 *Rhododendron* species and three outgroups

Authors: WenBao Ma, Yafei Ou, Buddhi Dayananda, HuiJuan Ji, Tao Yu

Data type: png

Link: https://doi.org/10.3897/compcytogen.18.119929.suppl1

Supplementary material 2

Ka/Ks ratios > 1 genes among *Rhododendron* chloroplast genomes

Authors: WenBao Ma, Yafei Ou, Buddhi Dayananda, HuiJuan Ji, Tao Yu

Data type: xlsx

Link: https://doi.org/10.3897/compcytogen.18.119929.suppl2

Supplementary material 3

Chloroplast genomes used for phylogenetic analyses

Authors: WenBao Ma, Yafei Ou, Buddhi Dayananda, HuiJuan Ji, Tao Yu

Data type: docx

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Karyotypic description and comparison of *Litoria* (*L.*) *paraewingi* (Watson et al., 1971), *L. ewingii* (Duméril et Bibron, 1841) and *L. jervisiensis* (Duméril et Bibron, 1841) (Amphibia, Anura)

Richard Mollard[1], Michael Mahony[2], Matt West[3]

1 *Melbourne Veterinary School, Faculty of Science, The University of Melbourne, Parkville, 3052, Australia* **2** *School of Environmental and Life Sciences, University of Newcastle, Callaghan, New South Wales, 2308, Australia* **3** *School of Biosciences, Faculty of Science, The University of Melbourne, Parkville, 3052, Australia*

Corresponding author: Richard Mollard (rmollard@unimelb.edu.au, mollard@amphicell.com)

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Abstract

The karyotype of *Litoria* (*L.*) *paraewingi* (Watson et al., 1971) (Big River State Forest, Victoria) is described here for the first time. It is prepared following tissue culture of toe clipping macerates, cryopreservation, reculture and conventional 4',6-diamidino-2-phenylindole (DAPI) staining. The *L. paraewingi* karyotype is then compared to similarly processed IUCN (International Union for the Conservation of Nature) least concern members *L. ewingii* (Duméril et Bibron, 1841) (southern Victoria) and *L. jervisiensis* (Duméril et Bibron, 1841) (Myall Lakes National Park, New South Wales), all members of the same *L. ewingii* complex/group. The *L. paraewingi* diploid number is 2n = 26, the same as for the other two species. *Litoria paraewingi* chromosomes 1, 2, 6 and 7 are submetacentric, chromosomes 3 and 5 are subtelocentric and the remainder are metacentric. No secondary constriction or putative nucleolus organiser region (NOR) was readily identifiable following conventional DAPI staining in any scored *L. paraewingi* metaphase spread. Conversely, a putative NOR was readily identifiable on the long arm of chromosome 1 in all examined metaphase spreads for the other two species. The karyotypes of *L. ewingii* and *L. jervisiensis* here further differ from *L. paraewingi* with chromosome 1 being metacentric and chromosomes 8 and 10 being submetacentric for both former species. The *L. jervisiensis* karyotype differs from those of *L. ewingii* and *L. paraewingi* by DAPI staining with: (i) apparent relative length inversion of subtelocentric chromosome 3 and metacentric chromosome 4 and (ii) chromosome 6 being metacentric rather than submetacentric. All three species have a highly conserved chromosome morphology with respect

to chromosomes 2, 5, 7, 9, 11, 12 and 13. The greatest gross morphological difference karyotypically is observed between *L. paraewingi* and *L. jervisiensis*. These karyotype data support the previous phylogenetic separation of these three species based upon genetic compatibility and behavioural, biochemical and molecular genetic analyses.

Keywords
Cell culture, cryopreservation, karyotype, Plains brown tree frog

Introduction

The current large scale existential threat to over 40% of amphibian species globally is well documented, making amphibians the most endangered vertebrate taxonomic class (Luedtke et al. 2023). Habitat loss facilitating disease spread, pollutant introduction and species invasion means that for many of these species, animal husbandry, assisted reproductive technologies and cryobanking programs, whether alone or in combination, are suggested requirements for their long-term survival (Kouba et al. 2013; Gillespie et al. 2016; Lampert et al. 2022). Cryobanking initiatives for amphibian assisted reproduction technologies, however, are currently restricted to sperm cells, with methods for oocyte or embryo cryopreservation remaining challenging (Clulow and Clulow 2016; Gagarinskiy et al. 2023). Numerous publications describe somatic cell culture of amphibian somatic cells (Fukui et al. 2003; Ferris et al. 2010; Strauß et al. 2011; Mollard 2018; Mollard et al. 2018; Bui-Marinos et al. 2022; Douglas et al. 2023). It is envisioned that cryopreservation of amphibian somatic cells will provide a necessary immediate resource for longer term genetic conservation initiatives including induced pluripotent stem cell technologies for cloning and gamete production (Kouba et al. 2013; Clulow and Clulow 2016; Codner et al. 2016; Oikawa et al. 2022). In respect to mouse ES cells, maintenance of > 50% euploid karyotype is essential for successful cloning outcomes and by proxy gamete production (Kusakabe et al. 2001; Olifent et al. 2002; Bonnet-Garnier et al. 2015). Cryobanking initiatives of somatic cells aimed at longer term conservation outcomes, therefore, must include steps to ensure recovery of karyotypically normal cells.

A generic level classification of taxa within the Australo-Papuan hyloid family Pelodryadidae has remained problematic largely due to the lack of a comprehensively sampled and well resolved phylogeny for these frogs. The family comprises 232 species split roughly half in Australia and half in Melanesia and eastern Indonesia and contributes 28% of anuran species diversity in the region. Molecular phylogenetic analysis indicates Pelodryadidae diverged approximately 50 million to 100 million years ago while the Australian/ New Guinean land mass and Antarctica were separating (Duellman et al. 2016; Feng et al. 2017; Brennan et al. 2023). Three genera,

Litoria (Tschudi et Agassiz, 1838), *Cyclorana* (Steindachner, 1867) and *Nyctimystes* (Stejneger, 1916) have been used to taxonomically allocate diversity within the Pelodryadidae, but the description of possible new genera remains the subject of debate (Wells and Wellington 1985; Duellman et al. 2016; Frétey and Dubois 2016). The Australian species of this family are found in all major habitats of the continent (Vidal-García and Keogh 2015). Despite this ecological breadth, morphological and life history variations are recognised to show a strong association with ecological specialisations. As such, previously applied informal sub-familial classification as species groups accommodating this variation remain widely recognised (Tyler and Davies 1978). One of the well characterised species groups is the *Litoria* (*L.*) *ewingii* group, which comprises nine species (Parkin et al. 2024): *L. callicelis* (Parkin et al., 2024), *L. ewingii* (Duméril et Bibron, 1841), *L. jervisiensis* (Duméril et Bibron, 1841), *L. littlejohni* (White et al., 1994), *L. paraewingi* (Watson et al., 1971), *L. revelata* (Ingram et al., 1982), *L. sibilis* (Parkin et al., 2024), *L. watsoni* (Mahony et al., 2020) and *L. verreauxii* (Duméril, 1853). Assignment to this complex is based upon a range of methods, including: genetic compatibility, mating call comparisons, biochemical analyses and most recently, detailed morphological, mitochondrial genetic and small nucleotide polymorphism (SNP) analyses (Mahony et al. 2020; Parkin et al. 2024).

Despite the in depth molecular analysis underpinning critical phylogenetic assignment within this complex, 2n = 26 karyotypes have been described in the literature for only *L. ewingii*, *L. jervisiensis*, *L. littlejohni* and *L. verreauxii* (Woodruff 1972; White et al. 1994; Schmid et al. 2018). Confirmation of diploid number, position of nucleolus organiser regions (NORs), centromeric positions and relative chromosomal length in karyograms of individual representatives of this complex remain wanting.

Here somatic cells from *L. paraewingi*, *L. ewingii* and *L. jervisiensis* were cultured and cryopreserved in liquid nitrogen (LN2) as a resource to safeguard against possible future existential threats. The previously undescribed karyotype of *L. paraewingi* is compared to that of *L. ewingii* and *L. jervisiensis* following recovery from cryopreservation. All three karyotypes show a 2n = 26 karyotype, yet also differ in several key respects. Most notably, the morphologies of chromosomes 1, 8 and 10 are common to *L. ewingii* and *L. jervisiensis* but not to *L. paraewingi*. A secondary restriction and potential NOR are identified on the long arms of chromosome 1 of both *L. ewingii* and *L. jervisiensis*, but not *L. paraewingi*. The obscure *L. paraewingi* secondary restriction perhaps more closely relates to the more obscure NOR of *L. littlejohni* which is located subterminally on the long arm of chromosome 11 and where satellites are not always observed (White et al. 1994). Karyotypes prepared from the cryobanking of cells from these three species reinforce their phylogenetic separation and provide assurance of relevantly cryopreserved cell types for any required future conservation initiative.

Methods

Ethics

This research was conducted in compliance with the EU Directive 2010/63/EU for animal experiments and according to The Declaration of Helsinki World Medical Association Code of Ethics. Prior to experimentation, all required Australian State governmental and institutional ethics, licenses and permissions were provided (Richard Mollard, Victorian Department of Environment, Land, Water & Planning Permit number 10008085). The *L. ewingii* specimen was collected from southern Victoria by Richard Mollard under an Animal Ethics Committee Notification of Scavenged Animal Tissue, University of Melbourne. The *L. jervisiensis* specimen was collected by Michael Mahony under the New South Wales National Parks Scientific Licence SL00190. The *L. paraewingi* specimen was collected from Big River State Forest, Victoria, Australia by Matthew West under the Victoria Wildlife Research Permit No. 10009587).

Tissue culture and cryopreservation

Toe clippings were obtained from deceased and unsexed *L. ewingii* and *L. jervisiensis* and a male *L. paraewingi*. Culture, cryopreservation, thawing and DAPI karyotyping were performed according to previously described methods (Mollard 2018; Mollard et al. 2018; Mollard and Mahony 2023). Chromosomes were arranged in size by descending order, with the largest chromosome designated chromosome 1 (King et al. 1990). Centromeric position and relative lengths were determined using Image J software with the Levan plugin (Levan et al. 1964). Metacentric, submetacentric and subtelocentric chromosomal morphologies were defined as long arm to short arm ratios of 1–1.69, 1.7–2.99 and 3–6.99, respectively (Levan et al. 1964). Four metaphase spreads each from *L. ewingii* and *L. paraewingi* and eight metaphase spreads from *L. jervisiensis* were arranged in descending order of size for putative NOR assignment, centromeric location and relative length measurements to chromosome 1, not including any secondary constrictions. An extra four *L. jervisiensis* metaphase spreads were scored to accurately compare relative lengths of chromosomes 3 and 4. Images were captured at 1000 × under oil immersion with an Olympus BX60 microscope, colour CCD Leica DFC425C camera, EL-6000 Leica light source and Leica LAS-AF and QCapture Pro7 Version 7.0.5 Build 4325 software (QImaging Inc, USA).

Cells were processed in culture from toe clippings of *L. ewingii*, *L. paraewingi* and *L. jervisiensis* (representative species images shown in Fig. 1). Following 15, 15 and 3 month LN2 cryopreservation periods, respectively, *L. ewingii*, *L. paraewingi* and *L. jervisiensis* cells were thawed into 24 well plates and passaged for alternate karyotyping and recryopreservation.

Figure 1. *L. ewingii*; photographed by Matthew West at Merri Creek, Australia, 2020. *L. paraewingi*; photographed by Stephen Mahony at Wangaratta, Victoria, Australia, 2017. *L. jervisiensis*; photographed by Stephen Mahony at Mungo Brush Park Myall Lakes National Park, New South Wales, Australia, 2021.

Results

Of the first 23 *L. ewingii* metaphases spreads scored, 16 (70%) showed a 2n = 26 chromosome count, with the remaining metaphase spreads showing 22 chromosomes (number of spreads = 2), 24 chromosomes (number of spreads = 2) and 25 chromosomes (number of spreads = 3) chromosomes. Of the first 15 *L. paraewingi* metaphase spreads, 13 (87%) showed a 2n = 26 chromosome count with the remaining showing either 23 or 25 chromosomes. Of the first 71 *L. jervisiensis* metaphase spreads scored, 67 (94%) showed a 2n = 26 chromosome count, with the remaining showing either 16, 21, 24 or 25 chromosomes. A higher number of *L. jervisiensis* metaphase spreads were prepared to accurately resolve this species' unique chromosomal relative length order as outlined below. Reconstruction of the anomalous karyotypes did not reveal obvious aneuploidies such as trisomies or chromosomal pair loss or repeated aneuploidies. Diversion from the 2n = 26 count is most likely technical, therefore, attributable to loss of individual chromosomes during cell dropping and spreading for preparation of DAPI staining and scoring.

For *L. ewingii*, chromosomes 2, 6, 7, 8 and 10 are submetacentric, chromo- somes 3 and 5 are subtelocentric and chromosomes 1, 4, 9, 11, 12, and 13 are metacentric (Table 1, Figs 2, 3A–C). A DAPI negative region was apparent on the long arms of chromosome 1 of all scored *L. ewingii* metaphase spreads and presumed to represent an NOR (Figs 2, 3A–C.) For *L. paraewingi* chromosomes 1, 2, 6 and 7 are metacentric, chromosomes 3 and 5 are subtelocentric and chromosomes 4, 8, 9, 10, 11, 12 and 13 are metacentric (Table 1, Figs 2, 4A–C). No DAPI negative chro- mosomal region was apparent in any of the 15 *L. paraewingi* metaphase spreads (Figs 2, 4A–C). For *L. jervisiensis*, chromosomes 2, 7, 8 and 10 are submetacentric, chromosomes 4 and 5 are subtelocentric and chromosomes 1, 3, 9, 11, 12, and 13 are metacentric (Table 1, Figs 2, 5A–C). A DAPI negative region was apparent on the long arms of chromosome 1 of all scored *L. jervisiensis* metaphase spreads and presumed to represent an NOR (Figs 2, 5A–C).

Table 1. Centromeric position (morphology) and relative lengths of chromosomes following DAPI staining of metaphase spreads. Measurements were taken from four *L. ewingii*, four *L. paraewingi* and eight *L. jervisiensis* metaphase spreads. Long arm to short arm ratios (A.R) and relative lengths (R.L.) are provided as average plus or minus standard deviation for all scored metaphase spreads of that species. R.L. is to chromosome 1, designated as length = 1. Chromosomal morphologies (Morph) in cells with light grey shading represent those differing to *L. ewingii*. Italicised chromosomal morphologies represent *L. jervisiensis* morphologies differing to those of *L. paraewingi*.

***Litoria ewingii* Chromosome Number**							
	1	**2**	**3**	**4**	**5**	**6**	**7**
A.R	1.33 ± 0.12	1.86 ± 0.26	3.38 ± 0.73	1.32 ± 0.10	3.35 ± 0.42	1.86 ± 0.25	1.92 ± 0.36
Morph	Metacentric	Submetacentric	Subtelocentric	Metacentric	Subtelocentric	Submetacentric	Submetacentric
R.L.	1	0.771	0.7198	0.6833	0.5977	0.5418	0.4185
	8	**9**	**10**	**11**	**12**	**13**	
A.R.	1.78 ± 0.58	1.59 ± 0.42	2.06 ± 0.50	1.30 ± 0.19	1.33 ± 0.15	1.21 ± 0.16	
Morph	Submetacentric	Metacentric	Submetacentric	Metacentric	Metacentric	Metacentric	
R.L.	0.4039	0.3533	0.3435	0.2797	0.2785	0.2539	
***Litoria paraewingi* Chromosome Number**							
	1	**2**	**3**	**4**	**5**	**6**	**7**
A.R.	1.81 ± 0.26	1.87 ± 0.33	3.78 ± 0.72	1.41 ± 0.30	3.43 ± 0.41	1.97 ± 0.44	1.97 ± 0.38
Morph	Submetacentric	Submetacentric	Subtelocentric	Metacentric	Subtelocentric	Submetacentric	Submetacentric
R.L.	1	0.8937	0.8609	0.7374	0.6351	0.6192	0.5297
	8	**9**	**10**	**11**	**12**	**13**	
A.R.	1.52 ± 0.24	1.54 ± 0.24	1.55 ± 0.35	1.46 ± 0.38	1.36 ± 0.20	1.45 ± 0.28	
Morph	Metacentric	Metacentric	Metacentric	Metacentric	Metacentric	Metacentric	
R.L.	0.4585	0.4108	0.3643	0.2909	0.2532	0.1966	
***Litoria jervisiensis* Chromosome Number**							
	1	**2**	**3**	**4**	**5**	**6**	**7**
A.R.	1.12 ± 0.09	2.27 ± 0.18	1.41 ± 0.14	3.93 ± 0.48	3.69 ± 0.62	1.36 ± 0.14	2.24 ± 0.29
Morph	*Metacentric*	Submetacentric	*Metacentric*	*Subtelocentric*	Subtelocentric	*Metacentric*	Submetacentric
R.L.	1	0.7927	0.7116	0.7098	0.6112	0.5978	0.5022
	8	**9**	**10**	**11**	**12**	**13**	
A.R.	1.92 ± 0.32	1.24 ± 0.16	2.25 ± 0.56	1.53 ± 0.32	1.59 ± 0.36	1.24 ± 0.21	
Morph	*Submetacentric*	Metacentric	*Submetacentric*	Metacentric	Metacentric	Metacentric	
R.L.	0.4187	0.3439	0.3087	0.2348	0.2067	0.1769	

Discussion

Somatic cells from *L. paraewingi*, *L. ewingii* and *L. jervisiensis* were successfully cryobanked in this study with respect to demonstrating recovery of karyotypically normal cells following freeze-thaw cycles. Karyotypes of all three species showed common morphologies for chromosomes 2, 5, 7, 9, 11, 12 and 13, but also unique morphologies. For example, *L. paraewingi* chromosomes 1, 8 and 10 differed morphologically to those of *L. ewingii* and *L. jervisiensis*. The *L. jervisiensis* karyotype differed from those of *L. ewingii* and *L. paraewingi* with respect to an apparent inverted relative length assignment for its metacentric chromosome 3 and subtelocentric chromosome 4. Furthermore, a secondary restriction was discernible on the long arms of chromosome 1 for *L. ewingii* and *L. jervisiensis* but not for *L. paraewingi*. The greatest number of chromosome morphological differences was observed between *L. paraewingi* and *L. jervisiensis*.

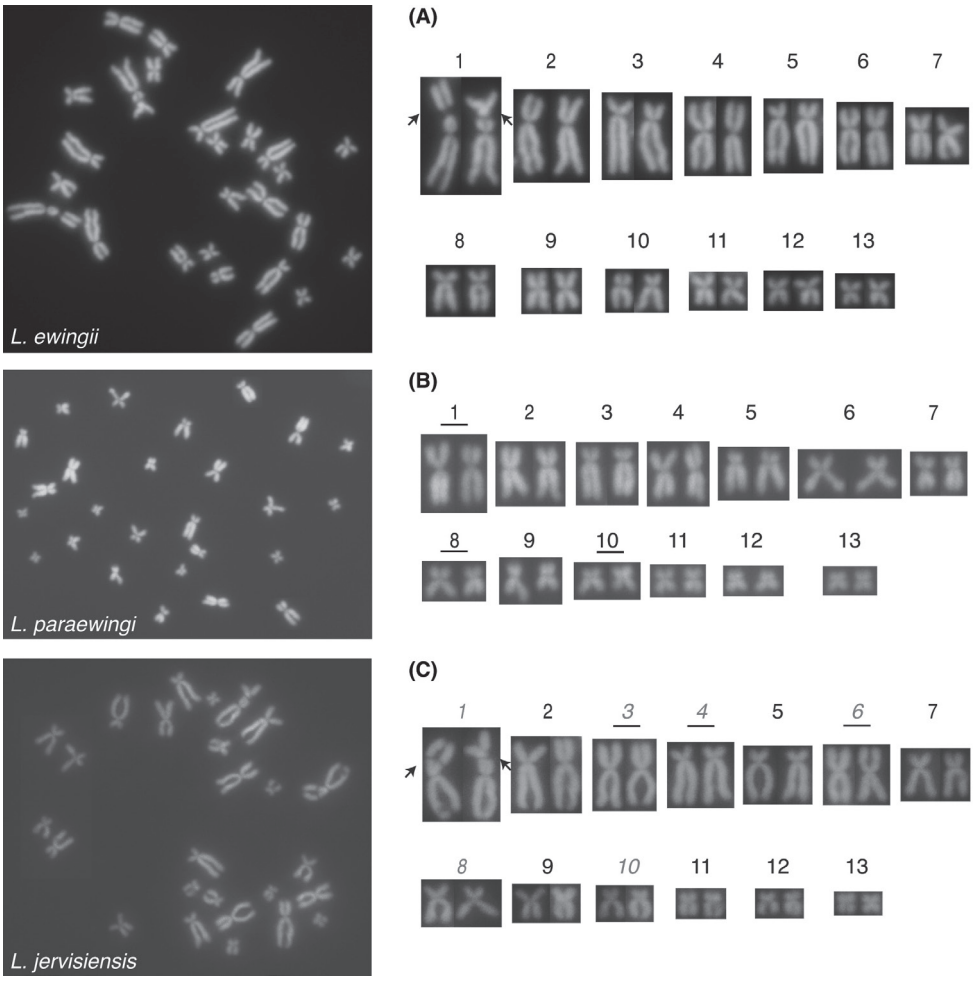

Figure 2. Representative metaphrase spreads of cryopreserved, thawed and cultured cells **A** *L. ewingii* **B** *L. paraewingi* **C** *L. jervisiensis*. Arrows indicate DAPI negative regions, or presumptive NORs. No DAPI negative regions were apparent in the *L. paraewingi* metaphase spreads. As per Table 1, underlined numbers represent those morphologies differing to *L. ewingii* and italicised (in red) numbers represent *L. jervisiensis* morphologies differing to those of *L. paraewingi*.

L. paraewingi is considered a cryptic species due to its high holotypic similarity to *L. ewingii*, with differentiation based upon detailed call analysis, genetic compatibility and molecular taxonomic analysis (Watson et al. 1971; Mahony et al. 2020). Here, by DAPI staining, *L. paraewingi* chromosomes 1, 8 and 10 showed marked divergence from that of *L. ewingii*, providing a further cytological differentiation of these species. The obvious *L. ewingii* and *L. jervisiensis* secondary constriction discernible on the long arms of all chromosome 1 metaphase spreads following DAPI staining is similar to that described previously for *L. verreauxii*, (Schmid et al. 2018). Apparent absence of a DAPI negative region, or secondary restriction, from the karyotype of *L. paraew-*

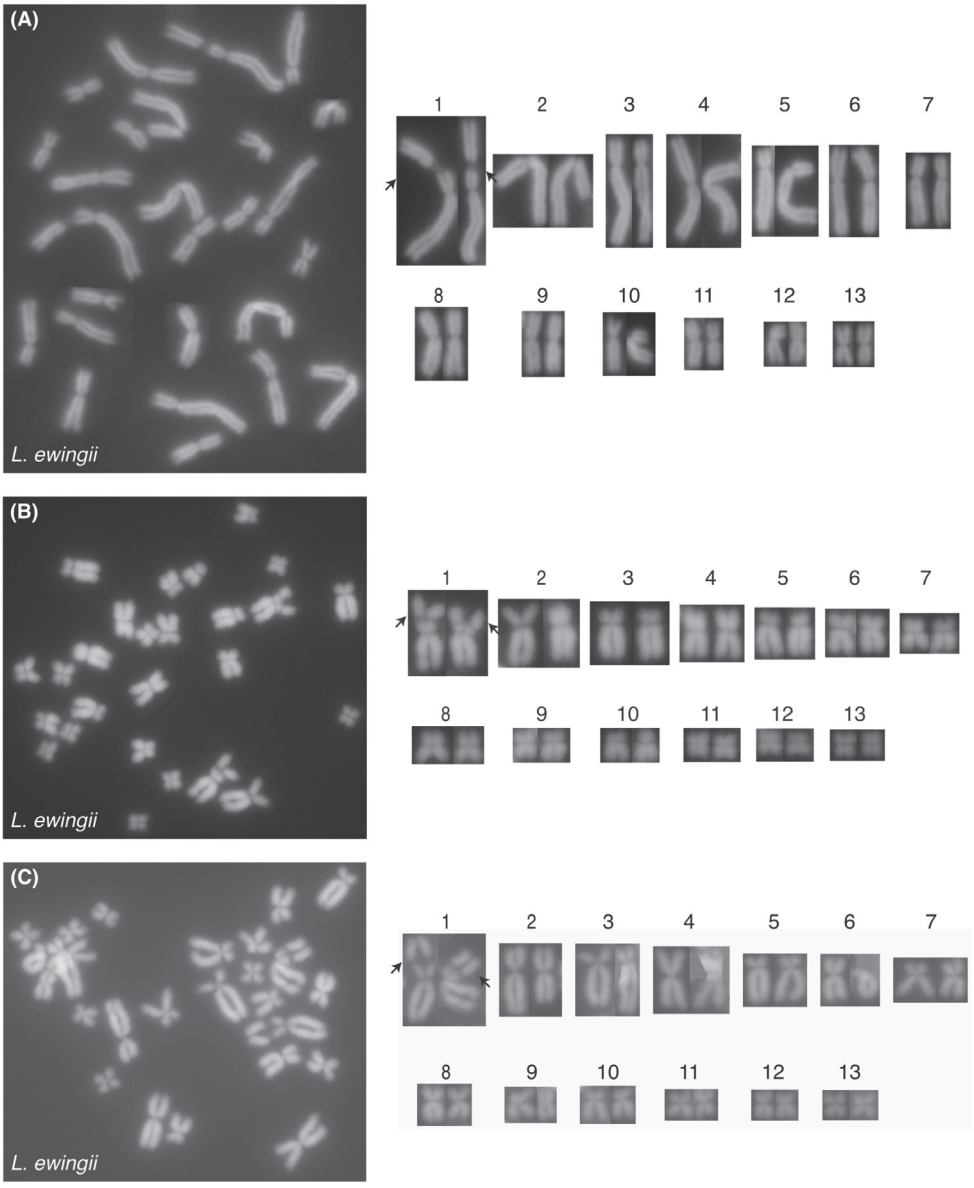

Figure 3. Metaphrase spreads of cryopreserved, thawed and cultured cells from *L. ewingii*. **A–C** three individual metaphase spreads. Arrows indicate DAPI negative regions, or presumptive NORs.

ingi may be a more similar observation to that reported for *L. littlejohni* (White et al. 1994). For *L. littlejohni* an NOR was discernible sub-terminally on the long arms of chromosome 11 most notably in silver nitrate (Ag-NO_3) stained chromosomes, with satellites not always observable with conventional aceto-orcein staining. Confirmation of an *L. paraewingi* secondary restriction or NOR location, therefore, may be better re-

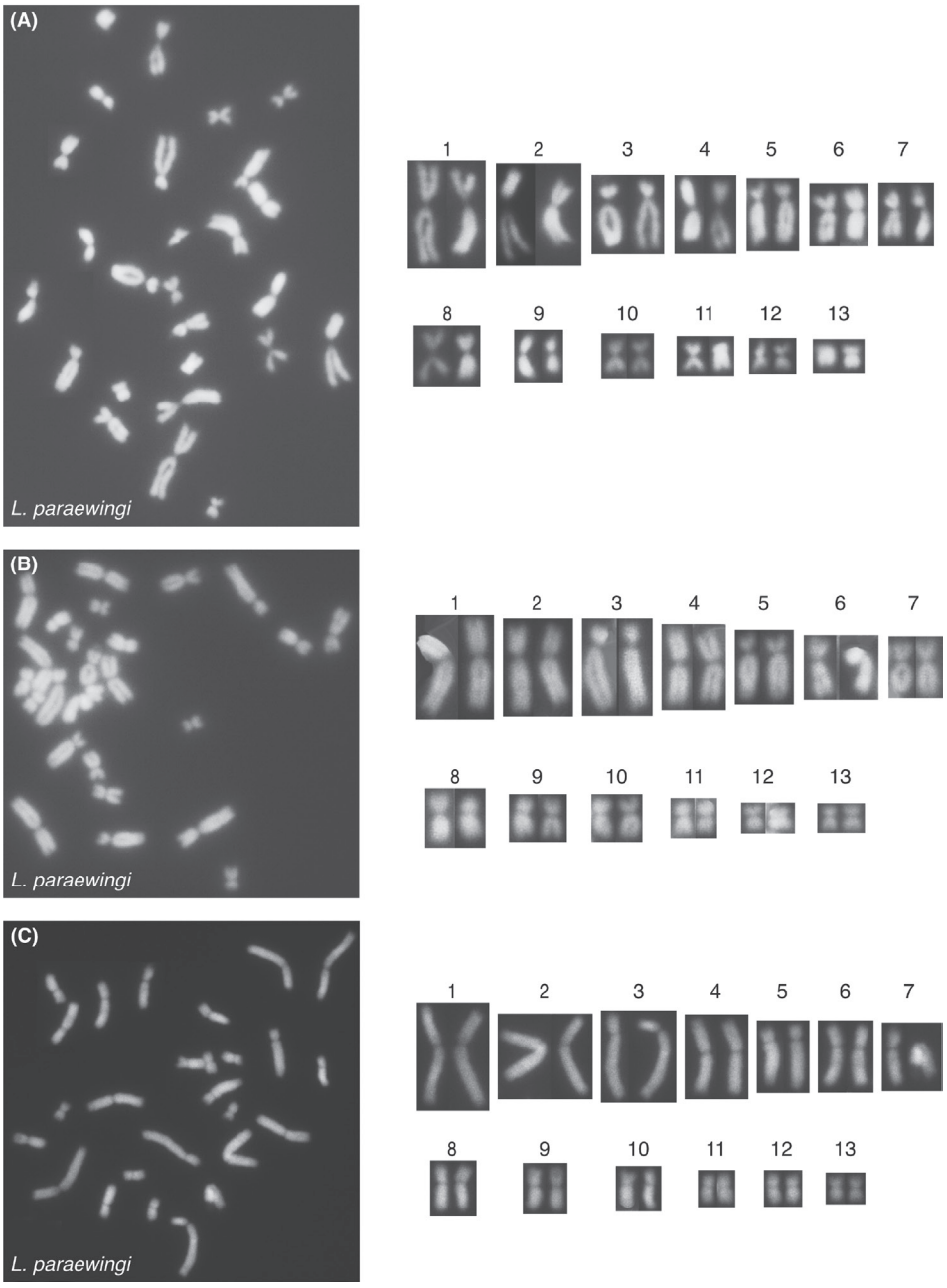

Figure 4. Metaphrase spreads of cryopreserved, thawed and cultured cells from *L. paraewingi*. **A–C** three individual metaphase spreads. No DAPI negative regions, or presumptive NORs, were apparent.

solved in the future by alternative staining techniques such as Ag-NO_3 staining or 18S or 28S rDNA FISH if also located more terminally without obvious satellites (White et al. 1994; Zaleśna et al. 2017).

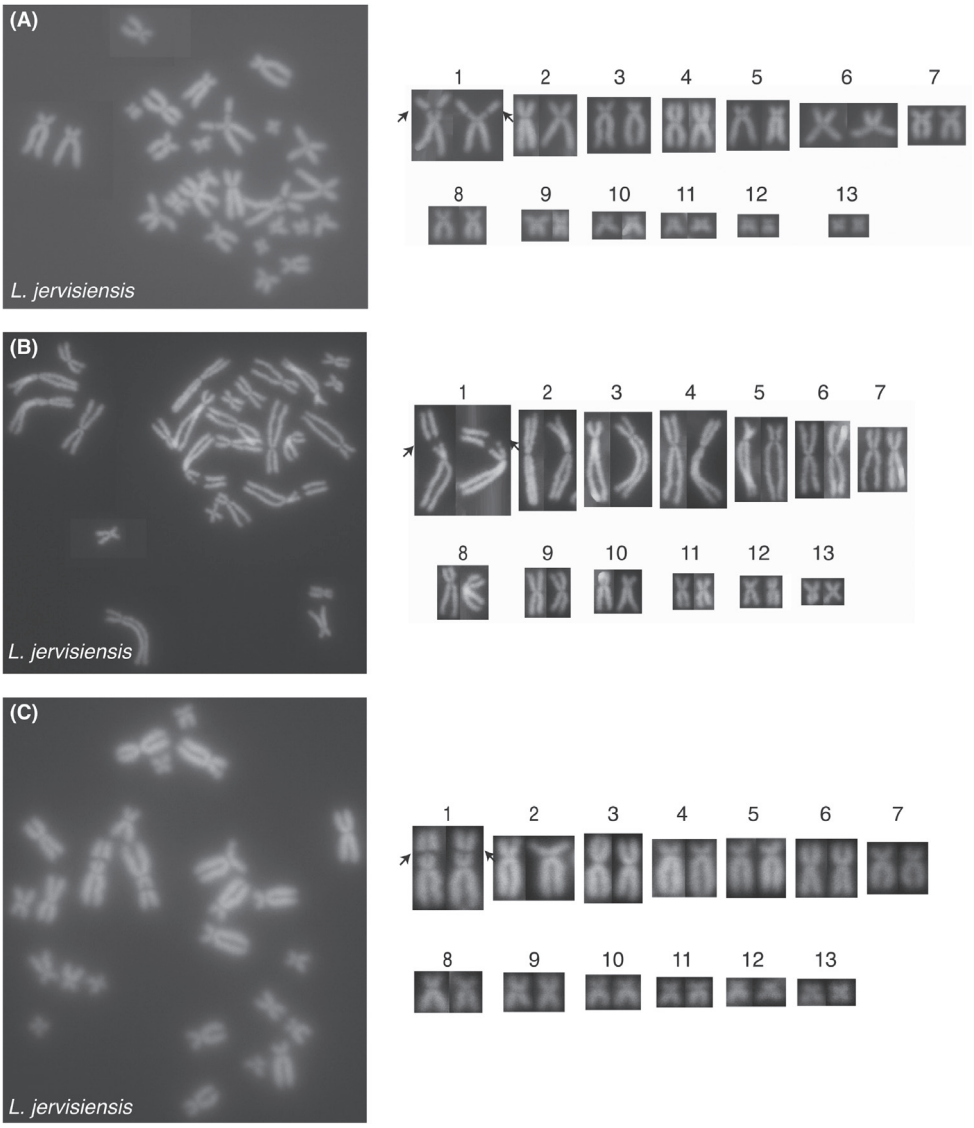

Figure 5. Metaphrase spreads of cryopreserved, thawed and cultured cells from *L. jervisiensis*. **A–C** three individual metaphase spreads. Arrows indicate DAPI negative regions, or presumptive NORs.

Conclusion

In conclusion, the karyotypes of *L. paraewingi*, *L. ewingii* and *L. jervisiensis* demonstrate a high level of morphological conservation yet also many unique attributes. These data support the phylogenetic separation of these species based upon previous behavioural, genetic compatibility, biochemical and molecular analyses (Mahony et al. 2020).

Competing interests

Richard Mollard has registered a company called Amphicell Pty Ltd (www.amphicell.com). Amphicell Pty Ltd received no funding for this work and privately provided the materials to execute the experimental procedures described in this study.

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ORCID

Michael Mahony https://orcid.org/0000-0002-1042-0848

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SHORT COMMUNICATION

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Different observers introduce not negligible biases in comparative karyomorphological studies

Lorenzo Peruzzi[1], Jacopo Franzoni[1], Manuel Tiburtini[1], Emanuela Abidi[1], Emiliano Alù[1], Giulio Barone[2], Elisabetta Bianchi[3], Chiara Cataudella[1], Emanuela Di Iorio[4], Maria Guerrina[5], Fabio Mondello[6], Luca Paino[4], Mario Pentassuglia[1], Manuela Porrovecchio[7], Giovanni Rivieccio[8], Eugenia Siccardi[3], Adriano Stinca[9], Alessio Tei[10], Virginia Volanti[3], Antonio Giacò[1]

1 *PLANTSEED Lab, Department of Biology, University of Pisa, Pisa, Italy* **2** *Department of Agricultural, Forest and Food Sciences, University of Palermo, Palermo, Italy* **3** *Department of Biology, University of Firenze, Firenze, Italy* **4** *Department of Biology, University of Napoli "Federico II", Botanical Garden, Napoli, Italy* **5** *DISTAV, University of Genova, Genova, Italy* **6** *Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences, University of Messina, Messina, Italy* **7** *Department of Biological, Geological and Environmental Sciences, University of Catania, Catania, Italy* **8** *Department of Chemical, Physical, Mathematical and Natural Sciences, University of Sassari, Sassari, Italy* **9** *Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania Luigi Vanvitelli, Caserta, Italy* **10** *Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy*

Corresponding author: Lorenzo Peruzzi (lorenzo.peruzzi@unipi.it)

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Abstract

Within a practical course of cytotaxonomy organized in Pisa (Italy) on February 2024 by the Group for Floristics, Systematics and Evolution of the Italian Botanical Society, we tested whether using image analysis softwares possible biases are still introduced by different observers. We conclude that observer bias selectively applies in possibly overestimating the length of short arms in a karyotype. As a consequence, the parameters most sensitive to these possible errors are CV_{CI} and CV_{CL}, and to a less degree M_{CA} and THL. To achieve more stable results among observers, a still lacking standardized measurement protocol could be helpful.

Keywords

Cytogenetics, cytosystematics, cytotaxonomy, karyotype asymmetry, karyotype structure

Introduction

Karyomorphology is an easy, cheap and powerful approach to obtain useful basic comparative information in systematic studies (Astuti et al. 2017). This usually implies the measurement of chromosomes (i.e. length of long arm [L], short arm [S], and other derived information) in spread metaphase plates, to describe the phenotypic aspect of the chromosome complement (Levin 2002; Guerra 2012). The most commonly used traits to characterize a karyotype structure and asymmetry are: the chromosome number ($2n$), the basic chromosome number (x), the total haploid (monoploid) chromosome length (THL), the mean centromeric asymmetry (M_{CA}), the coefficient of variation of chromosome length (CV_{CL}), and the coefficient of variation of centromeric index (CV_{CI}) (Peruzzi and Altınordu 2014).

However, while obtaining the chromosome number and basic chromosome number (see also Peruzzi 2013) should be a relatively easy task, it is well known that the reliability of karyomorphological measurements can be influenced by two main causes (Sybenga 1959; Bentzer et al. 1971): a) variation in actual chromosome length, b) variation caused by inaccuracy of the measurement. The first cause is biological and linked to several phenomena, which may alter the degree of chromosome condensation (e.g., Bentzer et al. 1971; Mártonfiová 2013; Mehravi et al. 2022; Franzoni et al. 2024). The second cause of variation is "artificial" and pertains to variation in methods and observer (Sybenga 1959; Essad et al. 1966; Bentzer et al. 1971). In particular, Bentzer et al. (1971) also addressed the question whether the same measurements made by different people produce consistent data, and showed that this is not the case, especially using camera lucida drawings of metaphase plates. Starting from the early 2000s, a new era of chromosome measurement through image analysis softwares started (e.g., Rasband 1997 onwards, Mirzaghaderi and Marzangi 2015; Altınordu et al. 2016; Kirov et al. 2017; Liu et al. 2023; Stossi and Singh 2023), certainly making the measurements more accurate than in the twentieth century, when scholars were forcedly based on camera lucida drawings or printed microphotographs. However, no information is available whether using image analysis softwares possible biases are still introduced by different observers in measuring the very same microphotographs.

We addressed this problem within a practical course organized in Pisa (Italy) between 6 and 9 February 2024 by the Group for Floristics, Systematics and Evolution of the Italian Botanical Society.

Material and methods

A metaphase plate of the diploid ($2n$ = 18) angiosperm *Santolina decumbens* Miller, 1768 subsp. *diversifolia* (Jordan et Fourreau,1869) Giacò et Peruzzi, 2022 (Asteraceae; Giacò et al. 2023) obtained from plants collected in Sisteron, Provence-Alpes-Côte d'Azur, France (Fig. 1) was taken from those used in the work by Giacò et al. (2022). This metaphase plate was given to all the participants to the course, who independently measured it, by using the software MATO (Liu et al. 2023).

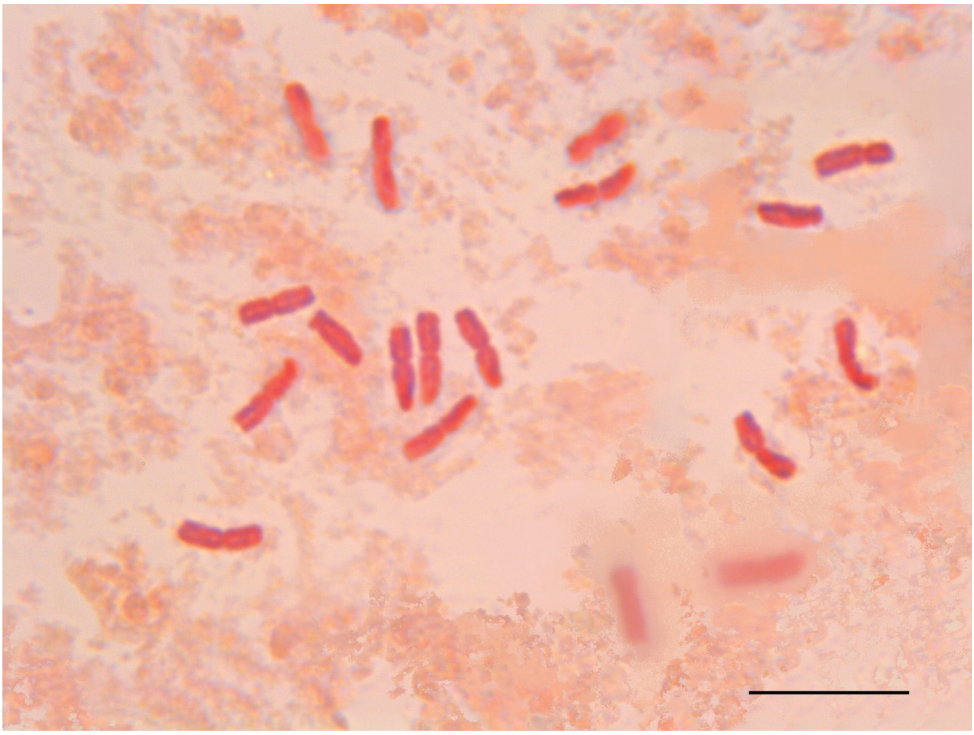

Figure 1. The metaphase plate of *Santolina decumbens* subsp. *diversifolia* (from Giacò et al. 2022) distributed to the participants for independent measurements. The image was built by pasting several images at different focus, in order to be able to see all the $2n = 18$ chromosomes in the same picture. Scale bar: 10 μm.

We focused on the following quantitative traits (Peruzzi and Altınordu 2014; Astuti et al. 2017):

- THL (total haploid [monoploid] length of chromosome complement). It is a gross proxy of genome size (Carta and Peruzzi 2016; Franzoni et al. 2024), and is obtained by the sum of the length of all the chromosomes in a metaphase plate, divided by the ploidy level.
- M_{CA} (mean centromeric asymmetry). It expresses the intrachromosomal karyotype asymmetry (Peruzzi and Eroğlu 2013), and is calculated as the mean value of the difference between the two (complementary) proportions L/(L+S) and S/(L+S), multiplied by 100.
- CV_{CL} (coefficient of variation of chromosome length). It expresses the interchromosomal karyotype asymmetry (Paszko 2006), and is calculated as the standard deviation of chromosome lengths (L+S) in a complement, divided by the mean chromosome length and multiplied by 100.
- CV_{CI} (coefficient of variation of centromeric index). It expresses the degree of heterogeneity in the position of centromere in a karyotype (Zuo and Yuan 2011), and is calculated as the standard deviation of centromeric index S/(L+S) in a complement, divided by the mean centromeric index and multiplied by 100.

The variation of each karyomorphological trait cited above was illustrated by means of boxplots. Then, to test which karyomorphological traits are more prone to biases introduced by different observers, for each trait a CV was calculated. Finally, correlations between parameters were tested by Pearson's correlation coefficient. All the analyses have been carried out in PAST 4.17 (Hammer et al. 2001; Hammer 2024).

Results

The variation of each karyomorphological trait is illustrated in Fig. 2, which is based on the data reported in Suppl. material 1: table S1. The coefficients of variation show the highest value concerning CV_{CI} (17.3%) and CV_{CL} (13.4%), and the lowest for M_{CA} (4.7%) and THL (9.4%).

According to Table 1, the only highly statistically significant ($p < 0.01$) and negative correlation is between THL and M_{CA} (Fig. 3). A positive correlation between CV_{CL} and CV_{CI} is only marginally significant ($p < 0.05$), while all other correlations are not significant ($p > 0.05$).

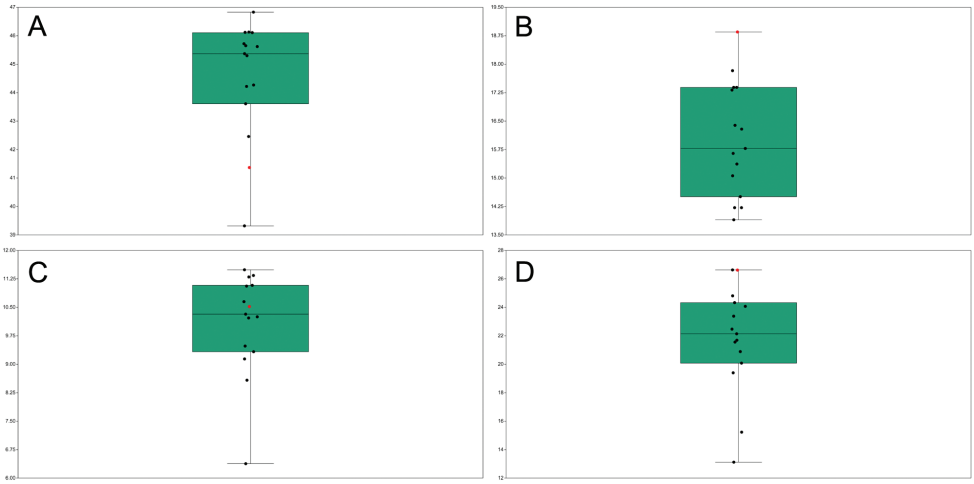

Figure 2. Boxplots with jitters illustrating the variability in the karyomorphological traits THL (A), M_{CA} (B), CV_{CL} (C), and CV_{CI} (D) independently calculated by the participants based on the same metaphase plate of *Santolina decumbens* subsp. *diversifolia* in Fig. 1. The red dot is the measurement n. 15 (see Suppl. material 1: table S1), used to build the karyotype of this population by Giacò et al. (2022).

Discussion

The significant negative correlation between THL and M_{CA} points towards selective observer bias that tends to overestimate the length of the short arm. Indeed, such an overestimation could at the same time cause an increment of THL and a decrease in MCA. Indeed, already Sybenga (1959) and Bentzer et al. (1971) evi-

Table 1. Pearson's correlation coefficients and p values among the considered quantitative karyomorphological traits based on 15 measurements independently made by different evaluators on the same metaphase plate of *Santolina decumbens* subsp. *diversifolia*. In bold are highlighted the significant correlations.

	THL	**M_{CA}**	**CV_{CL}**	**CV_{CI}**
THL		**p = 0.0022365**	p = 0.40465	p = 0.055789
M_{CA}	**-0.7248**		p = 0.38316	p = 0.056293
CV_{CL}	-0.23236	+0.38316		**p = 0.010211**
CV_{CI}	-0.50333	+0.63978	**+0.50225**	

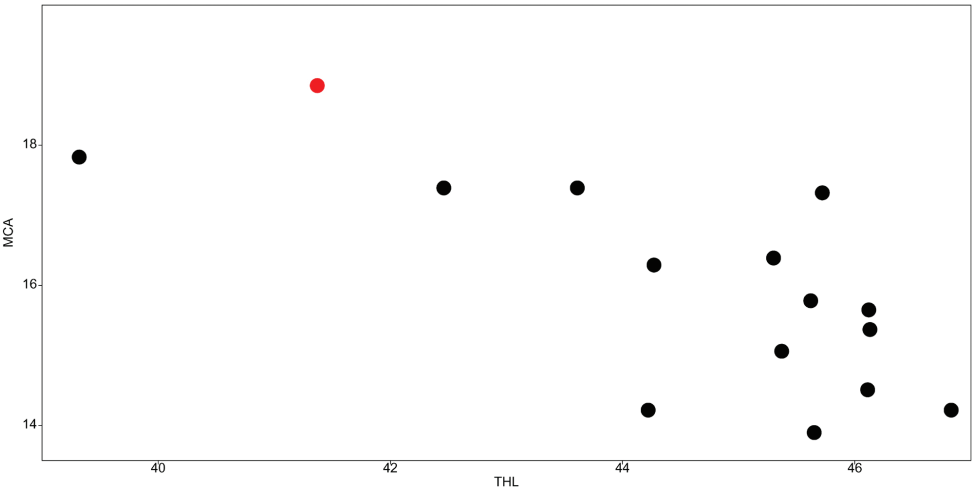

Figure 3. Scatter plot THL (x axis) vs. M_{CA} (y axis), highlighting the significant negative correlation among these two karyomorphological traits. The red dot is the measurement n. 15 (see Suppl. material 1: table S1), used to build the karyotype of this population by Giacò et al. (2022).

denced how possible measurement errors can become of increasing importance in case of small chromosomes / small chromosome arms. Possibly, the same correlation is not found in CV_{CI} because this parameter is based on centromeric index [S/(L+S)], so that an overestimation of short arm would have consequences both at the numerator and at the denominator of the centromeric index. On one side, this causes the lack of correlation between CV_{CI} and THL, while on the other side it causes a lot of further variation in this parameter, which is the most subjected to observer bias (up to 17.3% in our experiment). These errors may be due to the different decisions made when selecting the centromere, as no standardized protocol has ever been proposed.

We can conclude that, in karyomorphology, observer bias selectively applies in possibly overestimating the length of short arms in a karyotype. As a consequence, the parameters most sensitive to these possible errors are CV_{CI} and CV_{CL}, and to a less degree M_{CA} and THL.

Accordingly, we recommend special attention in recognizing and measuring correctly the short arms of chromosomes, which are the main source of observer bias in cytogenetics. To achieve this, a homogeneous approach among observers could be helpful. Moreover, the motto already claimed by Bentzer et al. (1971) "*in the course of an investigation all the measurements should be made by the same person*" also fully applies to the era of image analysis.

Author contributions

Lorenzo Peruzzi – methodology, Lorenzo Peruzzi – validation, Antonio Giacò, Emanuela Abidi, Emiliano Alù, Giulio Barone, Elisabetta Bianchi, Chiara Cataudella, Emanuela Di Iorio, Maria Guerrina, Fabio Mondello, Luca Paino, Mario Pentassuglia, Manuela Porrovecchio, Giovanni Rivieccio, Eugenia Siccardi, Adriano Stinca, Alessio Tei, Virginia Volanti – investigation, Lorenzo Peruzzi – writing and original draft preparation, Jacopo Franzoni, Antonio Giacò, Manuel Tiburtini – writing – review and editing, Lorenzo Peruzzi – funding acquisition. All authors have read and agreed to the published version of the manuscript.

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ORCID

Lorenzo Peruzzi https://orcid.org/0000-0001-9008-273X
Jacopo Franzoni https://orcid.org/0000-0002-0888-8396
Manuel Tiburtini https://orcid.org/0000-0003-4985-3058
Giulio Barone https://orcid.org/0000-0002-6345-3117
Elisabetta Bianchi https://orcid.org/0000-0003-1197-4081
Fabio Mondello https://orcid.org/0000-0002-9522-6277
Manuela Porrovecchio https://orcid.org/0009-0009-2349-4975
Giovanni Rivieccio https://orcid.org/0000-0003-0840-0212
Eugenia Siccardi https://orcid.org/0009-0008-4738-0633
Adriano Stinca https://orcid.org/0000-0002-8275-0184
Alessio Tei https://orcid.org/0009-0001-1326-609X

Supplementary material 1

Karyomorphological traits

Authors: Lorenzo Peruzzi, Jacopo Franzoni, Manuel Tiburtini, Emanuela Abidi, Emiliano Alù, Giulio Barone, Elisabetta Bianchi, Chiara Cataudella, Emanuela Di Iorio, Maria Guerrina, Fabio Mondello, Luca Paino, Mario Pentassuglia, Manuela Porrovecchio, Giovanni Rivieccio, Eugenia Siccardi, Adriano Stinca, Alessio Tei, Virginia Volanti, Antonio Giacò

Data type: docx

Explanation note: **table S1.** Karyomorphological traits independently calculated by the participants based on the same metaphase plate of *Santolina decumbens* subsp. *diversifolia* in Fig. 1.

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RESEARCH ARTICLE

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Description of the complete rDNA repeat unit structure of *Coturnix japonica* Temminck et Schlegel, 1849 (Aves)

Alina Zhukova[1], Gennadii Zakharov[2,3], Olga Pavlova[4,5], Alsu Saifitdinova[1,4,6]

1 *Herzen State Pedagogical University of Russia, Saint Petersburg, Russia* **2** *Pavlov Institute of Physiology, Russian Academy of Sciences, Saint Petersburg, Russia* **3** *EPAM Systems Inc., Saint Petersburg, Russia* **4** *International Centre for Reproductive Medicine, Saint Petersburg, Russia* **5** *Beagle Ltd., Saint Petersburg, Russia* **6** *Saint Petersburg State University, Saint Petersburg, Russia*

Corresponding author: Alsu Saifitdinova (saifitdinova@mail.ru)

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Abstract

Ribosomal RNA (18S, 5.8S, 28S) gene clusters in genomes form regions that consist of multiple tandem repeats. They are located on a single or several pairs of chromosomes and play an important role in the formation of the nucleolus responsible for the assembly of ribosome subunits. The rRNA gene cluster sequences are widely used for taxonomic studies, however at present, complete information on the avian rDNA repeat unit structure including intergenic spacer sequence is available only for the chicken (*Gallus gallus domesticus* Linnaeus, 1758). The GC enrichment and high-order repeats peculiarities within the intergenic spacer described for the chicken rDNA cluster may be responsible for these failures. The karyotype of the Japanese quail (*Coturnix japonica* Temminck et Schlegel, 1849) deserves close attention because, unlike most birds, it has three pairs of nucleolar organizer bearing chromosomes, two of which are microchromosomes enriched in repeating elements and heterochromatin that carry translocated terminal nucleolar organizers. Here we assembled and annotated the complete Japanese quail ribosomal gene cluster sequence of 21166 base pairs (GenBank under the registration tag BankIt2509210 Coturnix OK523374). This is the second deciphered avian rDNA cluster after the chicken. Despite the revealed high similarity with the chicken corresponding sequence, it has a number of specific features, which include a slightly lower degree of GC content and the presence of bendable elements in the content of both the transcribed spacer I and the non-transcribed intergenic spacer.

Keywords

Bendable DNA, nanopore sequencing, Japanese quail, nucleolar organizer, RNA polymerase I promoter

Introduction

Genomic elements responsible for conservative processes are of interest both from the point of view of studying the mechanisms of their implementation and for solving various molecular taxonomic problems. Protein synthesis or translation is one of the key and most ancient cellular processes. Translation takes place on ribosomes - complex particles of large and small subunits each consisting of two-thirds of ribosomal RNAs (rRNAs) and one-third of ribosomal proteins (Alberts et al. 2002). It is rRNA molecules, not proteins, that are responsible for the overall three-dimensional structure of the ribosome, the correct positioning of tRNA on the mRNA template, and enzymatic activity in creating peptide bonds (Ban et al. 2000; Alberts et al. 2002). In the animal cell there are four types of highly conservative rRNAs: 18S, a component of a small ribosomal subunit, and 5S, 5.8S, 28S forming a large ribosomal subunit. The 5S rRNA gene copies transcribed by RNA polymerase III typically make up a separate locus in the genome whereas the 18S, 5.8S, 28S rRNA genes are situated together in a cluster (Fig. 1), which is repeated many times forming loci of the ribosomal DNA (rDNA) generally referred to the nucleolus organizer regions. rDNA cluster the genes are separated by internal transcribed spacers (ITS1 and ITS2) and flanked by external transcribed spacers, 5'-ETS at the 5'-end the 18S rRNA gene and 3'-ETS at the 3'-end of the 28S rRNA gene. Transcription of rDNA clusters is operated by RNA polymerase I complex synthesizing a single precursor rRNA molecule (47S pre-rRNA), which is subsequently processed in three separate rRNAs. rDNA clusters are separated by an intergenic spacers (IGS), together they form rDNA repeated units (Alberts et al. 2002). The IGS comprises various inner repeats, and their deletion or amplification are the main causes of the IGS length variability, e.g. from ~3.3 kb in *Drosophila pseudoobscura* Frolova et Astaurov, 1929 (Mateos and Markow 2005) up to ~30 kb in human (Hori and Shimamoto 2021). The IGS contains rRNA promoter and terminator sites, regulatory elements, such as enhancer elements, which control pre-rRNA synthesis (Goodfellow and Zomerdijk 2013). Replication origins and replication fork barriers (RFBs) that prevent conflicts between the replication and transcription machineries are also located in the IGS (Brewer et al. 1992; Kobayashi et al. 1992; Akamatsu and Kobayashi 2015). Analysis of rDNA methylation showed that all the noncoding regions are heavily methylated, whereas about half of the coding regions are clearly unmethylated. But under certain circumstances, such as stress, repressive chromatin modifications can be lost provoking the IGS transcription by RNA polymerase II (Earley et al. 2010; Audas et al. 2012; Saka et al. 2013; Bierhoff et al. 2014). In contrast to highly conservative gene sequences, spacer sequences evolve rapidly, mainly through nucleotide substitutions as well as deletions, insertions, or duplications of DNA segments, thus being highly variable (Arnheim 1980). They are widely used for molecular systematics and species identification, but data on the organization of the rDNA repeat units in birds are very limited (Dyomin et al. 2017).

The repetition of individual motifs may create the preconditions for their isolation and study through cloning. Early studies were based on the accurate sequence analysis of rDNA-containing plasmids and cosmids (Gonzalez and Sylvester 1995; Gangloff 1996).

The routine technologies, such as Sanger sequencing or Next Generation Sequencing (NGS), generating relatively short reads, cannot provide sufficient overlap of repetitive regions. Long tandem repeated arrays with complex inner high-order repeat structures, which include rDNA repeat units, cannot be assembled from short-read sequencing data. The development of long-read sequencing technologies, such as the Pacific Biosciences (PacBio) or Oxford Nanopore Technologies systems, has made it possible to explore rDNA sequences. A good example of the use of such approach is the deciphering of the complete rDNA repeat unit of the chicken *Gallus gallus domesticus* Linnaeus, 1758, which has only recently been made possible by PacBio sequencing of a BAC clone with an rDNA insert (Dyomin et al. 2019). The structure of the chicken rDNA differs from that of rDNA in human, apes, clawed frogs or fish, due to the highly GC-rich (up to 80%) spacers and internal repeats in the IGS (Dyomin et al. 2016, 2019). To date, the chicken rDNA repeat remains the only fully annotated avian rDNA despite the growing number of genome sequencing data from various bird species based on all existing sequencing platforms. The complexity of working with avian rDNA is that they are very rich in GC pairs, at least in ITS1 and ITS2 (Dyomin et al. 2017), and conventional targeted sequencing with site-specific primers is practically impossible even using reagents aimed at sequencing GC-rich templates. The complex structure of high-order repeats within the IGS that we discovered earlier (Dyomin et al. 2019) may lead to the possibility of complex secondary structure and intermolecular connections, which, in combination with GC enrichment, may be the reason for the depletion of data on these DNA regions during whole-genome sequencing. Extending the list of bird species with a sequenced and annotated rDNA repeat units is important not only for the development of taxonomic studies like to other systematic groups of creatures, but also for understanding the modes of karyotype evolution involving the nucleolar organizer region.

Coturnix japonica Temminck et Schlegel, 1849 (Japanese quail) is a domesticated bird, widely used in the poultry industry for the production of meat and eggs. It also serves as a model species in biomedical, physiological, and embryological research, due to its small body size, short generation interval, and high fecundity (Lyte 2021; Strawn 2020; Molele 2022). *C. japonica* is closely related to *G. gallus domesticus*, belonging to the same family Phasianidae, order Galliformes. These two species separated ~35 Mya (van Tuinen and Dyke 2004). They have the same chromosome number 2n = 78, no interchromosomal rearrangements have occurred (Takagi and Sasaki 1974; Schmid et al. 2000; Shibusawa et al. 2001, 2004; Kayang et al. 2006; Zlotina et al. 2012). An interesting difference from the standard Galliformes chromosome-evolution model is the finding of three nucleolus organizers loci in Japanese quail microchromosomes in contrast to the single locus in chicken and turkey (Kretschmer 2018; McPherson et al. 2014). Two pairs of microchromosomes bearing terminal nucleolar organizers remain unidentified, information about their composition is completely absent from the chromosome-level genome assembly data for Japanese quail (GenBank accession: GCA_001577835.2) and molecular markers for them are not described. One of the loci is situated on the Japanese quail microchromosome orthologous to chicken chromosome 16 (GGA16), bearing genes of the major histocompatibility complex (MHC). It was specified that the

MHC-B and nucleolus organizer were localized at the q-arm. The MHC-B was more telomere proximal than the nucleolus organizer, similar to the genetic arrangement found in chicken. Furthermore, the q-arm is a DAPI-bright region, indicative of GC-rich DNA (McPherson et al. 2014). In the Japanese quail genome assembly *Coturnix japonica* 2.1 sequences related to nucleolus organizer are absent: CJA16 (orthologue of GGA16) comprises 344 kb and contains dozens of annotated genes not corresponding to rDNA. Thus, the aim of this study was to decipher rDNA repeat unit sequence in order to find conservative and nonconservative elements in the IGS of two bird species and to understand the evolutionary dynamics of the rDNA in birds. We took advantage of the Oxford Nanopore sequencing technology to overcome the problems associated with the high GC enrichment of rDNA and the abundance of internal repeats.

Material and methods

All manipulations with live animals and euthanasia were approved by Saint Petersburg State University Ethics Committee (statement # 131-03-2, issued on June 1, 2017).

Total genomic DNA was isolated from red blood cell nuclei of a Japanese quail female (Estonian breed) using standard phenol extraction procedures (Sambrook et al. 1991). The quality of the samples was assessed by capillary electrophoresis on a Qsep1 (BiOptic Inc., USA) device. DNA quantification was performed using a Qubit 4 fluorimeter (Thermo Fisher Scientific, USA). The libraries of the total genomic DNA were prepared using the NEBNext reagents (New England Biolabs, USA): NEBNext End repair / dA-tailing Module (E7546), NEBNext FFPE Repair Mix (M6630), NEB Blunt/TA Ligase Master Mix (M0367) following the manufacturer's protocol. We generated Oxford Nanopore long reads on the MinION device operating with MinKNOW nanopore sequencing software using SQK-LSK109 library preparation kit followed by sequencing on a SpotON Flow Cell (R9.4) Single (FLO-MIN106D) according to the manufacturer's instructions (Oxford Nanopore Technologies). The use of barcoded adapter BC02 (TCGATTCCGTTTGTAGTCGTCTGT) from Rapid Barcoding Sequencing Kit (SQK-RBK004) allowed the use of 1/10 GC-rich avian DNA samples in the flow cell load and improved the proportion of reads with complex secondary structure despite an overall decrease in coverage. The longest continuous raw reads containing in part or in whole the Japanese quail rDNA repeating unit (Suppl. material 1) allowed for the first time to obtain data on its organization, including the sequence of IGS, and to clarify sequencing and basecalling errors, as well as for sequence annotation, we performed additional alignments with all available data.

We began our bioinformatics analysis by searching for highly conserved sequences 18S–28S rRNA sequences based on data form chicken (Dyomin et al. 2016) in publicly available data of Japanese quail sequences and assembly data on NCBI database from the PRJNA292031 project (Nishibori et al. 2001). The fishing was carried out by using conserved sequences from the chicken rRNA gene cluster as the query. The contig derived from the incomplete Illumina read data was used to identify target data

among the raw nanopore reads. Guppy (version 4.2.5) software was used for advanced basecalling and Minimap2, a versatile pairwise aligner for genomic and spliced nucleotide sequences (Li 2016), was used to mapping reads and de novo assembly.

Selected raw nanopore reads were converted into fasta format using the AWK command-line script (Free Software Foundation, Inc.). Using the fasta files as the database for BLAST and the 18S–28S sequence of *C. japonica* as the query we created a separate file containing .fastq nanopore reads that have similarities with the quail rDNA genes. Finally, we used SPADES 3.15.3 with default parameters to assemble all these sequences into contigs (Meleshko et al. 2019). To verify the validity of this approach, we performed assembly of the original nanopore reads without filtering using SPADES with the same settings.

To validate our results, we used publicly available *C. japonica* whole genome sequencing data obtained on Illumina next generation sequencing platform (NCBI SRR2159508). We filtered the reads using the TRIMMOMATIC tool (Bolger et al. 2014). We then used the contigs obtained in the previous steps as reference sequences and aligned all Illumina reads using the BWA tool (Li and Durbin 2010). The corresponding reads were extracted using the SAMTOOLS package (Danecek et al. 2021) and de novo assembled using SPADES. Finally, we used GATK4.0 Haplotype Caller algorithms (Poplin et al. 2021) to check the corrections made by the Illumina data. rDNA sequence annotation was performed using GENEIOUS 9.1 (http://www.geneious.com).

Sequence annotation with gene boundary determination was performed based on conserved element data. Determination of the location of the promoter and transcription start site was accomplished based on transcript mapping from RNA-seq data (NCBI SRX9608520, SRX9608583, SRX574377) that were downloaded using SRA-toolkit (http://www.sthda.com). The reads were trimmed using the TRIM_GALORE program (https://www.bioinformatics.babraham.ac.uk). Mapping reads were conducted using BOWTIE2 (http://bowtie-bio.sourceforge.net). The resulting SAM file was converted, into a BAM file sorted from duplicated rows using SAMTOOLS (http://samtools.sourceforge.net). Then we converted BAM file to WIN format using the following script: https://github.com/MikeAxtell/bam2wig. The sequence coverage was calculated using SAMTOOLS. We visualized the coverage and found the starting point of transcription using IGV browser (https://software.broadinstitute.org) (Suppl. material 2).

Results

Because rDNA is typically underrepresented and fragmented in avian whole genome sequencing data, we first turned to transcriptome sequencing data, where ribosomal RNA is expected to be represented, to obtain primary information. Among Illumina reads from the PRJNA292031 project (Nishibori et al. 2001) we selected 510 reads by alignment to a reference chicken rDNA based sequence containing conservative rRNA regions. They were assembled into a three contigs with the length of 1065–3679 bp overlapping 18S–28S rDNA region, which was used to search for target reads in the following sequencing data obtained.

Using Oxford Nanopore Technology whole-genome sequencing we obtained 36524 nucleotide sequences, 74 of which were then aligned with 18S–28S rDNA region. The length of the aligned sequences ranged from 297 to 14126 bp. These sequences were then assembled into 13 contigs one of which had a length of 19964 bp, and included partial sequences of ETS and complete sequences of 18S–28S rRNA. After the pairwise alignment of each of the 74 reads with 18S–28S rDNA the sequence with length of 21166 bp which covers complete IGS region was obtained.

The contigs we obtained from this project were then aligned to three Illumina based contigs with the length of 1065–3679 bp. The comparison showed a high degree of similarity between 5'-ETS-28S sequences and the Japanese quail rDNA de novo assembly. We found 88.7% similarity to the 2,350 bp long region corresponding to 5'-ETS-ITS1 (completely overlapping the 18S rRNA gene), 90.3% similarity to the 1,085-bp-long region containing ITS1, and 88.4% similarity to the 3,735-bp long region of the 28S rRNA gene. These percentage indicates high quality of the consensus sequence obtained. Nevertheless, the 18S, 5.8S and 28S rRNA genes were edited according to the Illumina sequences.

The resulting 21166 bp sequence was accepted into GenBank under the registration tag BankIt2509210 Coturnix OK523374. The *C. japonica* rRNA genes boundaries were identified by comparison with the fully annotated sequence of the chicken *Gallus gallus* (MG967540). Comparison of the contigs we obtained with raw data from an alternative source (ERR11591487 and ERR11591488, French National Institute for Agriculture, Food, and Environment) showed the presence of raw reads with high homology – up to 90.6% and coverage – up to 99%.

C. japonica rDNA repeat unit was described through comparison with the rDNA repeat of the chicken, completely annotated previously (Dyomin et al. 2016, 2019). It includes 5'-ETS (1779 bp), 18S (1823 bp), ITS1 (2047 bp), 5.8S (157 bp), ITS2 (658 bp), 28S (4185 bp), and 3'-ETS (639 bp) followed by IGS (9878 bp) with the total length of 21166 bp (Fig. 1).

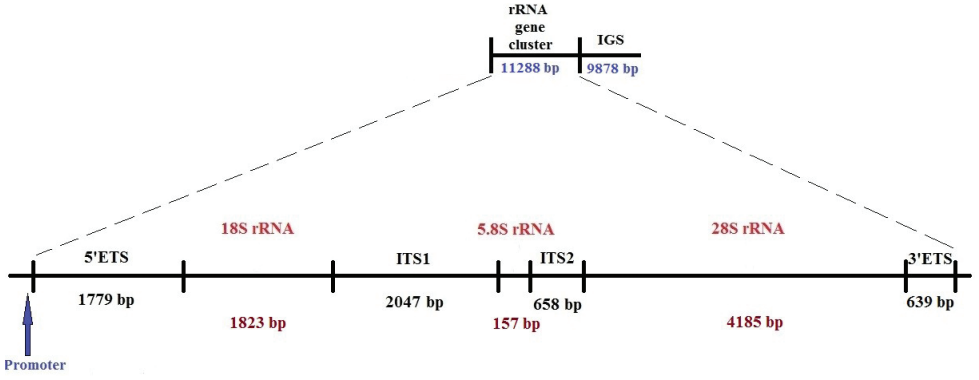

Figure 1. rRNA gene cluster of *C. japonica*.

The 5'-end of the 5'-ETS was determined by finding the transcription start point, using RNA-seq data from the NCBI (Suppl. material 2). The obtained RNA polymerase I promoter region of *C. japonica* rDNA (TTGCTCCGCAGGAGCGAGC) was compared with a similar chicken sequence MG967540, as well as with the one described by P. Massin and co-authors (Massin et al. 2005). The differences ranged from 1 to 4 nucleotides with a promoter length of 19 nucleotides (Fig. 2).

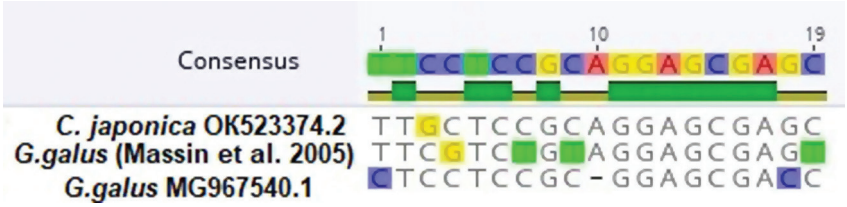

Figure 2. rDNA promoters operated by RNA polymerase I.

The primary sequence of the *C. japonica* rDNA genes - 18S, 5.8S and 28S - turned out to be conservative and demonstrates a high degree of similarity with the corresponding chicken regions. The determined Japanese quail rDNA gene sequences were 99.5% identical for 18S, 100% identical for 5.8S, and 85.7% identical for 28S described earlier for chicken. The sequence of the 5'ETS is highly GC-rich (74.1%), which is comparable with the chicken 5'-ETS (74.9%). The number of dispersed inverted repeats $(CGG)_{92}$ and $(GCC)_{78}$, as well as $(GCGA)_{22}$ and $(CGCT)_{10}$ was 28.4% and 7.1%, respectively. In addition, a direct microsatellite repeats $(GTGCC)_4$ and probably a degenerate repeat $(CAGM)_5$ are represented in the 5'ETS region: CAGACAGGACAGACAGGCAGA. ITS1 sequence (2047 bp) has high GC content - 76.8%, as well as ITS2 (658 bp) - 78.0% GC, which is higher than in 5'-ETS and 3'-ETS. However, compared to chicken corresponding regions, the GC content in each of them is lower (Table 1).

Table 1. Comparative characteristics of the CG composition of quail and chicken rDNA.

	5'ETS	3'ETS	IGS	ITS1	ITS2
			CG (%)		
Coturnix japonica	74.1	72.1	66.3	76.8	78.0
Gallus gallus	74.9	79.4	69.2	82.1	82.2

The dispersed inverted repeats in ITS1 were represented by $(CCG)_{12}$ and $(GGC)_3$, $(GCG)_3$ and $(CGC)_3$; in ITS2 - $(GCC)_3$ and $(CGG)_6$. Besides, there are several direct microsatellite repeats in ITS1: $(CGGG)_3$, $(GCC)_3$, $(GAG)_3$, $(CCT)_3$, $(TC)_5$ and in ITS2: $(CGA)_3$, $(GTTC)_4$, $(CG)_6$, $(CG)_5$ (Suppl. material 3: tables S1, S2). The repeating elements in ITS1 form extended polypurine / polypyrimidine bendable tracks that

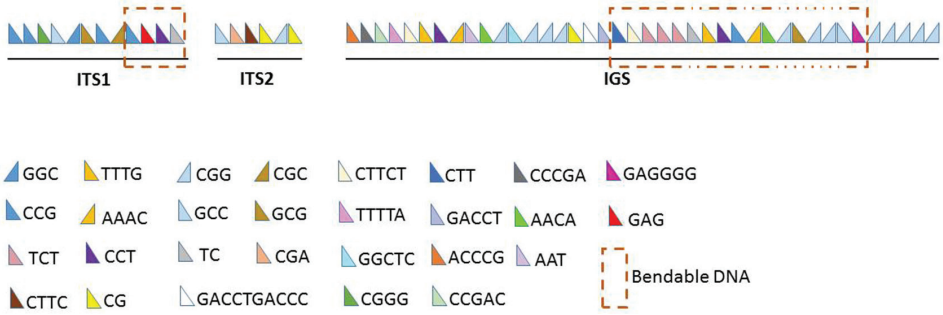

Figure 3. Distribution of repeating elements in *C. japonica* ITS1, ITS2 and IGS.

have the potential to form non-canonical secondary DNA structure, in particular the H-form (Fig. 3). The size of the 3'-ETS (639 bp) was determined by the localization of the transcription terminator, namely the poly-T region followed by inverted repeats. The boundaries of the second external transcribed spacer were determined this way, since transcriptome analysis demonstrated transcription attenuation on the border of the 28S gene. The CG content was 72.1%. In chicken, the same region contains 79.4% GC (Table 1). The dispersed inverted repeats are represented by $(CGG)_{17}$ and $(GCC)_{14}$. At the terminal part of this non-transcribed spacer, about 100 bases long, the following direct repeats (microsatellites) are found: $(ACCCG)_5$, $(CCCGA)_3$, and $(CCGAC)_3$. Probably, the last two represent a single sevenfold repeat with cytosine drop out at two positions: CCCGACCCGACCCGA**CCGA**CCCGACCCGA**CCGA**.

The Japanese quail IGS includes 9878 bp containing twenty tandem repeats (Suppl. material 3: table S3). The GC content in this region is 66.3% versus 69.2% in chicken (Table 1). In addition, the repeat $(GACCC/T)_{10}$ is probably degenerate because of the alternating nucleotide or there are two different tandem repeats $(GACCTGACCC)_3$ and $(GACCT)_4$. The extended non-transcribed region of the IGS is enriched in polypurine / polypyrimidine sequences prone to bend causing the formation of non-canonical secondary DNA structures (Fig. 3). The repeat element $(GAGGGG)_n$ may also contribute to the formation of the G-quadruplex.

Discussion

To better understand the biological processes occurring in the rDNA and the nucleolus, their common and particular aspects due to the physiology of a given species, reference rDNA sequence should be developed for every taxonomic group: human rDNA for Mammalia, *Xenopus* sp. for amphibia, *Danio rerio* F. Hamilton, 1822 for fish, terrapin for turtles and crocodiles. Most bird species are characterized by the presence of one pair of chromosomes carrying the nucleolar organizer regions with an ancestral interstitial localization. Despite advances in sequencing avian genomes using various platforms in recent years, most available datasets fail to provide the sequences

of the rDNA repeat unit suitable for comprehensive analysis. Before this work, the rDNA sequence of only one bird species had been deciphered – the rDNA of the chicken (Dyomin et al. 2019), so we compared our new results with it. The sequence of chicken ITS1 is longer than that of most animals, with few exceptions (Coleman 2013). A similar region of the Japanese quail genome was found to be 401 bp shorter. *C. japonica* ITS2 compared to the those of the chicken is also smaller. In this case, the difference was 76 nucleotides. Internal transcribed spacers are used as a convenient marker in phylogenetic studies. The recently described fact of the complication of ITS1 and ITS2 sequences in the evolution of Deuterostomia, the accumulation of GC nucleotides, and the elongation of sequences are surprising and currently unexplained (Dyomin et al. 2017). As defined by M. Gardiner-Garden and M. Frommer (1987), we assume that any stretch of DNA greater than 200 bp with a GC content greater than 50% and an observed to expected CpG ratio greater than 0.6 is a CpG island (Gardiner-Garden and Frommer 1987). A comparative analysis of Japanese quail and chicken rDNA repeat unit showed the comparable level of CpG island retention - 83.3% in rDNA of *C. japonica* and 89.7% in rDNA of *G. gallus*.

To date none of the regions of the quail rDNA cluster containing tandem repeats enriched with polypurine / polypyrimidine tracts of bendable DNA and extended repeats was represented in NCBI databases. According to our data (Fig. 3), *C. japonica* ITS1 and IGS are saturated with such elements to an even greater extent than the corresponding regions of the chicken rDNA repeat unit. Due to features of the secondary structure with the potential to form H-isoform DNA with sticky single-nucleotide arrays, bendable DNA of the polypyrimidine / polypurine tracts may play an important role in nucleolus formation involving nucleolus organizers from different chromosomes. We paid special attention to scattered inverted repeats, since RNA transcripts usually fold to form hairpins of different lengths (Singer and Berg 1998).

Functional state of such intracellular structures is one of the qualitative criteria of the physiological state of a cell as a whole. Along with the main function of ribosomal gene clusters - rRNA synthesis for ribosomes - there are now secondary or noncanonical functions associated mainly with transcription-inactive rDNA. A decrease in the number of inactive copies leads to instability of the entire nucleus chromatin, increases the sensitivity of the cell to damaging influences, and promotes accelerated cell aging (Paredes et al. 2011; Kobayashi 2014). It is known that nucleus can accumulate proteins which do not take part in ribosome biogenesis. The ability of rDNA copy to transcribe depends on its conformation and epigenetic modification - cytosine methylation in the CpG site (Ershova and Konkova 2020). Modern cytogenetic methods allow detection of such rearrangements and objective estimation of the activity degree of nucleus-forming regions of chromosomes. Morphological variants of the nucleus in tissue samples (including metastasized ones) with cancer dysplasia characterize the degree of functional activity of cells and reflect the level of processes related to ribosome biogenesis (Bolgova et al. 2012). Changes in functional state of loci due to such processes as activation or repression of transcription, DNA damage, and cell differentiation can be accompanied by relocation of this locus and large-scale changes in

the entire genome architecture. However, the data obtained using karyotype analysis methods is insufficient for a complete understanding of the mechanisms of transpositions and chromosomal rearrangements. Analysis of the primary rDNA sequence provides an opportunity to study not only the consequences, but also the sites responsible for such rearrangements - transposons and other mobile elements.

The best studied rDNA repeat unit is the human. It is about 43 kb, there are 100–500 copies in a cell (Agrawal and Ganley 2018; Parks et al. 2018). The IGS comprises three copies of the R repeat containing the Sal box terminator sites (Grummt et al. 1986). At the center of the IGS there is a repetitive region composed of a Long repeat, CT microsatellite, and Butterfly repeat. Some elements associated with noncoding RNA working in stress response, a cdc27 pseudogene, and putative c-Myc and p53 binding sites as well as conserved sites with unknown function have been annotated (Gonzalez and Sylvester 2001; Agrawal and Ganley 2018). The organization and the degree of divergence of the human rDNA units were studied using FISH, which allowed revealing rDNA units which were non-canonically oriented (Caburet et al. 2005). Deciphering of the entire individual nucleolus organizers on chromosomes 21 and 22 (Kim et al. 2018, 2021), studying variation by nanopore sequencing (Hori et al. 2021; Wang et al. 2023) evidenced the concerted evolution of rDNA units.

The Japanese quail genome as a model provides a good opportunity to study the specific processes occurring in the nucleolus organizer region related to genome activity of repeated DNA and, as a consequence, leading to the high risk of chromosomal rearrangements. Nucleolar organizers in *C. japonica* karyotype are localized on three different pairs of chromosomes. In addition to the ancestral interstitial nucleolus organizer on chromosome 16, there are two more terminal nucleolus organizers on the short arms of the acrocentric chromosomes (Solinhac 2010). We have not found any sequence variations in the primary ribosomal gene sequence, which may indicate characteristic features of rRNA gene clusters localized on different particular chromosomes. The previously shown involvement of transposons in the relocation of rRNA gene clusters to the terminal sites of the two microchromosomes may explain the lack of significant sequence changes in the clusters themselves (Saifitdinova et al. 2019). To identify the features of chromosome-specific nucleolar organizers, it is necessary to increase the collected amount of data based on long-read sequences that are more tolerant to high GC content and to improve methods for overcoming the difficulties caused by the presence of sticky regions enriched with repeats prone to the formation of non-canonical DNA forms and intermolecular connections.

Advanced techniques may finally allow the identification of two of GC-rich microchromosomes saturated with tandem repeats carrying terminal nucleolus organizers, which are still not represented in *C. japonica* chromosome-level genome assembly (GenBank accession: GCA_001577835.2), for which there are no molecular markers and no defined gene linkage groups. Knowing the characteristics of avian rDNA based on the previously studied chicken ribosomal cluster, in this work we used the possibilities of barcoding and diluted the Japanese quail genomic DNA sample with non-targeted DNAs with a lower GC content to reduce the likelihood of adhesion of the desired molecules. Although the

total number of reads and the coverage was not very high, we were able to obtain data on the *C. japonica* rDNA sequence for the first time. In this work, using nanopore long-read sequencing we identified and validated with NGS dataset the 21166 base pair of the complete Japanese quail ribosomal gene cluster sequence (GenBank under the registration tag BankIt2509210 Coturnix OK523374). This is the second deciphered avian rDNA cluster after the chicken, with some similarities to it as well as characteristic differences.

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ORCID

Alina Zhukova https://orcid.org/0000-0002-4416-1901
Gennadii Zakharov https://orcid.org/0000-0003-4850-3777
Olga Pavlova https://orcid.org/0000-0001-9488-6903
Alsu Saifitdinova https://orcid.org/0000-0002-1221-479X

Supplementary material 1

The longest obtained raw nanopore reads containing Japanese quail rDNA repeat unit sequences

Authors: Alina Zhukova, Gennadii Zakharov, Olga Pavlova, Alsu Saifitdinova

Data type: pdf

Link: https://doi.org/10.3897/compcytogen.18.127373.suppl1

Supplementary material 2

Localization of the promoter and transcription start site using RNA-seq data mapping

Authors: Alina Zhukova, Gennadii Zakharov, Olga Pavlova, Alsu Saifitdinova

Data type: pdf

Link: https://doi.org/10.3897/compcytogen.18.127373.suppl2

Supplementary material 3

Composition of tandem repeats in the noncoding spacer sequences of Japanese quail rDNA repeat units

Authors: Alina Zhukova, Gennadii Zakharov, Olga Pavlova, Alsu Saifitdinova

Data type: pdf

Explanation note: **table S1:** Characteristics of the Japanese quail ITS1 tandem repeats; **table S2:** Characteristics of the Japanese quail ITS2 tandem repeats; **table S3:** Characteristics of the Japanese quail IGS tandem repeats.

Link: https://doi.org/10.3897/compcytogen.18.127373.suppl3

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Chromosomes of *Pseudapanteles dignus* (Muesebeck, 1938) and a review of known karyotypes of the subfamily Microgastrinae (Hymenoptera, Braconidae)

Vladimir E. Gokhman[1], María Gabriela Luna[2,3], Consuelo Vallina[2], María José Bressa[4]

1 *Russian Entomological Society, Moscow, Russia* **2** *Centro de Estudios Parasitológicos y de Vectores (CEPAVE, CONICET-UNLP), La Plata, Argentina* **3** *Universidad Nacional de San Antonio de Areco, San Antonio de Areco, Argentina* **4** *Grupo de Citogenética de Insectos (GCI), Instituto de Ecología, Genética y Evolución de Buenos Aires (IEGEBA), Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales (FCEyN), Universidad de Buenos Aires (UBA), Buenos Aires, Argentina*

Corresponding author: Vladimir E. Gokhman (vegokhman@hotmail.com)

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Abstract

The karyotype of *Pseudapanteles dignus* (Muesebeck, 1938), an important parasitoid of a serious tomato pest *Phthorimaea* (= *Tuta*) *absoluta* Meyrick, 1917 (Lepidoptera, Gelechiidae), in the Neotropics and adjacent regions, was studied for the first time using morphometric analysis and several techniques of differential chromosome staining, i.e., C-banding and staining with base-specific fluorochromes, together with fluorescence *in situ* hybridization (FISH) with an 18S rDNA probe. We found n = 7 and 2n = 14 in *P. dignus*, with seven metacentric chromosomes of similar size in the haploid set. C-banding revealed various C-positive bands, either centromeric or interstitial, on most chromosomes. Both AT-specific and GC-specific fluorochromes, 4'6-diamidino-2-phenylindole (DAPI) and chromomycin A_3 (CMA_3) respectively, showed uniform staining of chromosomes. FISH visualized a single subterminal rDNA site on a medium-sized metacentric. A brief review of known chromosome sets of the subfamily Microgastrinae (Braconidae) is given; certain features of karyotype evolution of this group are discussed.

Keywords

Base-specific fluorochromes, Braconidae, C-banding, chromosomes, fluorescence *in situ* hybridization, karyotypes, Microgastrinae, parasitoids

Introduction

Parasitoid Hymenoptera are one of the most species-rich, taxonomically complicated and economically important groups of insects (Bebber et al. 2014; Forbes et al. 2018). In particular, the family Braconidae, with its high morphological and ecological diversity, contains more than 20,000 described species (Huber 2017). Moreover, Microgastrinae represent the second most speciose subfamily of Braconidae, which exceeds 3,000 described species, and up to 43,000 awaiting description, especially in the tropics (Rodriguez et al. 2013; Fernandez-Triana et al. 2020). Nevertheless, karyotypes of just a few members of this group are known so far, with only chromosomes of *Cotesia congregata* (Say, 1836) studied using differential staining (Belle et al. 2002; Gokhman 2009). We have examined the karyotype of another species from this subfamily, *Pseudapanteles dignus* (Muesebeck, 1938), an important solitary larval endoparasitoid of a serious worldwide tomato pest *Phthorimaea* (= *Tuta*) *absoluta* Meyrick, 1917 (Lepidoptera, Gelechiidae) in the Neotropics and adjacent regions (Fernandez-Triana et al. 2014), using several techniques of differential chromosome staining. The results of this work are given below. Several biological and ecological studies have shown that *P. dignus* can potentially control *P. absoluta*, either under natural conditions or by augmentative releases in tomato fields (Salas Gervassio et al. 2019; D´Auro et al. 2021; Vallina et al. 2022). Knowledge of genetic aspects of the parasitoid life history can therefore contribute to quality mass production of biocontrol agents, and consequently, to optimization of pest control (Lommen et al. 2017). In addition to the chromosomal study of *P. dignus*, we briefly review the current state of knowledge of karyotypic diversity of Microgastrinae.

Materials and methods

Origin of the material studied

The laboratory stock of *P. dignus* maintained at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE, CONICET and UNLP, La Plata, Argentina) originates from insects reared from cocoons of this parasitoid. These cocoons, containing pupae of *P. dignus*, were collected on tomato leaves infested with *P. absoluta* near La Plata (see Luna et al. 2007). This endoparasitoid species attacks second to fourth larval instars of gelechiid moths, particularly *P. absoluta*, depositing up to eight eggs per host during oviposition (D'Auro et al. 2021). However, only a single *P. dignus* larva survives to the third instar. At this stage, the parasitoid larva emerges from the dying host and pupates, typically spinning a silk cocoon (Luna et al. 2007). The preimaginal period lasts about 21 d; adults live ≥ 23 d in presence of the host (Vallina et al. 2022). To prepare the specimens for the chromosomal study, twenty cohorts of *P. dignus* were initiated by exposing a two-day-old, fertilized female to twenty larvae of *P. absoluta* inside leaf mines. The larvae were then kept in one-liter plastic containers and fed with 50% honey syrup

ad libitum. Cohorts were generated sequentially to synchronize the rearing process and to obtain material for dissections at the correct developmental stage. All cultures were maintained at 25 °C and 60 to 75% humidity, with a 14 h light: 10 h dark photoperiod in a walk-in environmental chamber. Voucher specimens from this study are deposited at CEPAVE (La Plata, Argentina).

Preparation and staining of chromosomes

Chromosomal preparations were obtained from cerebral ganglia of parasitoid prepupae generally following the protocol developed by Imai et al. (1988) with certain modifications (see, e.g., Gokhman et al. 2019). Ganglia were extracted from insects dissected in 0.5% hypotonic sodium citrate dihydrate solution containing 0.005% colchicine. The extracted ganglia were then transferred to fresh hypotonic solution and incubated for 30 min at room temperature. The material was transferred onto a pre-cleaned microscope slide using a Pasteur pipette and then gently flushed with Fixative I (glacial acetic acid: absolute ethanol: distilled water 3:3:4). The tissues were disrupted using dissecting needles in an additional drop of Fixative I. A drop of Fixative II (glacial acetic acid: absolute ethanol 1:1) was applied to the center of the area, and the more aqueous phase was blotted off the edges of the slide. The slides were dried for approximately 30 min and stored at room temperature.

For routine staining, chromosome preparations were stained overnight using a freshly prepared 3% Giemsa solution (Merck KGaA, Darmstadt, Germany). C-banding and sequential staining with AT-specific 4',6-diamidino-2-phenylindole (DAPI; Fluka BioChemika, Sigma Aldrich Production GmbH, Buchs, Switzerland) and GC-specific chromomycin A_3 (CMA_3; Fluka BioChemika) were carried out following Poggio et al. (2011). For C-banding, the pre-treated slides were stained with DAPI to improve the resolution of C-bands (Barros e Silva and Guerra 2010; Poggio et al. 2011).

Unlabeled 18S ribosomal DNA (rDNA) probe was generated by polymerase chain reaction (PCR) using universal arthropod primers: forward 5'-CCTGAGAAACGGCTACCACATC-3' and reverse 5'-GAGTCTCGTTCGTTATCGGA-3' (Whiting 2002). Total genomic DNA of *Dysdercus albofasciatus* Berg, 1878 (Hemiptera, Pyrrhocoridae), obtained by standard phenol-chloroform-isoamyl alcohol extraction, was used as a template. PCR was performed following the procedure described by Fuková et al. (2005) and Bressa et al. (2009). The PCR product displayed a single band of approximately 1,000 bp on a 1% agarose gel. The band was cut out from the gel, and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The 18S rDNA fragment was re-amplified by PCR and subsequently labeled with biotin-14-dUTP by nick translation using a BioNick Labeling System (Invitrogen, Life Technologies Inc., San Diego, CA, USA). FISH with biotinylated 18S rDNA probe was performed following the procedure developed by Sahara et al. (1999) with several modifications described by Fuková et al. (2005) and Bressa et al. (2009).

Image acquisition and analysis

Metaphase plates of *P. dignus* were examined and photographed with an optical microscope Zeiss Axioskop 40 FL fitted with a digital color camera Axiocam 208 (Carl Zeiss, Germany) as well as an epifluorescence microscope Leica DMLB fitted with a digital camera Leica DFC350 FX CCD (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK) respectively. To produce illustrations, the resulting images were processed with the image processing programs ZEN version 3.0 (blue edition), Leica IM50 version 4.0, Adobe Photoshop CC version 14.0, and GIMP version 2.10. Black-and-white images of chromosomes were captured separately for each fluorescent dye. Images were pseudocolorized (light blue, green, and red for DAPI, CMA_3, and Cy3, respectively) and processed with Adobe Photoshop CC version 14.0. KaryoType version 2.0 software (Altınordu et al. 2016) was also used for taking measurements from ten haploid metaphase plates of *P. dignus*. The chromosomes were classified following guidelines provided by Levan et al. (1964). All studies were conducted at CEPAVE (La Plata, Argentina), IEGEBA/DEGE of FCEyN of Universidad de Buenos Aires (Ciudad Autónoma de Buenos Aires, Argentina), and the Botanical Garden of Moscow State University (Moscow, Russia).

Results

The haploid karyotype of *P. dignus* contains seven metacentric chromosomes, which exhibit a gradual decrease in size (n = 7; Fig. 1A, B, Table 1). In prometaphase chromosomes, pericentromeric and interstitial heterochromatic segments are visible. In addition, a distinct secondary constriction is visible on a medium-sized chromosome (Fig. 1B). The diploid chromosome set of *P. dignus* consists of seven pairs of similar metacentric chromosomes (Fig. 1C; 2n = 14). Chromosome relative lengths of the

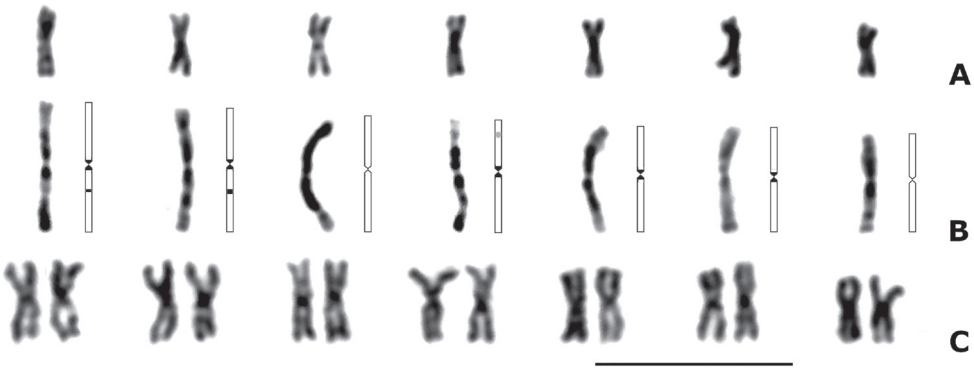

Figure 1. Karyograms of Giemsa-stained chromosomes of *P. dignus* **A** haploid, metaphase **B** haploid, prometaphase **C** diploid, metaphase. For **B** idiogram for each chromosome demonstrating heterochromatin distribution and position of the secondary constriction in black and grey respectively, is also shown. Scale bar: 10 μm.

Table 1. Relative lengths (RLs) and centromeric indices (CIs) of chromosomes of *P. dignus* (mean ± SD).

Chr. no.	RL, per cent	CI, per cent
1	16.15 ± 0.44	48.62 ± 0.86
2	15.42 ± 0.36	47.91 ± 2.68
3	14.67 ± 0.40	46.13 ± 2.74
4	14.35 ± 0.38	47.60 ± 1.71
5	13.87 ± 0.35	47.41 ± 2.03
6	13.19 ± 0.43	47.07 ± 2.38
7	12.35 ± 0.46	46.39 ± 2.43

haploid set (RLs) range from 16.15 ± 0.44 per cent for the longest chromosome to 12.35 ± 0.46 per cent for the smallest one (Table 1). Despite some difference in RLs between the longest and the shortest chromosome, the karyotype of *P. dignus* is fairly homogeneous in chromosome morphology and size, suggesting that this species possesses a highly symmetrical karyotype (Stebbins 1950).

C-banding reveals different patterns in the amount and location of constitutive heterochromatin on the chromosomes of *P. dignus*. Specifically, three pairs of chromosomes in the diploid set exhibit only centromeric C-positive bands. These bands are brighter and more conspicuous on chromosomes of two of the pairs than on the third one. On chromosomes of the two other pairs, strong C-positive centromeric bands are accompanied by small interstitial ones. No C-bands are detected on the remaining chromosomes (Fig. 2A, B). All mitotic chromosomes show relatively uniform fluorochrome staining with both DAPI and CMA_3 (Fig. 3).

In the diploid karyotype of *P. dignus*, FISH with an 18S rDNA probe reveals a single subterminal rDNA cluster on a pair of medium-sized metacentric chromosomes (Fig. 4). The location of the rDNA cluster apparently co-localizes with the secondary constriction observed on a specific medium-sized chromosome of this species (Fig. 1B).

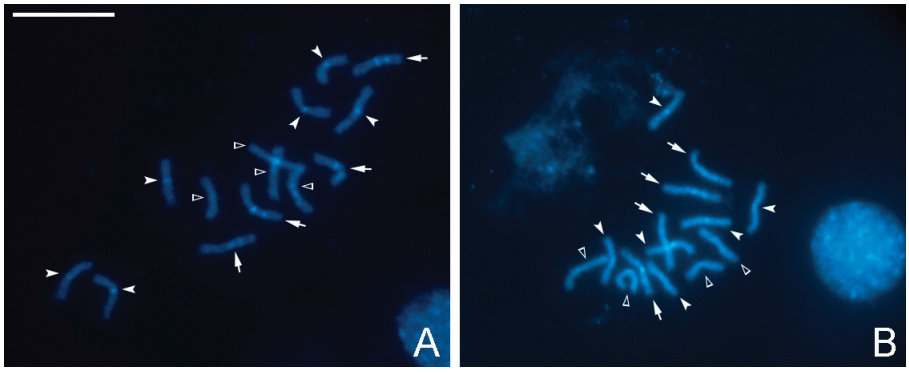

Figure 2. C-banded and DAPI-stained diploid metaphase plates of *P. dignus* (**A, B**). Arrows indicate chromosomes with both centromeric and interstitial C-positive bands, filled arrowheads indicate chromosomes with only centromeric C-positive bands, and empty arrowheads indicate lack of C-positive bands respectively. Scale bar: 10 μm.

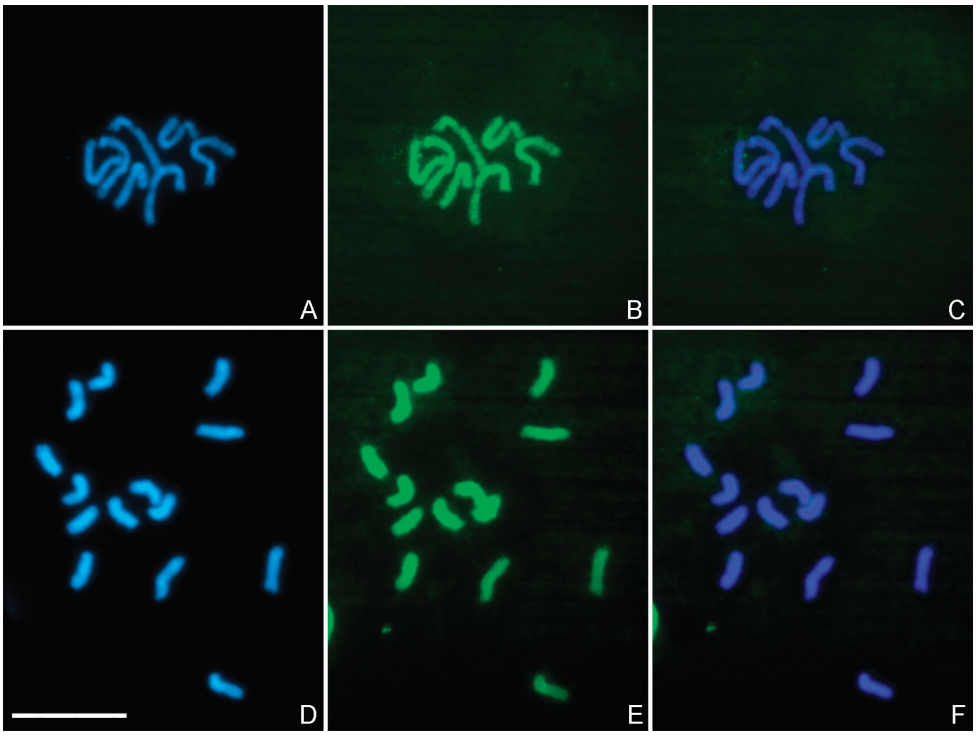

Figure 3. DAPI/CMA_3-stained metaphase haploid (**A–C**) and diploid (**D–F**) plates of *P. dignus* **A, D** DAPI staining **B, E** CMA_3 staining **C, F** merged images. Scale bar: 10 μm.

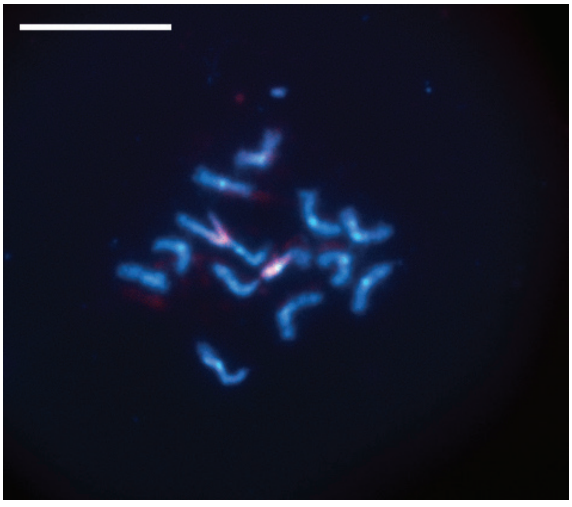

Figure 4. FISH with 18S rDNA probe on chromosomes of the diploid karyotype of *P. dignus*. Probe signals are indicated in red. Scale bar: 10 μm.

Discussion

The karyotype of *P. dignus* is characterized by the lowest chromosome number found in the subfamily Microgastrinae, with n values for other species ranging from 9 to 11 (Table 2). Moreover, members of this group exhibit considerable diversity in terms of chromosomal morphology. Specifically, karyotypes of most studied species of Microgastrinae, including *P. dignus*, *Cotesia glomerata* (Linnaeus, 1758) and *C. congregata*, predominantly contain biarmed chromosomes (Belle et al. 2002; Zhou et al. 2006). On the other hand, the chromosome set of "*Apanteles* sp." mostly harbors subtelocentric and/or acrocentric chromosomes (Hoshiba and Imai 1993). According to our phylogenetic reconstruction of karyotype evolution in Braconidae (Gokhman 2009), n values of 9 to 11 also prevail in the non-cyclostome lineage of this family; thus, the lower chromosome number, n = 7 found in *P. dignus*, is apparently derived. This lends further support from the suggested basal position of the genus *Microplitis* Förster, 1862 (Quicke 2015 and references therein), with n = 10–11 (Table 2).

Up to now, only three species of the family Braconidae have been studied using C-banding, "*Apanteles* sp." (Hoshiba and Imai 1993), *Aphidius ervi* Haliday, 1834 (n = 5, 2n = 10 and 12) (Gokhman and Westendorff 2003), and *Diachasmimorpha longicaudata* (Ashmead, 1905) (n = 20, 2n = 40) (Carabajal Paladino et al. 2013). In "*Apanteles* sp.", the karyotype consists of a single pseudoacrocentric chromosome with the fully heterochromatic shorter arm, along with nine more or less euchromatic subtelocentrics/acrocentrics together with an apparently euchromatic submetacentric chromosome (Hoshiba and Imai 1993). Chromosomes of *A. ervi* are also predominantly euchromatic; however, a few studied females carried an additional pair of almost fully heterochromatic acrocentric chromosomes (Gokhman and Westendorff 2003). In *D. longicaudata*, most chromosomes are pseudoacrocentric, and many of them carry large segments of pericentromeric heterochromatin (Carabajal Paladino et al. 2013). Our results obtained using C-banding in *P. dignus* thus revealed differences in the size and location of heterochromatic seg-

Table 2. Chromosome numbers of parasitoids of the subfamily Microgastrinae.

Species	n(2n)	Reference
Apanteles sp.†	11	Hoshiba and Imai 1993
Cotesia congregata (Say, 1836)	10	Belle et al. 2002
C. glomerata (Linnaeus, 1758)	10(20)	Zhou et al. 2006
Microgaster luctuosa Haliday, 1834 (= *curvicrus* Thomson, 1895)	(18)	Gokhman 2004
Microplitis demolitor Wilkinson, 1934	10	M. Strand, pers. comm., cited in: Gokhman 2009
M. ratzeburgii (Ruthe, 1858)	(22)	Gokhman 2009
M. tuberculifer (Wesmael, 1837)	(22)	Gokhman 2009
Pseudapanteles dignus (Muesebeck, 1938)	7(14)	Present paper

†Like many other identifications in the cited work, this one is dubious and may well refer to any other member of Microgastrinae, e.g., *Microplitis* sp.

ments compared to other Braconidae. Moreover, three different C-banding patterns were found within the diploid chromosome set of *P. dignus*, resulting in a species-specific distribution of constitutive heterochromatin. Various mechanisms have been proposed to account for the variation in the content and distribution of heterochromatin both within and between species, e.g., multiple replication, unequal exchanges, accumulation or elimination [reviewed by John (1988)], which could explain the differences observed among the four members of this family with known heterochromatin distribution.

Previously, multiple rDNA loci per haploid karyotype have been detected in certain Hymenoptera species using Ag-NOR, DAPI/CMA_3-banding and/or FISH; however, typically, only a single rDNA cluster is active (Hirai et al. 1994; Matsumoto et al. 2002; Gokhman 2009; Carabajal Paladino et al. 2013). In this order, as well as in other insects, CMA_3-positive bands co-localize with nucleolus organizing regions (NORs), suggesting that rDNA clusters are typically rich in GC base pairs (Camacho et al. 1991; Hirai et al. 1994; Vitturi et al. 1999; Maffei et al. 2001; Costa et al. 2004; Papeschi and Bressa 2006; Bolsheva et al. 2012; Gokhman et al. 2016; Gokhman and Kuznetsova 2024). However, in the parasitoid *D. longicaudata* (Carabajal Paladino 2011) and in certain true bugs (Hemiptera, Heteroptera) (Bressa et al. 2005; Severi-Aguiar and de Azeredo-Oliveira 2005; Morielle-Souza and Azeredo-Oliveira 2007; Poggio 2012), no such association has been demonstrated. The results of DAPI/CMA_3-banding indicate that all chromosomes of *P. dignus* lack specific regions enriched either in AT or GC base pairs. Thus, the NOR of *P. dignus* is apparently not associated with CMA_3-positive chromosomal segments as well.

To date, the only karyotypic study of Microgastrinae involving *in situ* hybridization was performed on *C. congregata* (Belle et al. 2002). Specifically, this technique visualized a single cluster of rDNA as well as certain DNA sequences coding for a symbiotic polydnavirus. In the haploid karyotype of this species, both sites appeared to have subterminal localization on shorter arms of the two different subtelocentric chromosomes (Belle et al. 2002). Contrary to *C. congregata*, all chromosomes of *P. dignus* are metacentric, but the single NOR is also located subterminally on a particular chromosome of the latter species. Interestingly, six rDNA clusters per haploid karyotype were earlier discovered in *D. longicaudata*, but, nevertheless, they all also have subterminal localization on chromosomes (Carabajal Paladino et al. 2013).

Most NORs in eukaryotic genomes are located in heterochromatic regions (Goessens 1984; Hadjiolov 1985; Babu and Verma 1987; Gokhman and Kuznetsova 2024), likely because certain heterochromatin-associated genes can silence repetitive DNA sequences and suppress recombination among them (Gottlieb and Esposito 1989). Based on the length and morphology of the chromosomes of *P. dignus*, we conclude that rDNA clusters in this species are located within the C-positive interstitial bands of one of the two chromosome pairs that carry these bands (Fig. 1B; see above). A similar pattern was previously observed in *D. longicaudata* (Carabajal Paladino et al. 2013). In this parasitoid species, hybridization signals with the 18S rDNA probe were also detected in heterochromatic regions. Nevertheless, both these regions and the rDNA clusters were CMA_3-negative and, consequently, not enriched in GC base pairs.

Although currently no karyotypically distinct groups of cryptic species of Microgastrinae are known, this situation may change as an increasing number of members

of this subfamily are examined, similarly to other taxa of parasitic wasps (Gokhman 2009, 2022). Moreover, chromosomal analysis of Microgastrinae will provide us further insights into their genetic features, which can, in turn, offer important information necessary for mass rearing and other aspects of applied use of these parasitoids. In addition, the results of the karyotypic study of this subfamily are already being used to verify the results of chromosome-level genome assemblies (Gokhman 2022, 2023). For example, this includes *C. congregata* and *C. glomerata* (Gauthier et al. 2021; Pinto et al. 2021). Furthermore, both genome assemblies of *Microplitis manilae* Ashmead, 1904 suggest n = 11 for this parasitoid (Shu et al. 2023; Yan et al. 2023). Since we report the same n value for two other *Microplitis* species (Table 2), these results appear plausible.

Author contributions

VEG: conceptualization, data curation, formal analysis, investigation, methodology, supervision, validation, visualization, writing – original draft, writing – review and editing. MGL: data curation, funding acquisition, methodology, resources, validation, writing – review and editing. CV: methodology, resources, writing – review and editing. MJB: conceptualization, data curation, formal analysis, investigation, methodology, resources, validation, visualization, writing – review and editing. All authors have read and agreed with the final version of the manuscript.

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ORCID

Vladimir E. Gokhman https://orcid.org/0000-0001-9909-7559
María Gabriela Luna https://orcid.org/0000-0001-5297-4833
Consuelo Vallina https://orcid.org/0000-0001-8733-2347
María José Bressa https://orcid.org/0000-0002-0191-6518

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Highlighting chromosomal rearrangements of five species of Galliformes (Domestic fowl, Common and Japanese quail, Barbary and Chukar partridge) and the Houbara bustard, an endangered Otidiformes: banding cytogenetic is a powerful tool

Yasmine Kartout-Benmessaoud[1,2], Siham Ouchia-Benissad[1], Leila Mahiddine-Aoudjit[1,3], Kafia Ladjali-Mohammedi[1]

1 *Faculty of Biological Sciences, University of Sciences and Technology Houari Boumediene (USTHB), Laboratory of Cellular and Molecular Biology, Team of Developmental Genetics. PO box 32 El-Alia, Bab-Ezzouar, 16110, Algiers, Algeria* **2** *Faculty of Nature and Life Sciences, Department of Physico-Chemical Biology, University Abderrahmane Mira, Campus Targa Ouzemour, 06000, Bejaia, Algeria* **3** *Department of Biology, Faculty of Science, M'Hamed Bougara University of Boumerdes, Boumerdes, Algeria*

Corresponding author: Yasmine Kartout-Benmessaoud (yasmine.kartout@univ-bejaia.dz; kartout_yasmine@yahoo.fr)

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Abstract

Birds are one of the most diverse groups among terrestrial vertebrates. They evolved from theropod dinosaurs, are closely related to the sauropsid group and separated from crocodiles about 240 million years ago. According to the IUCN, 12% of bird populations are threatened with potential extinction. Classical cytogenetics remains a powerful tool for comparing bird genomes and plays a crucial role in the preservation populations of endangered species. It thus makes it possible to detect chromosomal abnormalities responsible for early embryonic mortalities. Thus, in this work, we have provided new information on part of the evolutionary history by analysing high-resolution GTG-banded chromosomes to detect inter- and intrachromosomal rearrangements in six species. Indeed, the first eight autosomal pairs and the sex chromosomes of the domestic fowl *Gallus gallus domesticus* Linnaeus, 1758 were compared with five species, four of which represent the order Galliformes (Common and Japanese quail, Gambras and Chukar partridge) and one Otidiformes species (Houbara bustard).

Our findings suggest a high degree of conservation of the analysed ancestral chromosomes of the four Galliformes species, with the exception of (double, terminal, para and pericentric) inversions, deletion and the formation of neocentromeres (1, 2, 4, 7, 8, Z and W chromosomes). In addition to the detected rearrangements, reorganisation of the Houbara bustard chromosomes mainly included fusions and fissions involving both macro- and microchromosomes (especially on 2, 4 and Z chromosomes). We also found interchromosomal rearrangements involving shared microchromosomes (10, 11, 13, 14 and 19) between the two analysed avian orders. These rearrangements confirm that the structure of avian karyotypes will be more conserved at the interchromosomal but not at intrachromosomal scale.

The appearance ofa small number of inter- and intrachromosomal rearrangements that occurred during evolution suggests a high degree of conservatism of genome organisation in these six species studied. A summary diagram of the rearrangements detected in this study is proposed to explain the chronology of the appearance of various evolutionary events starting from the ancestral karyotype.

Keywords

Avian cytogenetics, chromosomal reshuffling, evolution, GTG-banding, Galliformes, Otidiformes

Introduction

Earth has experienced five major geoclimatic-induced extinctions, the last one was the disappearance of dinosaurs class from which only one family survived, represented by the birds. In fact, the Pseudosuchia (Crocodilians) and Ornithodira (dinosaurs, birds…) have a monophyletic origin in the same clade of Archosaurs (Archosauria). Birds evolved from theropod dinosaurs around 165 to 150 million years ago and separated from crocodiles about 240 million years ago (Brusatte et al. 2015; Pritchard et al. 2017; Benson 2018; Griffin et al. 2023; Olmo 2023). We are currently experiencing the irreversible sixth mass extinction, which could turn out to be, according to many parameters, more devastating than all others combined (Barlow et al. 2016; Maxwell et al. 2016; Betts et al. 2017; Ceballos et al. 2020).

There are approximately 11032 bird species worldwide, which represent the most diverse class of tetrapod amniote vertebrates. However, class Aves (birds) is particularly threatened by the impending mass extinction, and is also the least studied genetically compared to the others (Kretschmer et al. 2018; Wink 2019; Donsker and Rasmussen 2022).

The analysis of karyotypes to establish the phylogenetic relationships in birds is not as advanced as that of in mammals and is limited to only a few orders (Kiasim et al. 2021; Kretschmer et al. 2021a, 2021c; Intarapat et al. 2023). With the exception of Psittaciformes, Caprimulgiformes, Cuculiformes, Passeriformes and Ciconiiformes, the “signature” avian karyotype has remained largely unchanged in most groups. This remarkable conservation may be due to the more large number of diploid chromosomes and/or an increase in the recombination rate (O’Connor et al. 2024). On the other hand, knowledge of bird phylogenetics has greatly improved over the last ten years, despite, the difficulties encountered in studying the complex evolutionary of Neoaves, due to their fast divergence (Prum et al. 2015).

The domestic fowl *Gallus gallus domesticus* Linnaeus, 1758 (GGA) is considered as a model in phylogeny and comparative genomics and represents the only standardised bird karyotype (Ladjali-Mohammedi et al. 1999).

Domestic chicken chromosomes remain the best studied in birds. As this species shares several features with other avian species, it is considered the closest to the common ancestor of birds (Shibusawa et al. 2002, 2004; Derjusheva et al. 2004; Griffin et al. 2007).

Paradoxically, the sequencing and mapping of avian genomes are more developed than cytogenetic studies. The latter often remain partial in birds, despite their major contributions. Indeed, classical cytogenetics and banded cytogenetics have highlighted numerous characteristics of the avian karyotype, such as interchromosomal stability (Tegelstrom and Ryttman 1981; Belterman and De Boer 1984; Christidis 1990; Shibusawa et al. 2004) and intrachromosomal rearrangement within macrochromosomes (Stock and Bunch 1982; Hooper and Price 2017; Kretschmer et al. 2020). Comparative chromosomal mapping makes it possible to establish multi-species analysis in order to deduce the evolution of the karyotype, which is an essential element of phylogenomics (Graphodatsky et al. 2011; Seligmann et al. 2023; Ferreira et al. 2023; Nagao et al. 2023; O'Connor et al. 2024).

Cytogenetics has also allowed understanding of the chromosomal evolutionary process of plants (Liu et al. 2023), some mammal species (Di-Nizo et al. 2017; Rajičić 2022), insects (Farsi et al. 2020; Gokhman 2022), fishes (Araya-Jaime et al. 2022), amphibia (Dominato et al. 2022; Dudzik et al. 2023) and birds (Shibusawa et al. 2004; Nishida et al. 2008; Degrandi et al. 2020; Kretschmer et al. 2021a; Slobodchikova et al. 2022; Seligmann et al. 2023; Flamio and Ramstad 2024; O'Connor et al. 2024).

This is the case of avian species belonging to Phasianidae, order Galliformes as Common and Japanese quail, Barbary and Chukar partridge (Ouchia-Benissad and Ladjali-Mohammedi 2018; Kartout-Benmessaoud and Ladjali-Mohammedi 2018) and Houbara bustard, an endangered Otidiformes (Mahiddine-Aoudjit et al. 2019), of which the chromosomes are here described for the first time.

Regarding these recently studied species, farmed quails are economically important thanks to the production of eggs and meat, which are highly valued for their unique flavor (Lukanov 2019). The Common quail *Coturnix coturnix* Linnaeus, 1758 (CCO) is listed in 2018 as Least Concern (LC) in global and in 2020 as Near Threatened (NT) in Europe (IUCN 2024; BirdLife 2021).

The sharp decline in migratory populations observed in Western Europe led to its double legal registration in the Bonn (CMS) and Bern (1979) International Conventions on the protection and conservation of wild species. Thus, the introgressive hybridisation caused by the uncontrolled release of Japanese quails *Coturnix japonica* Temminck et Schlegel, 1849 (CJA) seems to induce a very worrying genetic shift (Guyomarc'h et al. 1998; Dérégnaucourt et al. 2005; Chazara et al. 2010; Puigcerver et al. 2013; Sanchez-Donoso et al. 2016; Kartout-Benmessaoud and Ladjali-Mohammedi 2018).

Besides, the Barbary partridge *Alectoris barbara* Bonnaterre, 1790 (ABA) is an endemic partridge in Algeria. It is a nesting sedentary bird found in different ecosystems. This common game bird is overhunted which leads to declining population size in some areas (Isenmann and Moali 2000). Although the Barbary partridge is listed as Least Concern on the IUCN Red List (2024), it is also nevertheless protected by several conventions (CITES, Bern Convention). In addition, the introduction of the exotic Chukar partridge *Alectoris chukar* Gray, 1832 (ACH) could lead to introgression in the wild genome of native partridge, which could give rise to infertile descendants (Barbanera et al. 2011).

Regarding the Houbara bustard *Chlamydotis undulata* Jacquin, 1784 (CUN) it is an endangered wild species, which is classified as vulnerable by the IUCN (2024). This species has recorded over the past thirty years, a significant decline in these natural populations, particularly due to poaching (BirdLife 2017). Although protected by CITES Appendix I and legislation in Algeria, the bustard is still hunted (Azafzaf et al. 2005). Additionally, the revision of the bird phylogenetic tree introduced a new order Otidiformes, to which the Houbara bustard was affiliated (Jarvis et al. 2014).

In the present study, we carried out a comparative cytogenetic analysis of six species belonging to the order Galliformes (GGA, CCO, CJA, ABA, ACH) and Otidiformes (CUN). The main aim of this work is to highlight inter or intrachromosomal rearrangements which would have occur during speciation. These results contribute to a better understanding of the phylogenetic relationships of these different species and the evolution of avian genome.

Material and methods

To carry out the comparison study, the same protocol was followed for the different species.

Biological material

For all species, embryos were collected during the laying period. Fertile eggs of Common quail (CCO) brought from the Tlemcen Hunting Centre, Algeria (34°53'24"N, 1°19'12"W) and those of the Japanese quail (CJA), Barbary partridge (ABA) and Chukar (ACH) were obtained from the Centre Cynégétique de Zéralda Algeria (36°42'06"N, 2°51'47"E).

Regarding the Houbara bustard (CUN) embryos, they were collected from Emirati Bird Breeding Centre for Conservation EBBCC (32°55'40.54"N, 0°32'33.71"E) in the region of Abiodh Sidi Cheikh (Wilaya d'El-Bayadh, south of Algeria).

The eggs were incubated in a ventilated incubator where the conditions of hygrometry (55%) and temperature (39.5 °C) are maintained in the Laboratoire de Génétique du Développement (Faculté des Sciences Biologiques, USTHB-Algeria).

Cell cultures and double synchronisation

Primary cell cultures were performed on embryos aged 6 to 19 days. These were stripped of their appendages and fibroblasts were isolated from different fragments (lung, heart, liver, kidneys and muscles) following treatment with a trypsin solution (0.05%, Sigma). The cells were incubated at 41 °C in RPMI 1640 culture medium (GIBCO) supplemented with 20 mM of HEPES, 1% of L-Glutamine (Gibco ref.: 22409-015, batch: 695608), 10% of foetal calf serum (FCS, Gibco ref.: 10270-106, batch: 41Q4074K), Penicillin-Streptomycin 1% and 1% of Fungizone (Gibco ref.: 15160-047, Batch: S25016D). Trypsinisation of cells was carried out to enhance division ability (Ladjali et al. 1995).

Cultures of fibroblasts were synchronised as described by Ladjali et al. (1995), using a double thymidine block during S phase in order to increase the yield of metaphase and early metaphase cells. The 5-bromo-2'-deoxyuridine (BrdU) (final concentration: 10 µg/ml, Sigma) was added to prepare chromosomes to the RBG staining (Zakharov and Egolina 1968; Ladjali et al. 1995). As a sufficient number of refractive mitotic cells was observed (after 6–8 h), they were treated with colchicine (final concentration: 0.05 µg/ml, Sigma) for 5 min at 37 °C. Cells were harvested by the addition of 0.05% trypsin-EDTA (Gibco). Hypotonic treatment was performed. In fact, cells were suspended for 13 min at 37 °C in hypotonic solution 1:5 (FCS- distilled water). Fixation and spreading were performed using standard methods (Dutrillaux and Couturier 1981; Ladjali et al. 1995).

Chromosomes staining

GTG-banding was carried out according to the Seabright modified method (1971). Aged (3–10 days) slides were incubated for 8–14 seconds in a fresh trypsin solution (final concentration: 0.25%, sigma). Slides were rinsed twice in PBS- (Phosphate Buffered Solution, pH = 6.8) and stained with 6% Giemsa (Fluka) for 8–10 minutes (Ladjali et al. 1995).

Chromosome classification

Slides were first observed with an optical microscope at objective magnification of 10× to estimate the mitotic index (AxioZeiss Scope A1). Slides, showing a higher mitotic index, were analysed and prometaphases and metaphases with decondensed and dispersed chromosomes, were photographed (CoolCube1 Metasystems).

According to the International System of Standardised Avian Karyotypes (ISSAK) (Ladjali-Mohammedi et al. 1999), macrochromosomes pairs were classified in decreasing size depending on the position of centromere (Shoffner 1974).

Comparative analysis

In order to highlight the similarities and divergences that occur during bird evolution, we proceeded to the comparison of the GTG bands obtained on macrochromosomes of the different species. Taking into consideration size of chromosomes, their morphology and GTG patterns.

Results

Comparative analysis of macrochromosomes and ZW sex chromosomes of five bird species (ABA, ACH, CCO, CJA, CUN) is undertaken, referring to the common karyotype of birds which is represented by the standard chicken karyotype (GGA).

The comparative study is carried out for the first eight macrochromosomes as well as the ZW gonosomes. This is based on three criteria, notably the GTG band patterns, the morphology of the chromosomes and the q/p ratio (Table 1).

This comparative analysis allowed us to show the presence of strong homologies between the compared different chromosomes and to identify the presence of certain rearrangements that would have taken place during speciation (Table 2).

Chromosome 1

The analysis of chromosome 1 in the six species studied allowed us to observe, on the one hand, that all the chromosome 1s of the species studied are submetacentric showing a great homology of GTG band patterns. On the other hand, differences in the ratio (q/p) are detected. Indeed, the size of the short arms (p) of chromosomes 1 of the Japanese quail and the Houbara bustard are smaller than in the other species (Fig. 1A). Arms ratios (q/p) are 2.15 and 2.46 respectively, whereas it is equal to 1.69 in chicken. On the other hand, the positions of the centromeres of the two species of partridge and of the common quail are similar to that of the chicken (Table 1).

Chromosome 2

There is a high conservation of CCO-2 and the two partridge species (ABA and ACH) in comparison with the ancestral chromosome 2. However, some rearrangements are detected in CJA and CUN. Indeed, the CJA-2 has a large region, whose GTG banding patterns are inverted. Also, with regard to CUN-2 we noted the absence of a terminal region on the long arm (q) showing the arm ratios (q/p) of 2.19 whereas it is equal to 1.94 in the chicken (Fig. 1B).

Chromosomes 3, 5 and 6

These chromosomes seem to be conserved in the all species analysed. They are morphologically similar (acrocentric in six species) and show conservation of GTG banding patterns. No rearrangement was detected in this work.

Chromosome 4

The GGA-4 chromosome is telocentric (r = 3.86) whereas it is subtelocentric in the two species of quail (rCCO = 6.16 and rCJA = 5.31). It is acrocentric in CUN (r = 10.98) and the two species of partridge studied (rABA = 4.24 and rACH = 5.38) (Table 1).

Table 1. Summary of the morphology, the ratio and the GTG patterns of macrochromosomes and ZW in the studied species.

Species	Morphology						(r)					
Chr	GGA	CCO	CJA	ABA	ACH	CUN	GGA	CCO	CJA	ABA	ACH	CUN
1	SM	SM	SM	SM	SM	SM	1,69	1.32	2.15	1.58	1.56	2.46
2	SM	SM	SM	SM	SM	SM	1,94	1.32	1.32	1.62	1.76	2.19
3	AC	AC	AC	AC	AC	AC	15,18	17.9	14.28	5.4	6.25	18.50
4	T	ST	ST	AC	AC	AC	3,86	6.16	5.31	4.24	5.38	10.98
5	AC	AC	AC	AC	AC	AC	9,39	8.25	7.4	3.8	6.28	13.37
6	AC	AC	AC	AC	AC	AC	21,83	8.18	9.5	3.41	4.46	15.86
7	T	T	AC	AC	AC	AC	3,18	4.38	6.6	2.42	4.28	41.89
8	SM	SM	SM	AC	AC	AC	1,46	1.96	1.95	2.96	3.76	92.52
Z	M	M	M	SM	SM	SM	1,12	0.49	1.09	1.24	1.12	2.17
W	SM	ST	ST	SM	SM	SM	1,59	5	5.11	1.37	1.47	3.01

GGA: *Gallus gallus domesticus*, **CCO**: *Coturnix coturnix*, **CJA** : *Coturnix japonica*, **ABA** : *Alectoris barbara*, **ACH** : *Alectoris chukar*, **CUN** : *Chlamydotis undulata*, **Chr** :Chromosomes, **M**: Metacentric, **SM**: Submetacentric, **AC** : Acrocentric, **T** : Telocentric, **ST** : Subtelocentric,**(r)** : Ratio (q/p).

Table 2. Chromosomal rearrangements that could have occur during speciation.

Studiedspecies	Commonquail	Japanesequail	Gambra	Choukarpartridge	Houbarabustard
Domestic chicken			partridge		
1	H	**NC**	H	H	**NC**
2	H	**DPI**	H	H	**TF**
3	H	H	H	H	H
4	**NC**	**NC**	**NC**	**NC+ PI**	**F**
5	H	H	H	H	H
6	H	H	H	H	H
7	H	**D (p) / NC**	**NC/Per. Inv.**	**NC/Per. Inv.**	**NC/Per. Inv.**
8	**Per. Inv.**	**Per. Inv.**	**NC/Per. Inv.**	**NC/Per. Inv.**	**NC/Per. Inv.**
Z	H	H	**Ter. Inv.**	H	**Ter. Inv. + Int. Del.**
W	**NC**	**NC**	H	H	H

H: Homology, **NC:** Neocentromere, **Per. Inv.:** Pericentric Inversion, **Para. Inv**.: Paracentric Inversion, **DPI :** Double Pericentric Inversion, **Ter. Inv.**: Terminal Inversion, **Int.Del. :** Interstitial Deletion**, D (p)** : Deletion of p arm**, F :** Fission, **TF :** Terminal Fission.

Moreover, a strong homology of G-banding patterns is observed on chromosomes 4 of all Galliformes species in the present work. Nevertheless, the presence of a larger short arm (p) is found in GGA compared to both quail species. However, we noted a clear difference in the size of chromosome 4 of the CUN compared to the other chromosomes. The CUN-4 correspond to the distal part of the long arm of chromosome 4 of the other species studied (Fig. 1C). Indeed, the CUN-4 would correspond to the distal part (q 2.1 – q 2.7) of the long arm (q) of the CCO-4 of and CJA-4. It would also correspond to the distal region q 2.1 – q 3.4 of the ABA-4 and to the region q 3.1 – q 4.7 of ACH-4.

Chromosome 7

The CCO-7 (r = 3,18) and GGA-7 (r = 4.38) chromosomes are telocentric. In contrast, chromosomes 7 in other species are acrocentric. Indeed, the measurable CCO-7 p-arm looks more similar to its GGA homolog than to the CJA, ABA, ACH and CUN (Fig. 1D). However, the comparative analysis of the GTG banding patterns of the different chromosomes 7 has made it possible to highlight a significant conservation between these species.

Chromosome 8

The chromosome CCO-8 is submetacentric (r = 1.96), CJA (r = 1.95) as in GGA (r = 1,46), while it is acrocentric in CUN, ABA and ACH (Fig. 1E). Despite significant conservation of the GTG banding pattern in quails and chicken, a rearranged region is observed which it is flanked by bands p 1.1 and q 1.2.

Chromosome Z

The chromosome Z is submetacentric in studied species except of CCO and CJA in which this gonosome is metacentric as for the chicken (Fig. 1F). However, a terminal inversion in the q arm is observed in each of CUN and ABA (corresponding to Zq2.1 in ABA and to Zq1.3-2.4 in CUN). A loss of an interstitial segment in the p arm of CUN-Z is also observed in this study and would correspond to the p1.1 → p1.3 region in GGA-Z.

Chromosome W

The W chromosome of the Partridges and the Houbara bustard is submetacentric, while it is subtelocentric in the two quails. High conservation of the GTG banding pattern is observed in all species (Fig. 1G). The W chromosome is ranked in the sixth position in quails, in the seventh position in Houbara bustard and in the ninth position in Barbary and Chukar partridges.

Discussion

In order to explore the chromosomal rearrangements that occurred in macrochromosomes during the evolution of the five species (CCO, CJA, ABA, ACH and CUN), a comparative analysis of the GTG morphological bands was carried out with chicken chromosomes, which represent the hypothetical ancestor of Neognathae. Indeed, we observed significant conservation between these species, but we also detected some rearrangements.

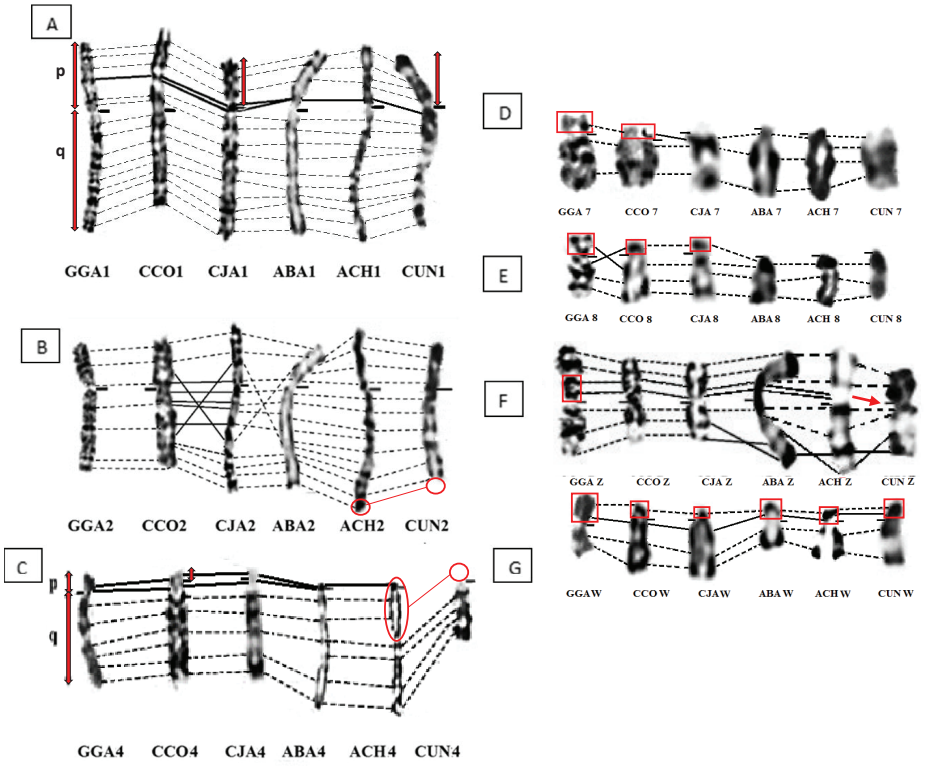

Figure 1. Comparison of chromosome (**A**) 1, (**B**) 2, (**C**) 4, (**D**) 7, (**E**) 8, (**F**) Z, and (**G**) W in GTG bands between the six species studied. The dotted lines indicate similarities, the full ones and the red circls/ frames show the differences. **GGA:** Domestic chicken, **CCO:** Common quail**, CJA:** Japanese quail, **ABA:** Gambra partridge, **ACH:** Chukar partridge, **CUN:** Houbara bustard.

Chromosomes 1

Chromosome 1 of the Gambra and Choukar partridges, as well as that of the Common quail and the Chicken are morphologically similar, showing strong homology of GTG banding profiles. On the other hand, the CJA-1 is identical to that of the CUN-1 and they have a shorter p arm. This result could be explained by the formation of an Evolutionary Neocentromere (ENC) on the ancestral chromosome of the CJA-1 and the CUN-1, which appeared during evolution (Fig. 2A).

However, high-resolution analysis of meiotic CJA-1 suggests that the difference in position of the centromere with that of the Domestic chicken is not caused by a pericentric inversion, but by the formation of a de novo centromere, which it was not accompanied by a rearrangement of the order of chicken-specific molecular markers (Zlotina et al. 2012). Although the mechanisms of ENC formation are poorly understood, they nevertheless seem to involve the inactivation of the old centromere and the formation of a new one in an euchromatic locus (Zlotina et al. 2012).

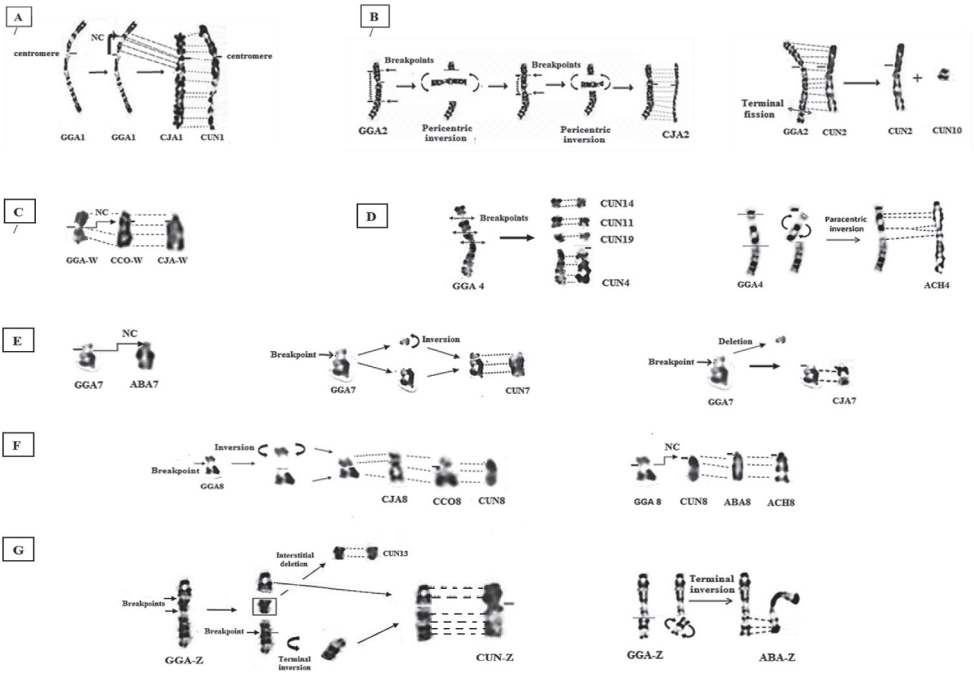

Figure 2. Representation of chromosomal rearrangements that could have occurred during the chromosomes formation of the six studied species **A** appearance of a neocentromere (NC) on the ancestral CJA1 and CUN1 **B** double inversion that could have occurred on chromosome 2 between GGA and CCO/CJA (left). Appearance of a possible terminal fission on ancestral GGA2, which would be at the origin of the formation of CUN2 and microchromosome CUN10 (right) **C** possible formation of a neocentromere during the evolution of GGAW and CCOW **D** appearance of several fissions on the ancestral chromosome 4, which would be at the origin of the formation of chromosomes 4, 11, 14 and 19 of the Houbara bustard (left). Appearance of paracentric inversion between GGA4 and ACH4 (right) **E** formation of a neocentromere between GGA7 and ABA7 (left) or the course of a pericentric inversion between GGA7 and CUN7 (in the middle), deletion of the short arm p of GGA7 and CJA7 could have occurred between during evolution (right) **F** pericentric inversion could have occurred between GGA8 and (CCO8, CJA8, CUN8) (left), possible formation of a NC between GGA8 and CUN8 as well as the both partridge species (right) **G** formation of CUNZ following a possible interstitial deletion (fragment corresponding to CUN13) occurring on the ancestral Z chromosome accompanied by a terminal inversion (left). A terminal inversion in Zq2.1 is observed in ABA (right).

This evolutionary phenomenon seems to be quite common. It has been reported in different taxonomic groups, particularly in birds. Indeed, it is thanks to the study carried out on red partridges that it was possible to show perfect conservation of the chicken Bacterial Artificial Chromosome (BAC) clones ordering themselves on chromosome 4 of *Alectoris rufa* and to introduce, for the first time in the class of birds, the term neocentromere (Kasai et al. 2003). This is also the case for pheasants (*Phasianus colchicus*, *Chrysolophus pictus*, *Lophura nycthemera* (Guttenbatch et al. 2003) and the *Peking duck* (Skinner et al. 2009).

This centromere repositioning is also reported in the ancestral CUN-1. The comparative mapping of the macrochromosomes of eight avian species including the Houbara

bustard, showed an almost total hybridisation of 17 BAC clones specific of GGA-1 (with the exception of the 5th marker which not found on the CUN-1). Nevertheless, it was noted that 6th marker is located on the short p arm of GGA-1 whereas it is found on the q arm of CUN-1 (Kiazim et al. 2021). According to the latest classification of birds, the orders of Colombiformes and Cuculiformes are very close to Otidiformes (Prum et al. 2015).

A similar result was observed on chromosomes 1 of the Mallard *Anas platyrhynchos* and the Helmeted guineafowl *Numida meleagris*. In this study additional of evidence for centromere repositioning in birds was reported (Kiazim et al. 2021). The use of chromosome painting with chicken-specific probes in five Columbidae species showed significant conservation of chromosome 1 organisation, notably in *Columbina talpacoti* and *Columbina passerina* (Kretschmer et al. 2020).

Chromosome 2

With the exception of CUN-2 and CJA-2, the chromosome 2 is fairly conserved in the species studied. The CJA-2, which has a large region with an inverted GTG banding pattern, could be explained by the appearance of a double pericentric inversion on its ancestral chromosome 2 (Fig. 2B). The latter has already been reported by Kartout-Benmessaoud and Ladjali-Mohammedi (2018). The identified inversions indicate the occurrence of double-stranded DNA breaks. Indeed, evolutionary breakpoint regions are fragile genomic regions favouring chromosomal rearrangements because they are found in genetically dense areas (Pevzner and Tesler 2003; Larkin et al. 2009).

This supports the result of previous studies which showed the presence of pericentric inversions on GGA-2 and CJA-2 using BAC clones (Schmid et al. 2005; Kayang et al. 2006), PAC (P1-derived Artificial Chromosome) clones (Fillon et al. 2003) and Cosmid clones (Shibusawa et al. 2001). Comparative mapping of meiotic CJA-2 by combination of immunodetection and FISH confirmed the presence of a double pericentric inversion (Zlotina et al. 2010, 2012).

Also, this result corroborates the study which reported pericentric inversions of the ancestral chromosome 2 in other species of birds belonging to the order of Galliformes. Indeed, this is the case of the duck *Anas platyrhynchos* whose BAC clones WAG42G5 and WAG9L1 were hybridised on GGA2q and APL2p, providing clear evidence of a pericentric inversion (Fillon et al. 2007; Skinner et al. 2009).

Furthermore, chromosome 2 of the bustard seems to have lost the terminal part of its long arm (q). Indeed, the end of the long arm (q) of CUN-2 is shorter than that of the Galliformes species studied and would be the consequence of terminal fission (Mahiddine-Aoudjit et al. 2019) (Fig. 2B).

The lost distal part could possibly be involved in another independent rearrangement process (Furo et al. 2015) or could correspond to the formation of a microchromosome. Deeper understanding of avian genomic structure permits the exploration of fundamental biological questions pertaining to the role of evolutionary breakpoint regions and homologous synteny blocks (O'Connor et al. 2024).

Thus, the comparison of the patterns of the GTG bands of the existing part in the chicken with the microchromosomes of the bustard allowed us to detect a similarity

with the microchromosome 10 (Fig. 2B). This leads us to consider the course of a terminal fission on the ancestral chromosome to give rise to macrochromosome 2 and microchromosome 10 of the Houbara bustard.

This hypothesis can only be confirmed by the hybridisation of molecular markers specific to the terminal (q) region of chromosome 2 of the Domestic chicken. Nevertheless, our result corroborates studies that have reported the fission of ancestral chromosome 2, particularly in Galliformes (Guttenbatch et al. 2003; Griffin et al. 2008; Kretschmer et al. 2018), Columbidae (Kretschmer et al. 2020) and Cuculiformes (Santos et al. 2020). Also as observed in Psittaciforme (parrots), Suliformes and Piciformes, a loss of chromosomal sequence and Fissions was reported on chromosome 2 (Huang et al. 2022; Barcellos et al. 2024).

Chromosome 4

The analysis of chromosome 4 in the species studied showed that it is acrocentric in CUN, ABA and ACH while it is telocentric in GGA and subtelocentric in CCO and CJA. The ratio q/p of chromosomes 4 of the both quails and chicken is different but we observed perfect conservation patterns in chromosome of the three species. This result could suggest repositioning of the centromere during the speciation event (Kartout-Benmessaoud and Ladjali-Mohammedi 2018). However, several hypotheses have been proposed to explain the differences between CJA-4 and GGA-4 (Shibusawa et al. 2001; Fillon et al. 2003; Schmid et al. 2005; Galkina et al. 2006).

Nevertheless, during the evolution of Galliformes karyotypes, centromeres appear to be formed *de novo* (Kasai et al. 2003; Galkina et al. 2006; Skinner et al. 2009). The profile of the bands is however preserved in the Gambra partridge and the Domestic fowl, while in the Choukar partridge, the subcentromeric region presents a different profile evoking a paracentric inversion (Fig. 2D) (Ouchia-Benissad and Ladjali-Mohammedi 2018).

In addition, comparison of GTG banding patterns revealed that CUN-4 would correspond entirely to the distal part (q 2.1 – q 2.7) of the long arm (q) of the CCO-4 of and CJA-4. It would also correspond to the distal region q 2.1 – q 3.4 of the ABA-4 and to the region q 3.1 – q 4.7 of ACH-4 (Ouchia-Benissad and Ladjali-Mohammedi 2018; Kartout-Benmessaoud and Ladjali-Mohammedi 2018; Mahiddine-Aoudjit et al. 2019). This indicates that the ancestral chromosome 4 may have lost its short arm and a part of the long arm during speciation. Similarly, we found that bustard microchromosome 14 (CUN-14) resembles the short arm (p) of GGA-4. While (CUN-11 and -19) microchromosomes would be similars to different regions of GGA-4 that are missing on CUN-4 (Fig. 2D).

Thus, CUN-4 seems to be derived from the fission of the ancestral chromosome 4, and corresponds only to the distal part of the long arm of chromosome 4 of the other species. Indeed, this chromosome is the result of a fairly complex evolutionary history (Chowdhary and Raudsepp 2000; Schmid et al. 2000; Shibusawa et al. 2004). This was shwon by the hybridisation of GGA-4 on the metaphases of 9 different species (Anseriformes, Gruiformes and Passeriformes) and revealed the existence of a partial homology with three different chromosomes of Gruiformes. Indeed, a segment of

GGA-4 would correspond to the short arm (p) of chromosome 4 of the Coot FAT-4 (*Fulica atra*, Gruiformes) while the other regions of GGA-4 are found on two other chromosomes (FAT-7 and FAT-13) (Nanda et al. 2011).

Hybridisation of chicken chromosome 4 on three different hummingbird chromosomes (*G. guira*, Cuculidae) has been noted, which represents a sister phylogenetic group with the Otidiformes already mentioned (Jarvis et al. 2014; Santos et al. 2020).

These events fission of the ancestral chromosome 4 could be explained by the fact that the DNA regions involved in the breaks are particularly fragile (Damas et al. 2019). Indeed, chromosomal regions likely to break have been identified and defined as being fragile (FS) and unstable sites (Sutherland 1979) and would be involved in chromosomal recombination events (Svetlova et al. 2001). This is also the case for *Geese* and the *Collared dove* (Shibusawa et al. 2002, 2004; Griffin et al. 2007).

Interchromosomal rearrangements involving microchromosomes are rare events in birds (Kretschmer et al. 2021a). The ancestral microchromosomal syntenies are conserved in Piciformes and Trogoniformes but chromosome reorganisation is observed in Suliformes included fusions involving both macro- and microchromosomes (Kretschmer et al. 2021a).

Contrary to chromosomes 5 and 6 which seem to be morphologically similar in all the species studied, chromosomes 7 and 8 would show rearrangements:

Chromosome 7 is telocentric in the Common quail and the Domestic fowl, whereas it is acrocentric in the other species analysed. It would seem that the deletion of the short arm (p) of the ancestral chromosome 7 would have occurred during evolution to give an acrocentric chromosome 7 like that of the Japanese quail (Kartout-Benmessaoud and Ladjali-Mohammedi 2018). The same rearrangement was proposed through the localisation of chicken-specific BAC clones on CJA-7 (Shibusawa et al. 2001; Fillon et al. 2003). Whereas, the formation of a neocentromere or the course of a pericentric inversion has been proposed to explain the current morphology of the CUN-7 and the two partridges (Fig. 2E) (Ouchia-Benissad and Ladjali-Mohammedi 2018; Mahieddine-Aoudjit et al. 2019).

Several studies have shown that chromosomes 7 and 8 are quite conserved in Galliformes (Kasai et al. 2003). While the hybridisation of specific probes of the GGA-7 on the metaphases of the Guinea fowl *Numida meleagris* revealed the presence of a pericentric inversion (Shibusawa et al. 2002). It would also seem to be the case of CUN-7 in which an inversion has been reported (Kiazim et al. 2021). Only molecular studies could elucidate such evolutionary events.

Chromosome 8

Comparison of GTG banding shows relatively conserved patterns in ABA-8, ACH-8 and CUN-8. However, CJA-8, CCO-8 and GGA-8 share the same morphology but not the same bands distribution. In fact, chromosome 8 of ABA/ACH/CUN is acrocentric while in CJA/CCO/GGA this chromosome is submetacentric (Fig. 2F). The morphological difference observed in these species could be explained by repositioning

of the centromere in common ancestor during divergence (Ouchia-Benissad and Ladjali-Mohammedi 2018; Mahieddine-Aoudjit et al. 2019).

In the other hand, double pericentric inversion may also have occured explaining differences in chromosomes morphology but the conservation of banding pattern is noted. In contrast, CCO-8 shows same morphology with GGA-8 but different disposition of GTG bands. This would be the result of a pericentric inversion in the region 8p 1.1- q1.2 (Kartout-Benmessaoud and Ladjali-Mohammedi 2018) as it has been reported in Japanese quail (Shibusawa et al. 2001; Fillon et al. 2003; Sasazaki et al. 2006).

Chromosome Z

The chromosome Z is submetacentric in the species ABA, ACH and CUN while it is metacentric in CCO and CJA, as in the chicken (Ouchia-Benissad and Ladjali-Mohammedi 2018; Kartout-Benmessaoud and Ladjali-Mohammedi 2018; Mahieddine-Aoudjit et al. 2019). Thus, a terminal inversion in the long arm is observed in each of CUN-Z and ABA-Z (corresponding to Zq2.1 in ABA) with loss of a region (p1.1–3) in the p arm of CUN-Z potentially corresponding to the microchromosome 13 (Fig. 2G) (Ouchia-Benissad and Ladjali-Mohammedi 2018; Mahieddine-Aoudjit et al. 2019). In addition, recurrent breakpoints evoking the presence of fragile sites have been detected on the Z chromosome of 15 species belonging to seven (07) different orders (Gerbault-Seureau et al. 2019). In fact, chromosome Z in birds contains high number of breakpoints and is particularly submitted to structural changes broadly represented by para or pericentric inversions (Fillon et al. 2007; Nanda et al. 2008; Skinner et al. 2009; Itoh et al. 2011) and rarely by Robertsonian translocation (Kretschmer et al. 2021b).

In addition, the Z chromosome presents a particularly high substitution rate in introns (Wang et al. 2014). The evolution of avian sex chromosomes was characterised by a complex process of inversions likely related to both Z and W (and/or other processes) (Yazdi and Ellegren 2018; Okuno et al. 2021). These rearrangements could explain the early divergence of Z chromosome than other chromosomes (Yazdi and Ellegren 2018; Degrandi et al. 2020; Hayes et al. 2020; Hooper et al. 2020; Huang et al. 2022).

Chromosome W

In both partridges and Houbara bustard, the W chromosome is submetacentric while it is telocentric in both quails, wich could be explained by an evolutionary new centromere (ENC) (Fig. 2C) (Ouchia-Benissad and Ladjali-Mohammedi 2018; Kartout-Benmessaoud and Ladjali-Mohammedi 2018; Mahieddine-Aoudjit et al. 2019).

The W chromosome is widely heterochromatic and contains high amounts of repetitive sequences, like that of Tataupa tinamou. In contrast, W chromosomes of Greater rhea and emu did not exhibit a significant buildup of either C-positive heterochromatin or repetitive DNAs. This indicates their large undifferentiation both at morphological and molecular levels (Setti et al. 2024). The W chromosome of birds, like that of snakes, seems to have degenerated during evolution, since it is morphologically small (Ellegren 2011). These repeats have been amplified in the pericentromeric region

of W chromosomes, which may have resulted from the disruption of meiotic recombination between the Z and W chromosomes at an early stage of sex chromosome differentiation (Ishishita et al. 2014). Hence, microsatellite sequences may play significant role in sex chromosome differentiation (Barcellos et al. 2019).

However, it exhibits much conserved gene content despite their independent evolution of recombination suppression (Graves 2014; Schartl et al. 2016; Xu and Zhou 2020). The sequencing of chicken W chromosome shows preservation of ancestral genes enriched for expressed dosage-sensitive regulators (Bellott et al. 2017).

The chameleons of the genus *Paroedura*, are considered excellent models for studies of convergent and divergent evolution of sex chromosomes (Rovatsos et al. 2023). We compared GTG-banded chromosomes of the species studied to trace the evolution of macrochromosomes. This type of analysis allows the identification of regions that have undergone possible events of neocentromere formation, deletions, inversions and fissions all of which contribute to rearrangements that influence speciation and phylogenetic relationships. A synthetic diagram is proposed to explain the chronology of appearance of the different evolutionary events since the ancestral karyotype (Fig. 3).

This study made it to highlight rearrangements linked to changes in morphology and profiles of GTG bands. Appearance of few inter- and intrachromosomal rearrangements

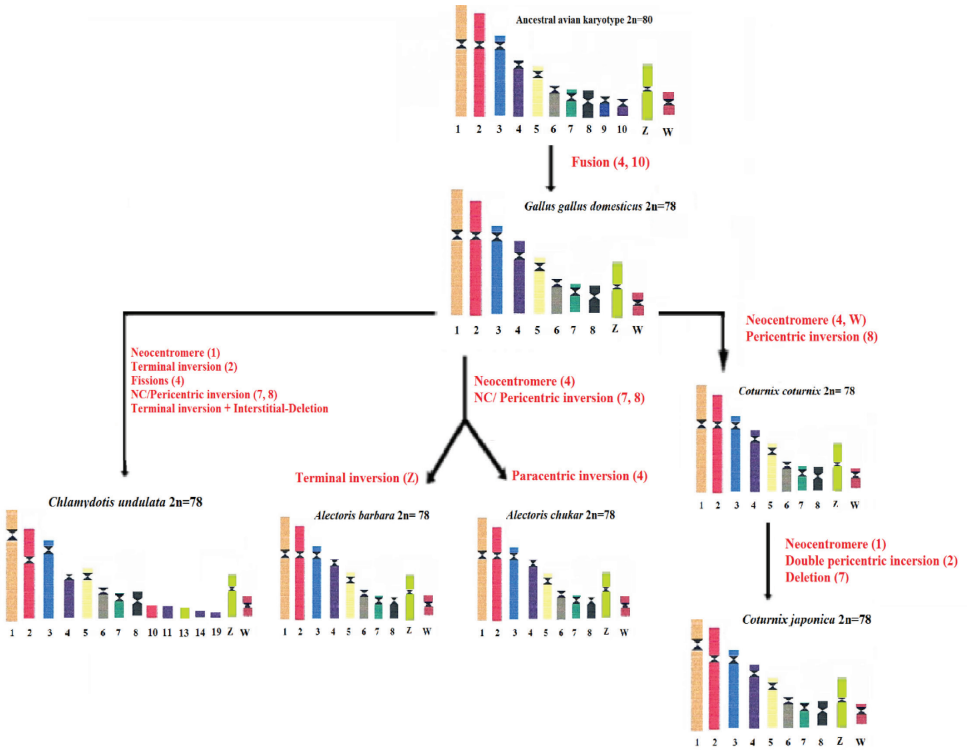

Figure 3. Evolutionary representation of partial karyotypes of some galliforms and of an otidiform as well as the inter and intrachromosomal rearrangements that would have occured during speciation, compared to the presumed ancestral avian karyotype.

that occurred during evolution suggests that the organisation of the genome is highly conserved between these six species studied. Of note, the Houbara bustard karyotype has the highest number of intrachromosomal and interchromosomal rearrangements (including fissions) compared to the ancestral avian karyotype. Also, found interchromosomal rearrangements involving shared microchromosomes between the two avian orders analysed. These rearrangements confirm that the structure of avian karyotypes would be more conserved at the interchromosomal but not intrachromosomal scale.

However, a comparison with phylogenetic species close to the bustard such as Cuculidae, Musophagiformes and Columbiformes would be interesting. Indeed, most Columbidae species showed at least one interchromosomal rearrangement (notably fissions). Nevertheless, intrachromosomal rearrangement remains the main driver of chromosome evolution in Columbidae. It is therefore fundamental to carry out interspecific hybridisations of chicken BAC clones to elucidate and confirm chromosomal rearrangements observed during this work.

Nevertheless, the conservation of endangered avian species is facilitated through the application of preservation and analysis of genomic data. The storage of chromosomes and nucleotides sequences is so a form of biobanking. Therefore, an analysis of sequence can identify genetically important individuals for breeding. Finally, avian genomics and stem cell approaches could not only offer hope of saving endangered species, such as the green peafowl but also other birds threatened with extinction.

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ORCID

Yasmine Kartout-Benmessaoud https://orcid.org/0009-0007-2658-2503

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SHORT COMMUNICATION

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Number and location of rDNA clusters in the superfamilies Tenthredinoidea and Cynipoidea (Hymenoptera): an update

Vladimir E. Gokhman[1], Valentina G. Kuznetsova[2], Boris A. Anokhin[2]

1 *Russian Entomological Society, Moscow, Russia* **2** *Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia*

Corresponding authors: Vladimir E. Gokhman (vegokhman@hotmail.com);
Valentina G. Kuznetsova (valentina.kuznetsova@zin.ru)

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Abstract

To identify nucleolus organizing regions (NORs), fluorescence *in situ* hybridization (FISH) with 18S rDNA probe was performed on chromosomes of *Tenthredo campestris* Linnaeus, 1758 (Tenthredinidae), *Arge ciliaris* (Linnaeus, 1767) (Argidae) (n = 10 in both) and *Aulacidea hieracii* (Bouché, 1834) (Cynipidae) (2n = 20). In all these species, a single pericentromeric rDNA cluster per haploid karyotype was detected. This number of NORs is confirmed as ancestral for the order Hymenoptera.

Keywords

Apocrita, Argidae, chromosomes, Cynipidae, Hymenoptera, rDNA-FISH, Symphyta, Tenthredinidae

Hymenoptera represent one of the largest insect orders, with the approximate number of described species far exceeding 150,000 (Huber 2017). However, the overwhelming majority of this taxonomic diversity belongs to the suborder Apocrita, or higher Hymenoptera (see, e.g., Forbes et al. 2018), whereas the substantially less speciose Symphyta (= lower Hymenoptera) harbor less than nine thousand members (Huber 2017).

Nevertheless, Symphyta include the least advanced Hymenoptera, and therefore studying these taxa is necessary to reconstruct ancestral character states for the order in general. Among other characteristics, this also applies to different karyotype features of Hymenoptera (Gokhman 2023a). For example, the ancestral nature of the canonical "insect-type" telomeric repeat in this order, TTAGG, was proven only when telomere structure of certain sawflies has been studied (Gokhman and Kuznetsova 2018; Lukhtanov and Pazhenkova 2023). However, other characteristics of symphytan chromosome sets, i.e., the number and location of clusters of 45S ribosomal DNA (rDNA), were studied in just a few members of the superfamily Tenthredinoidea, which belong to the families Tenthredinidae, Athaliidae and Diprionidae (Rousselet et al. 1999, 2000; Kuznetsova et al. 2001; Matsumoto et al. 2002). During these studies, different techniques for revealing 45S rDNA sites, which represent nucleolus organizing regions (NORs), i.e., AgNOR-banding, staining GC-enriched chromosome segments with chromomycin A_3 (CMA_3) and *in situ* hybridization with rDNA probes, including that using fluorescent dyes (FISH), were employed (Gokhman and Kuznetsova 2024). Nevertheless, the ancestral number and location of these rDNA clusters in the Symphyta remain ambiguous, since haploid karyotypes of two members of the genus *Tenthredo* Linnaeus, 1758, *T. velox* Fabricius, 1798 and *T. arcuata* Forster, 1771, were shown to have one and two 45S rDNA sites, respectively (Kuznetsova et al. 2001). Moreover, another species of the family Tenthredinidae, *Rhogogaster viridis* (Linnaeus, 1758), as well as *Diprion pini* (Linnaeus, 1758) and *Neodiprion abietis* (Harris, 1841) (Diprionidae) also have single 45S rDNA clusters (Rousselet et al. 1999, 2000; Kuznetsova et al. 2001), but the haploid karyotype of *Athalia rosae* (Linnaeus, 1758), which is now placed in a separate family Athaliidae (Wutke et al. 2024), carries four sites of that kind (Matsumoto et al. 2002).

Although much more is now known about the number and location of NORs in Apocrita (Gokhman and Kuznetsova 2024), reconstruction of the ancestral number and location of these parameters in this suborder is also far from straightforward. Specifically, both the number and location of these sites vary substantially across parasitoids as well as across aculeate Hymenoptera. Whilst the only 45S rDNA cluster per haploid karyotype is a widespread condition in Apocrita (see, e.g., Gokhman et al. 2024), chromosome sets of these insects can harbor up to six and even 15 NORs in parasitic wasps and Aculeata, respectively (Gokhman and Kuznetsova 2024). FISH also visualized single 45S rDNA clusters in all studied members of the superfamily Cynipoidea, i.e., in *Diplolepis rosae* (Linnaeus, 1758) (Cynipidae) (Gokhman et al. 2014) as well as in four species of the family Figitidae (Gokhman et al. 2016). In addition, karyotypes of three other members of Cynipidae, *Aulacidea hieracii* (Bouché, 1834), *Isocolus jaceae* (Schenck, 1863) and *I. scabiosae* (Giraud, 1859), have been recently examined using CMA_3 (Gokhman 2021). Although single CMA_3-positive sites per haploid chromosome set were detected in all these species, these results could be corroborated using FISH, since CMA_3-positive chromosome segments do not always represent NORs (see, e.g., Gromicho et al. 2005; Gokhman et al. 2017). We have therefore undertaken the present study to further identify the number and location of rDNA clusters in the superfamilies Tenthredinoidea and Cynipoidea. The results of this study are given below.

Material and methods

Origin of insects

Adult sawflies and galls containing immature stages of *A. hieracii* were collected in 2022 by the first author near Ozhigovo, Russia (about 60 km SW Moscow) as well as by M.I. Nikelshparg (Saratov State University, Saratov, Russia) near the city of Saratov, Russia (about 730 km SE Moscow), respectively. All sawflies were identified by S.A. Basov (Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia).

Preparation of chromosomes

Chromosome preparations were obtained according to the guidelines provided by Naito (1982) and Imai et al. (1988) with a few modifications. Adult female sawflies were dissected in small Petri dishes in distilled water, unfertilized mature eggs were extracted from their bodies, placed into the dishes on a filter paper soaked with distilled water, and then incubated for 3–4 days at room temperature (RT). Haploid embryos and cerebral ganglia were extracted from the eggs and gall wasp prepupae, respectively, and dissected in 0.5% hypotonic sodium citrate solution containing 0.005% colchicine. The embryos and ganglia were then transferred to a fresh portion of hypotonic solution and incubated for about 30 min at room temperature. The material was transferred onto a pre-cleaned microscope slide using a Pasteur pipette and then gently flushed with Fixative I (glacial acetic acid: absolute ethanol: distilled water 3:3:4). The tissues were disrupted using dissecting needles in an additional drop of Fixative I. A drop of Fixative II (glacial acetic acid: absolute ethanol 1:1) was applied to the center of the area, and the more aqueous phase was blotted off the edges of the slide. The slides were then dried and stored at RT for a few weeks.

FISH procedure

Genomic DNA from a male *Pyrrhocoris apterus* (Linnaeus, 1758) (Hemiptera, Heteroptera, Pyrrhocoridae) was isolated using CTAB extraction method. FISH with the 18S rRNA gene probe was carried out on chromosomes of all studied species. The target 18S rRNA gene was PCR amplified (see Grozeva et al. 2011 for the table of primers) from the genomic DNA of *P. apterus*, and labeled by PCR with biotin.

In situ hybridization was performed as described by Schwarzacher and Heslop-Harrison (2000) with modifications. Chromosome preparations were dehydrated through 70, 80 and 96% ethanol at RT and treated with 100 μg/ml RNAse A (Sigma) for 30 min at 37 °C in a humid chamber; washed three times in 2× SSC (5 min each) at RT; dehydrated through 70, 80 and 96% ethanol at RT; incubated in 5 mg/ml pepsin in 0.01 N HCl for 10 min at 37 °C; washed sequentially in PBS,

in PBS containing 0.05 M $MgCl_2$ for 5 min each, in 1% PFA in PBS containing 0.05 M $MgCl_2$ for 15 min, in PBS for 5 min, in PBS containing 0.05 M $MgCl_2$ for 5 min at RT each; dehydrated through 70, 80 and 96% ethanol at RT and finally, dried. After pretreatment, 6.5 µl hybridization mixture containing about 100 ng of labeled probe, 50% formamide, 2× SSC, 10% (w/v) dextran sulfate, 1% (w/v) Tween 20 and 10 µg salmon-sperm DNA was added on preparations. Slides were mounted using glass coverslips and rubber cement. The slides were denatured for 5 min at 75 °C. The chromosome slides were then incubated for 42–44 h at 37 °C. Following hybridization, the slides were washed in 2× SSC for 3 min at 45 °C, then in 50% formamide in 2× SSC for 10 min at 45 °C, two times in 2× SSC (10 min each) at 45 °C, blocked in 4× SSC containing 1.5% (w/v) BSA and 0.1% Tween 20 for 30 min at 37 °C in a humid chamber. 18S rRNA gene probe was detected with 5 µg/ml Avidin-FITC (Invitrogen). Detection was performed in 4× SSC containing 1.5% BSA and 0.1% Tween 20 for 1 h at 37 °C. Slides were washed three times in 4× SSC containing 0.02% Tween 20 (10 min each) at 45 °C and dehydrated through 70, 80 and 96% ethanol at RT. Chromosomes were mounted in an antifade medium (ProLong Gold antifade reagent with DAPI, Invitrogen) and covered with a glass coverslip.

Image acquisition and analysis

Metaphase plates and interphase nuclei were analyzed under a Leica DM 6000B microscope with a 100× objective. Fluorescence images were taken with a Leica DFC 345 FX camera using Leica Application Suite 4.5.0 software with an Image Overlay module. To prepare illustrations, the resulting images were arranged and enhanced using GIMP 2.10. Chromosomes were classified according to the guidelines provided by Levan et al. (1964).

Results

Superfamily Tenthredinoidea
Family Tenthredinidae

Tenthredo campestris Linnaeus, 1758

A detailed description of the karyotype of this species can be found in Gokhman (2024). The haploid set of *T. campestris* includes 10 biarmed chromosomes. In the karyotype of this species, the first chromosome is about 1.3 times longer than the second one, which is, in turn, also 1.3 times longer than the third chromosome. The remaining chromosomes more or less gradually decrease in size. The haploid karyotype of *T. campestris* harbors the only pericentromeric rDNA cluster on a particular medium-sized chromosome (Fig. 1A).

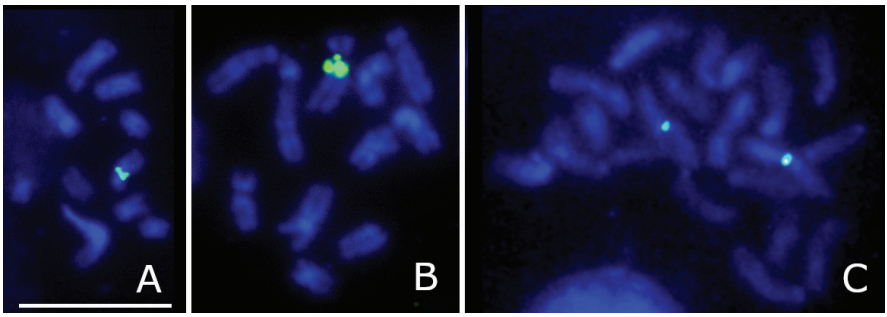

Figure 1. FISH with 18S rDNA probe on Hymenoptera chromosomes. *Tenthredo campestris*, n = 10 (**A**), *Arge ciliata*, n = 10 (**B**), *Aulacidea hieracii*, 2n = 20 (**C**). Probe signals are indicated in green. Scale bar: 10 μm.

Family Argidae

***Arge ciliaris* (Linnaeus, 1767)**

A detailed karyotypic description of this species is given by Gokhman (2023b). The haploid set of *A. ciliaris* harbors 10 biarmed chromosomes. The first chromosome is about 1.8 times longer than the second one; the remaining chromosomes gradually decrease in size. On some metaphase plates, the second metacentric bears an obvious pericentromeric secondary constriction on its longer arm (see Gokhman 2023b). It is therefore not surprising that the only 18S rDNA site revealed in this species by FISH is apparently co-localized with this constriction (Fig. 1B).

Superfamily Cynipoidea
Family Cynipidae

***Aulacidea hieracii* (Bouché, 1834)**

A detailed description of the karyotype of this species, including results of CMA_3 staining can be found in Gokhman (2021). The diploid set of *A. hieracii* includes 20 chromosomes, which gradually decrease in size, with a single pericentromeric CMA_3-positive band located on the largest metacentric. FISH with the 18S rDNA probe revealed the only paired site in place of the CMA_3-positive band (see Gokhman 2021), thus suggesting that both used techniques visualized the same rDNA cluster (Fig. 1C).

Discussion

Haploid karyotypes of both studied members of Tenthredinoidea, *T. campestris* (Tenthredinidae) and *A. ciliaris* (Argidae), harbor single rDNA clusters. Moreover, this character state predominates within Tenthredinoidea (see above), and this therefore sug-

gests that the single rDNA site per haploid chromosome set is ancestral at least for the whole superfamily. This is further corroborated by the fact that *A. ciliaris* remains the only examined member of the Argidae + Pergidae clade, a sister one to the remaining Tenthredinoidea except Blasticotomidae (Wutke et al. 2024), the most basal family which chromosomes are totally unknown. If this is true, then higher numbers of rDNA clusters found in *Tenthredo arcuata* and *Athalia rosae* (Kuznetsova et al. 2001; Matsumoto et al. 2002) apparently represent apomorphic conditions. Anyway, studying these clusters in other superfamilies of the Symphyta could either confirm or reject this assumption.

In turn, Tenthredinoidea also represent a sister clade to all other Hymenoptera, except for the family Xyelidae (Wutke et al. 2024), in which the structure and location of rDNA sites are still unknown (see Gokhman 2023a). Nevertheless, a single 45S rDNA cluster per haploid karyotype can be found in the majority of parasitoid Hymenoptera including all known Cynipoidea (Gokhman et al. 2014, 2016; Gokhman 2022; present paper). Furthermore, the same character state is considered ancestral for Aculeata, despite wide variation in the number and location of these clusters in aculeate Hymenoptera (Menezes et al. 2021). Taken together, all this information suggests that a single rDNA site per haploid genome is also ancestral for the whole order Hymenoptera. Again, this does not seem surprising, since a similar pattern is apparently characteristic of the class Insecta in general (Gokhman and Kuznetsova 2024).

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ORCID

Vladimir E. Gokhman https://orcid.org/0000-0001-9909-7559
Valentina G. Kuznetsova https://orcid.org/0000-0001-8386-5453
Boris A. Anokhin https://orcid.org/0000-0002-4110-6704

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RESEARCH ARTICLE

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Gallophilous theory of cyclical parthenogenesis in aphids (Homoptera, Aphidinea)

Ilya A. Gavrilov-Zimin[1]

1 *Zoological Institute of the Russian Academy of Sciences, Universitetskaya Emb. 1, Saint Petersburg, 199034, Russia*

Corresponding author: Ilya A. Gavrilov-Zimin (coccids@gmail.com)

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Abstract

The paper elaborates theoretical basis of the origin of aphid cyclical parthenogenesis in view of the original life of these insects in strobiloid galls on *Picea* spp. The period of gall opening is greatly extended in time, which prevents normal panmixia and creates a selective advantage for parthenogenetic reproduction. Migration of aphids to secondary host plants, on which closed galls never form, parthenogenetic reproduction on these plants, and the subsequent simultaneous return of "remigrants" to the main host plant make it possible to synchronize the development of the bisexual generation and achieve mass panmixia at the end of the life cycle only; it coincides with the end of summer growth shoots or the autumn end of the vegetation period as a whole. The evolutionary transition of aphids from conifers to angiosperms in the Cretaceous period in parallel meant the possibility of development in more spacious galls accommodating several consecutive parthenogenetic generations, the transition to viviparity and telescopic embryonization, significantly accelerating the propagation.

Keywords

Adelgidae, Eriosomatidae, evolution, galls, oviparity, Pemphigidae, Phylloxeridae, unisexual (virgin) reproduction, viviparity

Introduction

Insects of the suborder Aphidinea (aphids), among all organisms, are characterized by one of the most aberrant reproductive modes, combining both extremely rare and absolutely unique features. An extensive literature is devoted to this topic, but a reader

can check the most general modern reviews (Blackman 1987; Moran 1992; Hales et al. 1997; Simon et al. 2002; Wilson et al. 2003; Wool 2005; Davis 2012; Gavrilov-Zimin et al. 2015; Yan 2020; Gavrilov-Zimin 2021) to start. The complicated reproduction system of aphids is based on cyclic parthenogenesis, which combines strict, regular alternation of one bisexual generation with one or more parthenogenetic generations with the appropriate regular seasonal change of host plants.

Such alternation is an apomorphic character of the entire suborder Aphidinea and among the approximately 5000 modern species of aphids not a single one is known that does not have parthenogenetic generations. Moreover, all species of aphids known only from parthenogenetic generations are considered to have lost their bisexual generation, usually coincident with loss of the primary host plant (Mordwilko 1901; Popova 1967; Moran 1992, etc.). Cyclic parthenogenesis of aphids is operated by a unique aphidoid cytogenetic mechanism, in which exclusively parthenogenetic females emerge from fertilized eggs, whereas males and amphigonous females, on the contrary, are produced by parthenogenesis only (Blackman 1987; Dixon 1987; Hales et al. 1997; Gavrilov-Zimin et al. 2015). In the more archaic aphid superfamily Phylloxeroidea, females of all generations always lay eggs outside of their bodies, whereas in the more advanced superfamily Aphidoidea, parthenogenetic females exhibit placental viviparity. In the latter case, the situation is complicated by paedogenesis (reproduction at immature instars) and telescopic embryonization (see more about embryonization of different groups of organisms in a special article: Gavrilov-Zimin 2024), in which the embryo developing inside mother's body already contains embryos of the next generation. The bisexual generation of aphids always differs from parthenogenetic generations in reduced fertility (down to a single egg that fills the entire abdominal cavity of the female), usually smaller in size, underdevelopment of some organs, and in many cases also complete aphagia. All these features have been discussed many times and in great detail in the aphidological literature, and by now, the theory of the connection between cyclical seasonal migrations of aphids to different host plants with the different nutritional value of these plants in different months of the warm season of the year can be considered quite well substantiated, including experimentally (Mordwilko 1901; Popova 1967; Havill and Foottit 2007, etc.). This theory connects the origin of aphids and their main diversity with life on plants of the temperate climate of the Holarctic and is in good agreement with the extreme taxonomic paucity of aphids in the tropical zone and throughout the southern hemisphere of the planet, as well as the complete absence of the most archaic groups (Adelgidae and Phylloxeridae) in the tropical climate. Here it is appropriate to cite R. Blackman and V. Eastop (https://aphidsonworldsplants.info/Introduction/): "*Cyclical parthenogenesis is a very successful way of exploiting the short-lived growth flushes of temperate plants, and aphids are thus a very successful group in temperate climates, using seasonal clues to time the alternation of the sexual and parthenogenetic phases of their life cycles. Such life cycles cannot however be readily adapted to tropical conditions. Aphids moving from temperate zones into the tropics simply lose the sexual phase of the life cycle, and in doing so they lose the potential to evolve and diversify that is dependent on the recombination of genes. The tropics may also have acted in this way as a barrier to aphid*

colonization of southern temperate regions, which also have very small indigenous aphid faunas". However, the question of the origin of the aphid cyclic parthenogenesis itself and their paradoxical reproductive system still remains open. I also cannot agree with the above quote about "very successful way" of reproduction through cyclic parthenogenesis (and parthenogenesis in general) in the conditions of a short growing season in a temperate climate, because parthenogenesis, in itself, does not lead to an acceleration of individual development and/or propagation. Such acceleration in any climate can be achieved, for example, due to the loss of some stages of ontogenesis or due to telescopic embryonization, but archaic groups of aphids (adelgids and phylloxeras) do not have these; the last feature appears only in the advanced superfamily Aphidoidea. Among the huge group of Arthroidignatha (= Hemiptera s.s.) insects, which unites about 120,000 species, not a single example of cyclic parthenogenesis is known outside of Aphidinea.

Menwhile, among Heteroptera (about 50 000 species), in Cicadinea (about 50 000 species) and Psyllinea (about 3,500 species), examples of usual thelytokous parthenogenesis occur as rare exceptions (Kuznetsova et al. 2021). In Aleyrodinea (about 1500 species), arrhenotokous parthenogenesis is known, combined with the usual regular panmixia. In Coccinea (more than 8,000 species), the sister group to aphids, parthenogenesis (of the thelytoky, arrhenotoky or deuterotoky type), with rare exceptions, is facultative and combined with irregular bisexual reproduction (Gavrilov-Zimin 2018). At the same time, true bugs, cicads, psyllids and scale insects, unlike aphids, are characterized by significant taxonomic diversity in different climatic zones of both hemispheres of the planet, including in the temperate climate of the Palaearctic, where among these groups there are species with both one and several generations per year. For example, the scale insect *Diaspidiotus perniciosus* (Comstock, 1881) (Homoptera: Diaspididae) in southern Europe has up to 5 generations per year with obligate bisexual reproduction (Danzig 1993: 192).

In general, for insects and other animals, constant bisexual reproduction is the main, absolutely dominant method of reproduction, the selective advantage of which is undoubtedly due to increased heterozygosity and a corresponding increase in the range of variability in populations. The theoretical basis for the selective advantage of bisexual reproduction has been developed in detail in numerous publications on population genetics and evolutionary theory (see, for example, Felsenstein 1974; Smith 1978; Otto 2009).

The names of higher taxa of aphids and other related insects are given according to the nomenclature-taxonomic system from Gavrilov-Zimin et al. (2015, 2021).

Origin of cyclic parthenogenesis in Adelgidae

Since all modern species of aphids possess parthenogenetic generations in their life cycle, and it is not possible to reliably judge the nature of reproduction of extinct species, the time of transition from obligate bisexuality to heterogony can only be determined very approximately. Heie (1987: 384–385) suggested that such a transition could have occurred at the beginning of the Cretaceous. This hypothesis was based

solely on the fact that among the remains of the extinct Cretaceous superfamily Canadaphidoidea (sister to modern superfamilies of aphids), individual body parts of one male are known, which supposedly had a copulatory organ: "*One among five known specimens carries a ventral process which hardly can be anything else than a male's copulatory organ. Males are rare today, and no other male has ever been found among fossils. This may mean that parthenogenetic reproduction did not occur in Canadaphidoidea, but arose in its sister group after the separation had taken place*". Since copulatory organs are also present in males of all modern families of aphids, it is rather difficult to understand Heie's idea. Other authors (for example, Moran 1992) preferred vaguer statements that parthenogenesis arose very early in the evolution of aphids.

The first remnants of aphids are known from the Triassic period (Heie 1987) or even from the Permian period (Shcherbakov 2007). At that time, angiosperms did not yet exist. The latter became a noticeable component of ecosystems only in the Cretaceous period, although their first representatives could have appeared somewhat earlier (Han et al. 2023). Consequently, at the initial stage of their evolution, aphids were associated with gymnosperms and, possibly, with some higher spore plants. This is in perfect agreement with the fact that among living aphids, the most archaic in their reproductive biology are representatives of the family Adelgidae, obligately connected with gymnosperms. It is the adelgids that, of all aphids, have a normally developed ovipositor and are characterized by obligate oviparity in all generations of the life cycle. Oviparous aphids also include the sister family Phylloxeridae, but in them the ovipositor is lost (or represented by a vestigium) and there is not a single example of connections with gymnosperms. All other aphids (superfamily Aphidoidea) exhibit viviparity in parthenogenetic generations, complete loss of the ovipositor, and feed primarily on flowering plants (Fig. 1); their few connections with gymnosperms are clearly apomorphic (see below). Thus, it is logical to consider adelgids as a group that has preserved the original lifestyle and mode of reproduction for all aphids. Various morphological apomorphic characters of modern adelgids, of course, do not contradict the preservation of the archaic reproductive system, since the evolution of any taxon is mosaic.

The life of aphids on bark or needles is not fundamentally different from the life of other related groups of insects living on gymnosperms, but do not have cyclic parthenogenesis (for example, coccids, true bugs, cycads). A unique feature that distinguishes adelgids from all these insects is the ability to induce the formation of closed galls, very similar and probably homologous to strobili (Fig. 2). Such galls are formed exclusively on spruce trees (*Picea* spp.), despite the regular seasonal migration of many adelgid species to other genera of gymnosperms. This means that the formation of the gall is determined not only by the special chemical composition of adelgid saliva, but by the specific response of the growing spruce shoot to the penetration of this saliva. It is noteworthy that living on the same plants (and even on the same spruce branches) monophagous soft scales *Physokermes* spp. (family Coccidae) do not cause the formation of any galls. The difference in feeding behavior between these soft scale insects and adelgids is that the former never live on annual growing shoots, while the latter, on the contrary, settle on the bud and provoke its total or partial transformation of a growing shoot into an open

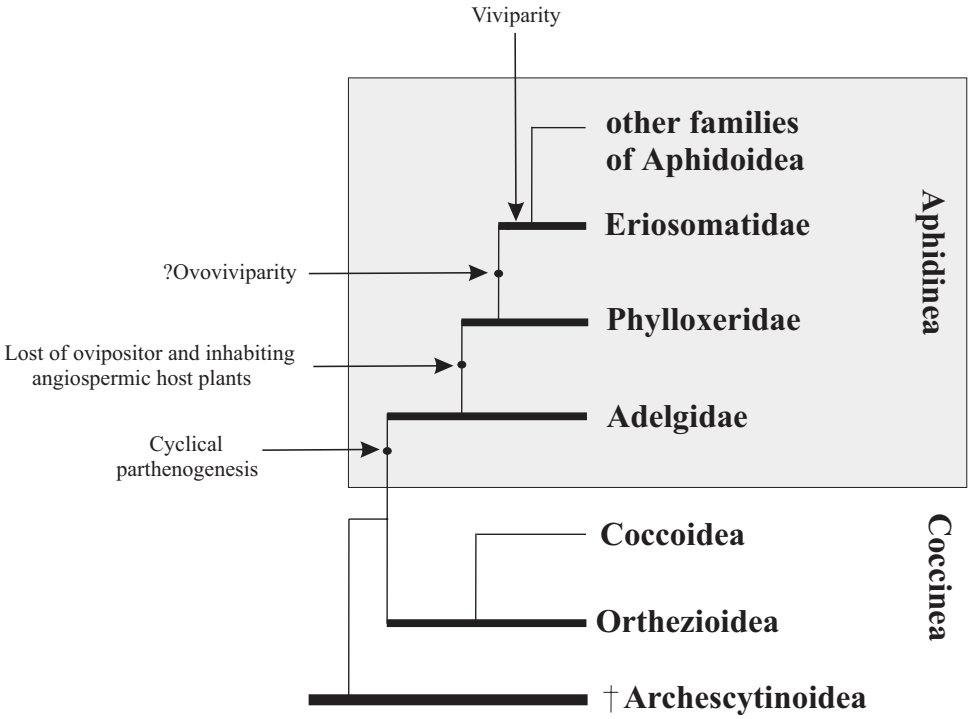

Figure 1. Hypothetical scheme of the evolution of reproductive peculiarities in aphids. The bold lines designate presumable paraphyletic taxa.

and then a closed gall. In this case, the gall is not of an arbitrary shape, but always resembles a spore-bearing shoot (strobilus), i.e. the transformation occurs within the usual range of morphological variation of the plant. In this regard, the galls of adelgids, unlike the galls of other aphids (and most other gall-forming animals), should not be considered as a kind of "neoplasm", a plant "tumor". Both the strobilus and the adelgid gall are in fact a shortened shoot with thickened needles transformed into covering scales.

It is logical to speculate that in the early stages of aphid evolution, when the first adelgids or their ancestors began to live on the ancestors of modern spruce trees, the formation of galls occurred in the same way as now. Reliable paleontological finds attributed to the modern genus *Picea* Dietrich, 1824 have been known since close to the beginning of the Cretaceous period, 136 million years ago (Klymiuk and Stockey 2012). It is clear that the appearance of modern spruce taxa was preceded by a long evolution of their ancestral forms. Probably, even then, the spring generation of aphids was locked in the closed cavities of the galls (Fig. 3) for a period that was not and could not be strictly fixed. Spruce galls are opened (due to the drying of their "leaves"), not when the aphids sitting inside need it, but in accordance with the physiological characteristics of a particular plant and a particular tree branch. It is clear that even spruce trees of the same species growing in the same area are differently lit and shaded, are able to obtain

Figure 2. Mature gall of *Adelges* sp., Poland. Photo & copyrights: https://www.flickr.com/photos/hedera_baltica/52949379151/.

moisture and nutrients from the soil to varying degrees, have different ages, heights, crown spans, etc. Also, different parts of the crown of the same tree are illuminated and supplied with moisture to varying degrees. As a result, the opening of adelgid galls, even those growing in the same locality, lasts for months. For example, in the vicinity of St. Petersburg (North-West Russia) this lasts from mid-June to the late August (Cholodkowski 1915; Popova 1967), and in Japan (Hokkaido) — from mid-June to early September (Tabuchi et al. 2009). It is also a well-known fact that adelgid galls "*ripe in the crown of trees (spruces) not simultaneously, but as the lower and then the upper branches mature*" (Popova 1967: 184). In some cases, according to my observations, even the opening of different cavities of the same adelgid gall does not occur simultaneously, but individual cavities of dried galls remain closed, or the resulting hole is not wide enough, and then the nymphs inside die. There are also well known examples of time-extended opening of galls in other groups of aphids. Thus, in *Phylloxera devastatrix* Pergande, 1904 (Phylloxeridae), the galls on the shoots and petioles of hickory (*Carya* spp.) leaves

Figure 3. Transverse section of gall of *Adelges cooleyi* (Gillette, 1907) with nymphs inside, USA. Photo & copyrights: Whitney Cranshaw, https://www.insectimages.org/browse/detail.cfm?imgnum=5422255.

open from the first half of May to mid-June (Baker 1935), in *Colopha compressa* (Koch, 1856) (Eriosomatidae) the females fly out from the galls (even on the same branches of one elm-tree) throughout July (Gavrilov-Zimin, personal observations in Leningrad Prov. of Russia), and in *Pemphigus spyrothecae* Passerini, 1860 (Eriosomatidae) the galls open from late August to early November and in some cases remain completely closed, dooming their entire population to death (Popova 1967: 108; Gavrilov-Zimin, own observations). All of these examples, as well as many others discussed below, clearly demonstrate the inconsistency of the hypothesis that aphids allegedly secrete certain chemical substances that stimulate the gall to open at the right time for the aphids (Tabuchi et al. 2009: 459): "…*opening of adelgid galls is induced by stimuli of larvae in the galls (Rohfritsch 1992), suggesting that the longer larval period is not because the larvae are waiting for the galls to open*" Moreover, the size and the speed of maturation of the adelgid gall also depends on how many fundatrices participated in its formation and how many larvae of the daughter generation (gallicolae) inhabit it (Ozaki 2000).

In the splitting approach to the taxonomy of adelgids, it is often believed galls that formed in different parts of the crown and open at different times are caused by different "cryptic" species from 7 different genera of adelgids (Cholodkowski 1915; Shaposhnikov 1964, etc.). Fortunately, in the latest taxonomic catalog of adelgids (Favret et al. 2015), the number of genera is reduced to two: *Adelges* Vallot, 1836 and *Pineus* Shimer, 1869, but the number of nominal species still remains quite large (about seventy).

It is extremely difficult to understand how most of these "species" differ from each other, since the existing identification keys (for example, Cholodkowski 1915; Shaposhnikov 1964; Mamontova 1991, etc.) are not built on clear morphological characteristics, but on vague, overlapping descriptions of lifestyle and association with a specific part of the host plant. That is, in such reasoning, galls located on different branches of the same tree open at different times because they contain aphids of different "cryptic species," and these "species" are "independent" because their galls open at different times... No one has carried out any crossing experiments to objectively confirm the independence of these "species," although recently (Havill et al. 2020) an attempt was made to reconstruct the pattern of hybridization between several nominal species of adelgids using indirect methods of molecular genetics. Most of the nominal "species" of adelgids have been described based on parthenogenetic lines, which, according to the authors of these species, do not engage in the sexual process (see more below). In any case there is no doubt that initially different nominal species of *Adelges* and *Pineus* were represented with a single ancestral species, because the monophyly of adelgids is doubtless and is not denied by anyone. There is also no doubt that this ancestral species initially reproduced in a normal, regular bisexual mode, like most other insects. The fundatrices then began feeding and ovipositiing on the growing spruce shoots (or spruce ancestors). The shoot grew, gradually isolating the hatched larvae inside the galls from the external environment. The larvae molted several times and grew, filling with their bodies the entire cramped space of the gall cavities (Fig. 3). The subsequent extremely unsynchronous opening of galls on different branches and on different spruce plants inevitably led to the fact that the simultaneous mass emergence of male and female nymphs from different galls was impossible. A significant or even the majority of female and male nymphs in the population always found themselves locked in the cavities of "immature" galls, while a smaller part emerged from the galls that had already opened on a particular day or several days. These circumstances led, at least, to the following consequences. 1) The small number of males and females emerging from the galls on any day or several days could not ensure effective panmixia in view of the small size of the aphids and their relatively weak abilities for independent purposeful flight. 2) Sexually mature nymphs of females, located in galls that had not yet opened, found themselves physically isolated from males that had already emerged from other galls. 3) As the appearance of adult winged females spread over time, they scattered to various suitable food plants (spruce, larches, fir, pine, etc.) and most of the females remained unfertilized. In such situation, which regularly repeated every warm season for millions of years, females capable of parthenogenetic reproduction received a selective advantage. Parthenogenesis allowed each female to not synchronize the development of her reproductive system and subsequent reproduction with the development of other individuals of the population. After early or late emergence from the gall (or even directly inside the gall, as in the modern species *Pineus similis* (Gillett, 1907)), adelgids laid parthenogenetic eggs. These eggs hatched into larvae that either managed to feed on their own and go through one or two molts before the end of the warm season, or without feeding they crawled into cracks in the bark and overwintered

there. With the onset of the next warm period of the year, larvae of different stages began feeding, moulted, and asynchronously turned into adult females and males. In this case, it again turned out to be impossible to achieve effective panmixia, and parthenogenetic females that laid eggs without fertilization again received a selective advantage in the second generation.

The fate of the third generation of adelgids that settled on the main host plant and on secondary plants turned out to be different. On spruce trees, females of the third generation began feeding on young shoots (as the most favorable place for feeding) and their progeny again found itself locked inside the galls. The change of generations on spruce trees, thus, turned out to be cyclic and exclude bisexual reproduction entirely.

On secondary host plants, sap sucking did not lead to the formation of galls and, accordingly, there were no physical obstacles to the free mating of males and females. However, due to the asynchrony of the two previous generations, the problem remained of re-synchronizing the appearance of males and females in mass numbers and their meeting in the same place, which is necessary for effective cross-fertilization. The only opportunity for such secondary synchronization was the time of approximately the same end of growth of shoots of coniferous trees by the middle of the warm season. In modern conditions, depending on the specific region and the conditions of a particular year, shoot growth ends by the end of June-beginning of July (Popova 1967). By this time, many desynchronized parthenogenetic lines of adelgids are already developing on secondary food plants. The simultaneous sharp deterioration in nutrition leads to the fact that winged "sexupares" begin to appear en masse in these lines. It is this fourth or fifth generation that moves from thelytoky to deuterotoky, that is, it forms eggs in itself, both female and male. The sexupares fly to the main host plant (spruce), where they lay eggs on the underside of the needles. From these eggs, a bisexual generation develops relatively synchronously and, finally, the possibility of mass cross-fertilization with all its genetic advantages is achieved. Under these conditions, it is important that sexuparae return from various secondary host plants to spruce, since in this way a high concentration of males and females is achieved in the same place. Due to the fact that the development of the bisexual generation always occurs in the second half of the year, when the conditions for feeding aphids on woody plants become unfavorable, the small size of the sexual individuals and their reduced fertility (often only one egg per female) are understandable, in comparison with parthenogenetic generations of the first half of the year. The loss of wings in the sexual generation, as well as in the parthenogenetic generations developing in the first half of the year on secondary plants, also becomes clear. To achieve synchronicity and mass cross-fertilization, it is important that both generations remain in their places — the first until the process of fertilization, and the second until the synchronous end of shoot growth.

Thus, the occurrence of a complicated cyclical change of parthenogenetic and bisexual generations in aphids can be explained by the same basic biological reasons that appeared at the initial stage of the evolution of oogamous multicellular organisms and continue to operate up to now; namely, the problems of mass synchronous production of gametes and their concentration in the same place of space (Gavrilov-Zimin

2023). In aphids, parthenogenetic generations absolutely dominate in the life cycle. In this case, the oocytes of the parthenogenetic female undergo only one meiotic division, with the formation of a single polar body (Fig. 4); recombination of homologous chromosomes is sometimes assumed, but has not been sufficiently studied and only a few species were ivolved in such investigations (Blackman 1987). Parthenogenetic females are capable of producing embryos with a single or double set of X chromosomes, through a special cytogenetic mechanism ("mini-meiosis") that ensures appropriate elimination of one set of X-chromosomes in oocytes, developing into male embryos (Orlando 1974; Blackman and Hales 1986).

Aphid spermatogenesis occurs in a unique way. Instead of the usual male meiosis producing four identical gametes, aphids produce only two sperm, both with X chromosomes (Blackman 1987). Thus, in the bisexual generation of aphids, all individuals turn out to be homogametic, carrying X chromosomes, and as a result of subsequent fertilization, all offspring have paired X chromosomes and become females. For this reason, the bisexual generation is necessarily replaced by parthenogenetic generation (heterogony). Probably, such a system was formed over hundreds of millions of years of forced alternation of generations (for the reasons discussed above) and was so firmly entrenched in the aphid genome that abandoning it became impossible even after more advanced groups of aphids switched to feeding on angiosperms, including those on which closed galls are not formed (see below).

Below I shall consider the real life cycles of modern adelgid species, which seem to be rather similar with the hypothetical cycle of their ancestor.

Life cycles and ontogenesis of extant species of Adelgidae

Amongst about 70 recent nominal species of adelgids, combining in the genera *Adelges* and *Pineus*, only 24 species are considered as holocyclic (or probably holocyclic): *Adelges cooleyi* (Gillette, 1907), *A. glandulae* (Zhang, 1980), *A. isedakii* Eichhorn, 1978, *A. karafutonis* Kono et Inouye 1938, *A. kitamiensis* (Inouye, 1963), *A. knucheli* (Schneider-Orelli et Schneider, 1954), *A. lariciatus* (Patch, 1909), *A. laricis* Vallot, 1836, *A. merkeri* (Eichhorn, 1957), *A. nordmannianae* (Eckstein, 1890), *A. pectinatae* (Cholodkovsky, 1888), *A. prelli* (Grosmann, 1935) *A. roseigallis* (Li et Tsai, 1973), *A. tardoides* (Cholodkovsky, 1911), *A. torii* (Eichhorn, 1978), *A. tsugae* Annand, 1924, *A. viridis* (Ratzeburg, 1843), *Pineus armandicola* Zhang et al., 1992, *P. cembrae* (Cholodkovsky, 1888), *P. floccus* (Patch, 1909), *P. orientalis* (Dreyfus, 1888), *P. pinifoliae* (Fitch, 1858), *P. sichunanus* Zhang, 1980, *P. strobi* (Hartig, 1839).

The detailed information on all mentioned species can be easily found on the site of R. Blackman and V. Eastop: https://aphidsonworldsplants.info/, and also in the main monographs on Nearctic and Palaearctic species: Marchal 1913; Cholodkowski 1915; Annand 1928; Popova 1967, Steffan 1968, etc.

The larvae of these holocyclic species form closed galls on the spring shoots of various spruce species (*Picea* spp.), and during the summer the larvae emerge from the opened

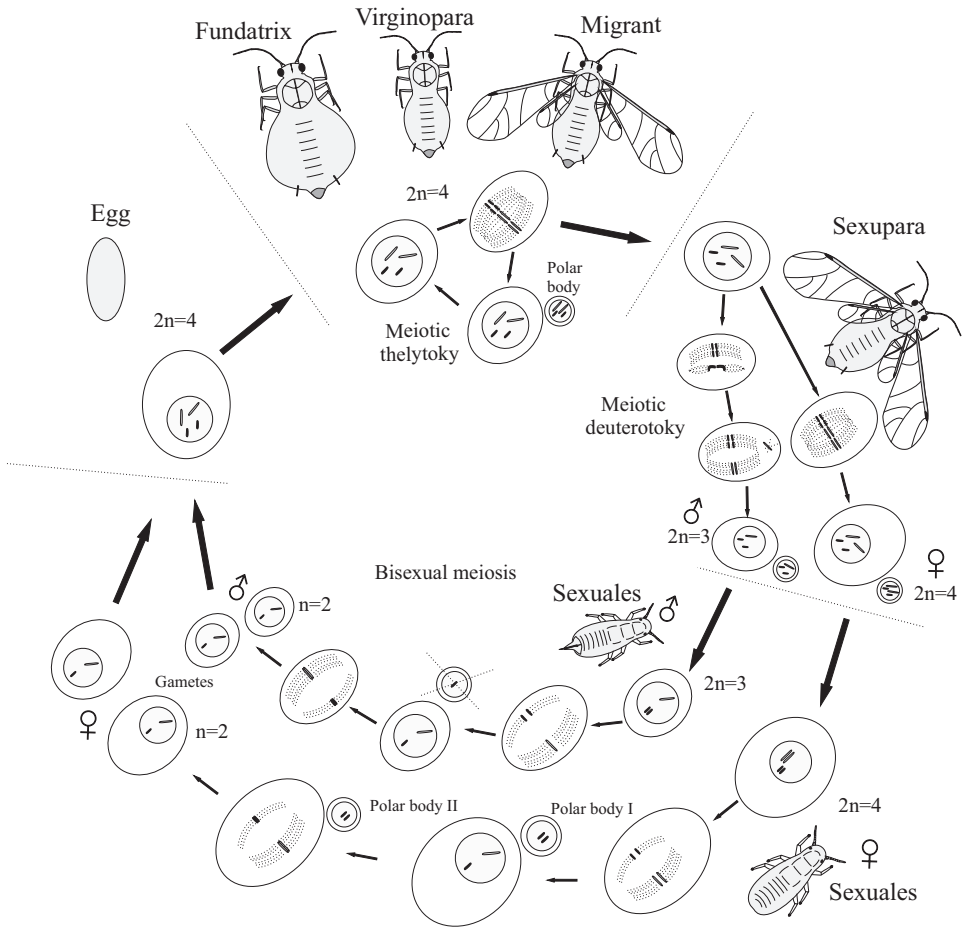

Figure 4. Generalized scheme of cyclical parthenogenesis in holocyclic aphids with diploid chromosome number 4 (after Gavrilov-Zimin 2021).

galls, molt for the last time and become winged migrants (Fig. 5). It is believed that among species of the genus *Adelges*, migration occurs mainly to various species of larch (*Larix* spp.), less often to various species of fir (*Abies* spp.) and very rarely (in *A. cooleyi* and *A. tsugae*) to Douglas-fir (*Pseudotsuga* spp.) and hemlock (*Tsuga* spp.). In full-cycle species of the genus *Pineus*, migration always occurs to various species of pine trees (*Pinus* spp.).

The life cycles of most of these species fully correspond to the supposed cycle (described above) of the ancestral species for adelgids and aphids in general. However, several nominal species of the genus *Pineus* (less archaic than *Adelges*) show interesting nuances and deviations. Thus, in the American nominal species *Pineus strobi* (Hartig, 1839) (allegedly widely distributed throughout the Palaearctic), in the USA, the usual migration occurs between primary food plants (*Picea* spp.) and Weymouth pine (*Pinus strobus* Linnaeus, 1753) (Popova 1967: 203). But there is complete confusion in the

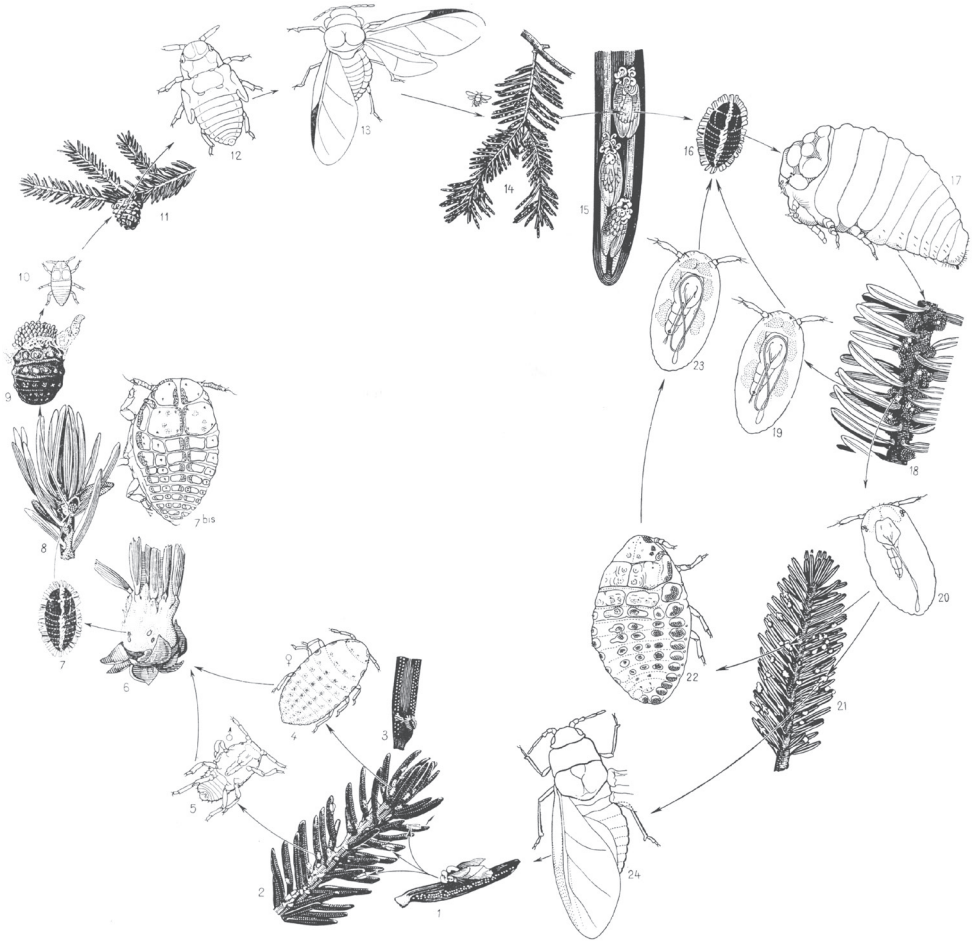

Figure 5. Biennial life cycle of *Adelges nordmannianae* (Eckstein, 1890) (Aphidinea) (after Pesson 1951; Gavrilov-Zimin 2021), with changes. Stages 1–13 occur during first year on *Picea orientalis* (Linnaeus, 1763): 1 female "sexupara", migrated from fir (June) 2, 3 larval instars on spruce (July) 4, 5 female and male (July) 6 oviposition (July) 7 wintering larva (August-March) 7^{bis}, 8 female "fundatrix" (April) 9 oviposition (April) 10, 11 larva, producing a gall on twig of spruce (Mai) 12 nymph (June) 13 migrating female (July). Stages 14–24 occur during second year on *Abies nordmanniana* (Steven, 1838): 14, 15 females, migrating from spruce lay eggs (July) 16 wintering larva (August-April) 17, 18 parthenogenetic female and it oviposition (Mai) 19–23 new parthenogenetic generations (Mai-June) 24 alate female, migrating to spruce (June).

relevant literature regarding the details of the life cycle, due to the above-mentioned taxonomic chaos of aphid "species" that have no discrete differences from each other. Firstly, *Pineus strobi* itself has no clear differences from the previously described and very widespread *P. pini* (Goeze, 1778). Secondly, the later described American "species" *P. floccus* (Patch, 1909) is no different from *Pineus strobi* (Popova 1967: 203). All three of these supposedly "independent" species develop mainly on secondary host plants

(pine trees), producing several parthenogenetic wingless generations of females per year. However, from the end of spring to mid-July, winged sexuparae appear in greater or lesser numbers and migrate to the spruce if suitable spruce species are available nearby. According to Raske and Hodson (1964), the sexuparae of *P. strobi* fly to spruce trees in late June and early July, lay eggs on the needles, but their offspring do not survive. In *P. floccus*, according to Walton (1980), generations of fundatrices, sexuparae and sexuales are probably completely absent (i.e. the life cycle is represented exclusively by parthenogenetic generations, one of which develops in galls on spruce, and the next several on pine). In the early spring of the second year, overwintered parthenogenetic females lay eggs from which both wingless parthenogenetic generations develop, remaining on the pine tree, and winged parthenogenetic females, which fly to the spruce in May, lay eggs there, and from these eggs a new generation of gall-forming larvae emerges (Walton 1980). In addition, all three of the mentioned nominal species (*Pineus pini*, *P. strobi*, and *P. floccus*) are not fundamentally different from *P. orientalis* (Dreyfus, 1888), except that the latter is characterized by a two-year life cycle typical for adelgids with a regular change of food plants (Marchal 1913; Havelka et al. 2019). Moreover, for *P. orientalis* it is known that on some species of spruce its sexual generation develops normally, while on others it dies (Popova 1967: 200). Thus, it would be logical to consider all four nominal species as a single widespread species under the oldest name *Pineus pini* s.l., and the indicated deviations in reproductive behavior to consider as a manifestation of intraspecific variability in different climates and on different food plants. A notable feature of this variable species can be considered the very early (starting from the end of May) appearance of winged females on the secondary host plant (pine trees), when the nutritional value of the growing shoots of coniferous trees is still high. This peculiarity may be due to the fact that pines often grow in arid habitats and at least in some southern parts of the extensive range of *Pineus pini* s.l. the growth of shoots of the host plant stops by the end of spring. Similar examples of the very early appearance of winged sexuparae are known for *Adelges viridis* in the vicinity of St. Petersburg (Russia) on larches under conditions of abnormally warm spring (Popova 1967: 188).

The remaining "species" (about 50–55) from the genera *Adelges* and *Pineus* were described according to parthenogenetic generations, living on secondary host plants, less often on spruce trees (in galls or cracks in the bark). It is known about many of these "species" that in the summer winged sexuparae appear among the wingless parthenogenetic females, but their further fate has not been traced. In other cases, a detailed examination of the morphology and lifestyle makes it easy to guess from which full-cycle species the corresponding parthenogenetic lineages originate. Thus, unholocyclic *A. aenigmaticus* Annand, 1928, *A. diversis* Annand, 1928, *A. geniculatus* (Ratzeburg, 1844), *A. japonicus* (Monzen, 1929), *A. karamatsu* Inouye, 1945, *A. lapponicus* (Cholodkovsky, 1889), *A. oregonensis* Annand, 1928, and *A. tardus* (Dreyfus, 1888), as well as holocyclic *A. isedakii* Eichhorn, 1978, *A. lariciatus* (Patch, 1909), and *A. tardoides* (Cholodkovsky, 1911), in fact are the variations of *Adelges laricis* Vallot, 1836 – see more detail comments on R. Blackman's and V. Istop's site: (https://aphidsonworldsplants.info/d_APHIDS_A/#Adelges).

An interesting feature is known in the gall-inhabiting generation of *Pineus similis* (Gillett, 1907), which supposedly develops only on spruce trees, without migrating to secondary host plants. Two variants of larvae feed in the galls. Usual larvae give rise to winged migrants that fly to the branches of the same spruce or neighboring spruce trees. Other larvae moult directly inside the gall onto wingless females, which lay eggs there (Cumming 1962). This is so far the only known example of reproduction inside galls among adelgids, although similar examples, as will be shown below, are rather often in more "advanced" groups of aphids.

Evolution of cyclic parthenogenesis in Phylloxeridae

The second group of aphids characterized by obligate oviposition in all generations is the family Phylloxeridae — phylloxeras. The cyclic parthenogenesis of these aphids, combined with the loss of the ovipositor and the complete absence of connections with gymnosperms, allows to say that phylloxeras are not just a sister group to adelgids (as is often indicated in aphidological literature, e.g. Heie 1987), but originated from a certain ancient species of adelgids after the appearance and widespread distribution of angiosperms in the Holarctic, that is, not earlier than the Cretaceous period (Fig. 1). All modern phylloxera species (about 70) are associated exclusively with woody plants, mainly beech trees (Fagales) and willows (Salicaceae). Most phylloxeras live on Nearctic flora, where they feed on various species of hickory (*Carya* spp.). On the leaf blades, petioles and twigs of these plants, phylloxera provoke galls of very diverse structure (Figs 6, 7), including closed ones (Pergande 1904; Stoetzel 1985a). In at least some of these species, the complete life cycle includes regular migration of winged females emerging from galls to secondary host plants — oaks (*Quercus* spp.) and chestnuts (*Castanea* spp.), where galls are not formed (Stoetzel 1985a). In other phylloxera species living on hickory, the development is unholocyclic or the cycle is unknown. The grape phylloxera, *Daktulosphaira vitifoliae* (Fitch, 1855), which is widespread throughout the world in grape-growing areas, is also of North American origin and, moreover, is believed to descend from an ancestral species that once lived on hickory (Popova 1967: 229). On American grape varieties, this phylloxera develops as holocyclic with a regular migration from the leaves, where it lives in open galls, to the roots and back. Open galls on the edges of leaves of *Nyssa sylvatica* Marschall, 1785 (Cornaceae) in the USA are also formed by *Phylloxerina nyssae* (Pergande, 1904). The species lives without host change; the bisexual generation develops in cracks in the bark. The host connection of *N. sylvatica* also appears to be of a secondary origin, since all species of the genus *Phylloxerina* Börner, 1908 are characterized by exclusively wingless generations, unholocyclic development and association mainly with willows (*Salix* spp.) and poplars (*Populus* spp.).

In the Palearctic fauna, the diversity of phylloxeras is in all respects significantly lower than in the Nearctic. Firstly, among the Palaearctic species, not a single one is known to migrate to unrelated host plants. Only for *Phylloxera quercus* Boyer de Fonscolombe, 1834 in the Mediterranean is migration between different (evergreen and deciduous) oak species known. Secondly, with the exception of *Olegia ulmifoliae* (Aoki,

Figure 6. Gall of *Phylloxera* sp. on leaf of hickory, USA. Photo & copyrights: Judy Gallagher, https://www.flickr.com/photos/52450054@N04/50955746943/.

Figure 7. Gall of *Phylloxera* sp. on leaf of hickory, USA. Photo & copyrights: Katja Schulz, https://www.flickr.com/photos/treegrow/48516072207/.

1973), which lives in closed galls on elm leaves, there are no gall-forming phylloxeras in the Palearctic. Thirdly, the range of food plants of Palaearctic phylloxeras is limited mainly to oaks, willows and poplars. One species is also known from elm, pear and chestnut. All three of these species are represented exclusively by wingless generations, as are the species living on willows and poplars. Among Palearctic species that feed on oaks, winged females are known only for a few species from the type genus *Phylloxera* Boyer de Fonscolombe, 1834 (Popova 1967: 227).

Thus, analysis of food connections and life cycles allows to conclude that the origin of phylloxeras as a taxonomic group was connected with hickories in North America. Probably, the evolutionary transition from gymnosperms, on which the adelgid ancestor of phylloxeras lived, to hickory was due to the fact that, of the Nearctic angiosperms common in the Cretaceous period, only hickory formed galls and this allowed the first phylloxeras to develop in the usual cycle of alternation of gall and freeliving generations. It is believed that *Carya* spp. appeared in North America in the second half of the Cretaceous period, and related extinct plant genera even earlier (Zhang et al. 2013). In addition to preserving the usual reproductive cycle, the life of phylloxeras in closed galls at the initial stages of their evolution apparently provided effective protection from predators and unspecialized parasites and made it possible to save from them several most proliferous generations of aphids, developing in the spring and in the first half of summer. As is well known, Adelgoidea does not have specialized hymenopteran parasites (Mamontova 2008: 109), just as almost all primitive archaeococcids (Orthezioidea) do not have such parasites, with the exception of only some species from their most "advanced" group Iceryini (Gavrilov-Zimin and Danzig 2012).

The proposed transition from living in closed galls on spruce trees to living in closed galls on hickory leaves, petioles or shoots would inevitably lead to changes in the reproductive biology of the first phylloxera. 1) Unlike the cramped internal cavities of spruce galls, hickory galls, as they grow, form a large space that far exceeds the body volume of an adult aphid. This circumstance allowed the first generation of the inhabitants of the galls not to wait for their opening, but to lay eggs just inside the gall, with the subsequent development of the second and even third generations there. In some modern species, the productivity of gall inhabitants turns out to be extremely high: in *Phylloxera devastatrix* Pergande, 1904 — from 300 to 1300 individuals per gall, depending on its size (Baker 1935), in *Olegia ulmifoliae* — up to 1500 individuals in one gall (Shaposhnikov, 1979). 2) Laying eggs inside the gall does not require the presence of an ovipositor, since there is no need to hide the egg in any additional shelter, and thus it turns out to be possible to explain the loss of this organ in phylloxeras. 3) The development of several generations, including bisexual, inside the gall, do migration to secondary host plants unnecessary, since the life cycle with heterogony in this case is finished on the same plant. Probably for the same reason, phylloxeras does not have a two-year life cycles.

On the other hand, all these changes do not cancel other circumstances that played an important role in the evolution of adelgids and remained factors in the evolution of more advanced groups of aphids. These circumstances are the impossibility

of synchronization and mass cross-fertilization during development in closed galls and a sharp decrease in the nutritional value of tree shoots by the middle of the warm period of the year. The action of these factors, combined with the possibility of the development of several gall generations on hickory, led to a significantly greater diversity of phylloxeras life cycles and host connections compared to adelgids. Some phylloxera species have maintained regular migrations from hickories to secondary host plants (oaks, chestnuts, and possibly some others). Other phylloxeras have switched to permanent holocyclic development on hickory. The third groups of species began to develop exclusively on secondary host plants, forming certain leaf deformations on them or forming open galls that do not interfere with the free synchronous emergence of sexuparae and/or bisexual generations. A fourth group of species, probably due to the gradual evolution of the chemistry of their saliva, began to form open galls on the hickories themselves. On the other hand, the formation of closed or open galls on hickory apparently depended not only on the evolution of phylloxeras, but also on the gradual physiological and morphological evolution of different species of these trees. It cannot be ruled out that at the very beginning of the evolution of phylloxeras, females of their common ancestor had already formed both closed and open galls, depending on what type of hickory and on what part of it (leaf blade, petiole or base of a young shoot) they started to feed. In any case, the obvious multidirectionality of the reproductive evolution of phylloxeras and the sharp expansion of their host connections in comparison with adelgids allows us to speak of their significant similarity in these parameters with Aphidoidea aphids. At the same time, neither phylloxeras nor Aphidoidea can return to constant bisexual reproduction, which was in the ancestors of aphids, due to the developed specific features of gametogenesis and the exclusively parthenogenetic method of formation of the bisexual generation (see above). Their life cycle must include at least one parthenogenetic generation, alternating with a bisexual one. Such a reproductive "minimum" is actually achieved in some modern species, for example, in the European oak phylloxera *Acanthochermes quercus* Kollar, 1848 (Fig. 8), in the cycle of which only parthenogenetic wingless fundatrices and wingless non-feeding individuals of the second, bisexual generation remain. The development of these two generations takes place in April-May, and the rest of the year the species is represented by resting eggs preserved inside the bodies of dead females (Grassi 1912: 69–70).

It is also necessary to note two interesting ontogenetic features of phylloxeras, the evolutionary significance and prevalence of which remain poorly understood. Firstly, according to the observation of M. Stoetzel (1985b), the larval instars of the bisexual generation of phylloxeras are pupal-like ("*pupiform larvae*") due to their immobility. Such "pupae" appear after shedding the egg shell, molt four times and turn into adult mobile females and males. Secondly, for many phylloxera species, the laying of eggs of different sizes by the phyllocarcas is noted — large female and small male. Unfortunately, at present it is impossible to say with certainty whether these features are inherent in all phylloxeras and only them, or whether they are also found in some more advanced aphids (Aphidoidea).

Figure 8. Larvae of fundatcises of *Acanthochermes quercus* Kollar, 1848 on oak leaf, Abkhazia. Photo of A.S. Kurochkin.

Further evolution of cyclical parthenogenesis and the origin of viviparity in Aphidoidea

All modern species of aphidoid aphids are characterized by the loss of the ovipositor. Taking into account this fact, reproductive characteristics and the nature of host connections, it is logical to believe that aphidoid aphids originated in the Cretaceous from a certain ancient species of phylloxeras. Otherwise, we would have to admit that the complex of characters (cyclical parthenogenesis, a unique cytogenetic system, loss of the ovipositor, the transition from gymnosperms to angiosperms) arose independently several times in the evolution of aphids; the first time in the adelgid-phylloxera branch for the reasons discussed above, and at least twice in aphidoid aphids for some other unknown reasons. Such an extraordinary combination of a number of evolutionary

coincidences seems absolutely incredible. All the few connections between aphidoid aphids and gymnosperms are clearly of a secondary nature. Such connections are found in a number of genera of lachnids (Lachnidae), in representatives of the genus *Neophyllaphis* Takahashi, 1920 (Drepanosiphidae) and in some genera of eriosomatids (Eriosomatidae). These examples require somewhat more detailed consideration.

Lyachnids of the subfamily Cinarinae, widespread in the Holarctic and associated with various species of pines (*Pinus* spp.), spruce (*Picea* spp.), fir (*Abies* spp.), larch (*Larix* spp.), and cypress (Cupressaceae), are considered either by different aphidologists as one of the youngest, most advanced groups of aphidoid aphids, or, conversely, as one of the most archaic (see review of competing opinions in Mamontova 2008). These contradictions are due to the fact that the morpho-anatomical characteristics of lachnids give a mosaic picture of plesiomorphy vs. apomorphy. However, none of the aphidologists consider lachnids to be more archaic and ancient in comparison with adelgids and phylloxeras. Moreover, within the family Lachnidae itself, aphids associated with angiosperm trees (type subfamily Lachninae) appear to be more primitive than cynarines based on their morphological characters (Mamontova 2008: 104–164). In any case, all lachnids are characterized by telescopic embryonization based on placental viviparity of parthenogenetic generations. Not a single example of oviparous parthenogenetic generations is known among lachnids (as well as all aphidoid aphids) and, accordingly, it is impossible to discern a direct evolutionary connection between them and any oviparous species ancestral to all aphids.

Aphids of the genus *Neophyllaphis* of the monotypic subfamily Neophyllaphidinae are represented in the modern fauna by 18 species associated with gymnosperms of the families Podocarpaceae and Araucariaceae, mainly in the Southern Hemisphere, including in the mountainous regions of the tropical zone of the planet. All these species develop unholocyclically, but in a number of cases they demonstrate holocycly, with the appearance of winged (rarely wingless) individuals of the bisexual generation (Blackman & Eastop, https://aphidsonworldsplants.info/d_APHIDS_N/#Neophyllaphis). The very fact that these aphids, like their host plants, in their distribution are separated from the obvious center of diversity and origin of aphids, i.e. from the temperate climate zone of the Holarctic, does not in itself allow us to consider them an ancestral group in relation to other aphidoid aphids. For one of the species, *N. brimblecombei* Carver, 1971, a feeding relationship with eucalyptus (*Eucalyptus robusta* Smith, 1792) was indicated in southern China, where the species was apparently introduced from Australia (Qiao et al. 2001); it may additionally testify the secondary nature of the connection between these aphids and gymnosperms. The parthenogenetic generations of all these aphid species are characterized by obligate viviparity, which, as in the case of lachnids, excludes a direct evolutionary connection of *Neophyllaphis* spp. with the hypothetical ancestors of aphids.

Phylogenetic reconstructions proposed by various authors for other groups of aphidoid aphids are extremely contradictory (Heie 1987; Shaposhnikov 1987; Wegierek 1992; Wojciechowski 1992; Heie and Wegierec 2009; Ortiz-Rivas and Martinez-Torres 2010; etc.) and do not allow to make unambiguous judgments about aphid evolutionary history. Based on morpho-anatomical characters, Eriosomatidae (= Pemphigidae) are usually considered as one of the archaic families of aphidoid aphids (Shaposhnikov

1987; Heie and Wegierec 2009). However, when constructing evolutionary reconstructions, none of the aphidologists-phylogeneticists pay attention to the fact that only for eriosomatids, among all Aphidoidea, the birth of parthenogenetic offspring was noted in shells, which were soon discarded by the hatched larva (Mordwilko 1901: 58; Hille Ris Lambers 1950). Since eriosomatids have the same number of larval instars as other aphids, there is no reason to assume any additional "embryonic molt". So, these shells are of maternal origin and are homologous to the egg membrane, i.e. chorion, as Mordwilko (1901: 58) directly wrote about. This peculiarity should probably be considered as plesiomorphic, indicating a transition from ovoviviparity to placental viviparity. In general, ovoviviparity is a common intermediate stage between oviparity and viviparity in the evolution of various groups of animals, including the sister group of scale-insects (Ivanova-Kazas 1995; Ostrovsky et al. 2016; Gavrilov-Zimin 2022). In addition, the development of embryos in the body of the migrating generation in the studied eriosomatids does not occur sequentially, as in other Aphidoidea, but simultaneously, so that a female that has flown to a secondary host plant lays all her offspring in a very short period of time, just as many oviparous animals do, in particular, females of oviparous and ovoviviparous scale-insects (Coccinea). This feature of pemphigids was previously noted by Hille Ris Lambers (1950). I myself verified the simultaneity of embryo development by dissecting migrating females in such species as *Colopha compressa* (Koch, 1856), *Prociphilus fraxini* (Fabricius, 1777), *P. xylostei* (De Geer, 1773), and *Pemphigus spyrothecae* Passerini, 1860. Considering that the placental viviparity of aphids, combined with pedogenesis and telescopic embryonization, could hardly have arisen suddenly, it is logical to recognize the eriosomatids as a possible "transitional form" in the evolution from the oviparous Phylloxeroidea to the viviparous Aphidoidea (Fig. 1).

The family Eriosomatidae is divided into three subfamilies: Eriosomatinae, Fordinae and Pemphiginae. Aphids of Eriosomatinae (genera *Aphidounguis* Takahashi, 1963, *Byrsocryptoides* Dzhibladze, 1960, *Colopha* Monell, 1877, *Colophina* Börner, 1931, *Eriosoma* Leach, 1818, *Gharesia* Stroyan, 1963, *Hemipodaphis* David et al., 1972, *Schouteden*, 1906, *Paracolopha* Hille Ris Lambers, 1966, *Schizoneurata* Hille Ris Lambers, 1973, *Schizoneurella* Hille Ris Lambers, 1973, *Siciunguis* Zhang et Qiao, 1999, *Tetraneura* Hartig, 1841, *Zelkovaphis* Barbagallo, 2002) mainly use as their primarily host plants various *Ulmus* spp. and *Zelkova* spp., on the leaves of which they form closed or open galls (Fig. 9). Several generations of parthenogenetic females develop in the galls. By mid-summer, winged females appear in the galls and migrate to the roots (less often above-ground parts) of various woody or herbaceous flowering plants. Several parthenogenetic generations also develop on secondary host plants, but never forming galls on these plants. In autumn, winged remigrants return to elms, where they hatch bisexual generation larvae in cracks of the bark. These larvae lack mouthparts, do not feed, molt four times, and then mate and each female lays one overwintering egg.

For the subfamily Fordinae (genera *Aloephagus* Essig, 1950, *Aploneura* Passerini, 1863, *Asiphonella* Theobald, 1923, *Baizongia* Rondani, 1848, *Chaetogeoica* Remaudière et Tao, 1957, *Dimelaphis* Zhang, 1998, *Floraphis* Tsai et Tang, 1946, *Forda*

von Heyden, 1837, *Geoica* Hart, 1894, *Geopemphigus* Hille Ris Lambers, 1933, *Inbaria* Barjadze et al., 2018, *Kaburagia* Takagi, 1937, *Meitanaphis* Tsai et Tang, 1946, *Melaphis* Walsh, 1867, *Nurudea* Matsumura, 1917, *Paracletus* von Heyden, 1837, *Qiao* Hébert et al., 2022, *Rectinasus* Theobald, 1914, *Schlechtendalia* Lichtenstein, 1883, *Slavum* Mordvilko, 1927, *Smynthurodes* Westwood, 1849, *Tramaforda* Manheim, 2007) the primarily host plants are *Pistacia* spp. and *Rhus* spp. Closed or open galls are formed on the leaves of these plants (Fig. 10). Life cycles are similar to those of Eriosomatinae, but the taxonomic diversity of secondary host plants is much wider and includes (in species of the American genus *Melaphis*) even mosses. In many species of Fordinae, the opening of the galls occurs only at the end of summer or even in autumn (in September-October). For this reason, the whole life cycle extends over two years. In some Fordine species, the founders first form a small "temporary" gall, and then a significant part of the females of the daughter generation leave the maternal gall and form new, more spacious galls on the same plant (Wool 2005: 87–88). Species distributed in regions where pistachios and sumacs are currently absent are represented only by parthenogenetic generations on secondary host plants. Among such populations, cases of mosaicism are sometimes encountered, when winged parthenogenetic females feeding on the roots of secondary host plants contain both thelytocous (with mouthparts) and bisexual (without mouthparts) embryos (Mordwilko 1901: 83, 214; Popova 1967: 117). Gavrilov-Zimin (2024) proposed to call this phenomenon as "mosaic embryonization."

Figure 9. Galls of *Tetraneura ulmi* (Linnaeus, 1758) on elm leaf, USA. Photo & copyrights: Judy Gallagher, https://www.flickr.com/photos/52450054@N04/33994074962/.

Figure 10. Galls of two different species of Fordinae on twigs of *Pistacia terebinthus* Linnaeus, 1753. Photo & copyrights: Gene Selkov, https://www.flickr.com/photos/selkovjr/45002126051/.

The aphids of the subfamily Pemphiginae (genera *Ceratopemphigiella* Menon et Pawar, 1958, *Ceratopemphigus* Schouteden, 1905, *Clydesmithia* Danielsson, 1989, *Cornaphis* Gillette, 1913, *Diprociphilus* Zhang et Qiao, 1999, *Epipemphigus* Hille Ris Lambers, 1966, *Formosaphis* Takahashi, 1925, *Furvaphis* Hong, 2002, *Gootiella* Tullgren, 1925, *Grylloprociphilus* Smith et Pepper, 1968, *Mimeuria* Börner, 1952, *Mordwilkoja* Del Guercio, 1909, *Neopemphigus* Mamontova et Kolomoets, 1981, *Neoprociphilus* Patch, 1912, *Pachypappa* Koch, 1856, *Pachypappella* Baker, 1920, *Patchiella* Tullgren, 1925, *Pemphigus* Hartig, 1839, *Prociphilus* Koch, 1857, *Thecabius* Koch, 1857, *Tiliphagus* Smith, 1965, *Uichancoella* Calilung, 1975) use mainly *Populus* spp. as primarily host plants, but sometimes inhabit also the other arboral angiosmerms. Spring generations feed inside closed or open galls on the leaves or petioles of poplar leaves, and winged migrants, emerging from the galls, usually fly to the roots of coniferous trees, less often to herbaceous angiosperms. The genus *Prociphilus* differs from other genera of the subfamily in an unusually wide range of primary host plants (from the families Rosaceae, Caprifoliaceae, Oleaceae, etc.), but summer migration is still carried out to the roots of coniferous trees. Some species, for example, *Pemphigus spyrothecae* Passerini, 1860, which lives in closed galls on poplars (Fig. 11), have a cycle without changing of host plants. Only a few pemphigines live in tropical climate, such as the monotypic genus *Ceratopemphigus*, whose members form closed galls on *Ligustrum robustum* (Roxburgh, 1832) in southeast Asia; the life cycle of this species is unknown (Cock et al. 2010).

Thus, in all three subfamilies of Eriosomatidae, many species demonstrate an archaic life cycle, characteristic of adelgids, with development inside closed galls. In the subfamily Fordinae a number of species even demonstrate a two-year cycle, again characteristic of adelgids. This fact further illustrates the extreme dependence of the aphid life cycle on the specific gall formation on specific plants. It is impossible to imagine that such a prolongation of the cycle would be evolutionarily beneficial for the corresponding aphid species, in any way "controlling" the development of the gall. However, it is quite logical to explain this situation by the simple impossibility of leaving the closed galls before the end of the summer season. The appearance of placental viviparity and telescopic embryonization, which occurred for the first time probably among eriosomatids, makes it possible to significantly accelerate the change of generations, and, consequently, increase the number of descendants, regardless of the time of gall opening. Of course, this is only possible if there is sufficient internal space in the gall, which also depends on the exact host plant peculiarities.

Among other aphidoid aphids, life in closed galls is known only for a number of genera/species of Hormaphididae, assigned to the tribe Cerataphidini (Aoki and Kurosu 2010). Gall-forming cerataphidins use *Styrax* spp. trees as primary host plants, on which they form single-chambered or multichambered closed galls. Bamboo, palm and ginger plants are usually used as secondary host plants. In temperate climate, the cycle of gall-forming cerataphidins is quite similar to that of eriosomatids (Aoki and Kurosu 2010). This similarity is not surprising, given that hormaphidids are considered by some researchers to be a group closely related or even sister to eriosomatids (Heie 1987;

Figure 11. Galls of *Pemphigus spyrothecae* Passerini, 1860 on petioles of poplar, Samara Prov. of Russia. Photo of A.S. Kurochkin.

Wojciechowski 1992). In the Asian subtropics and tropics, the cerataphidin cycle either becomes incomplete on secondary (less often primary) host plants, or migration from secondary to primary plants becomes facultative (Aoki and Kurosu 2010). The galls of *Ceratoglyphina styracicola* (Takahashi, 1921) on *Styrax suberifolius* Hooker et Arnott, 1837 in Taiwan reach an extraordinary longevity (up to 20 months!); the gall population can reach 100,000 individuals, approximately half of which are non-breeding individuals — "soldiers" performing a guard function (Aoki and Kurosu 2010).

In aphidoid aphids of the families Aphididae, Drepanosiphidae, Mindaridae, some species form different "pseudogalls", which are curled leaves or needles of host plants. Such shelters do not pose problems for the free exit of migrating winged individuals of aphids, and this exit occurs as the growth of the shoots of the corresponding plants ends and their nutritional value decreases (Popova 1967).

Conclusion

From the above consideration of the life cycles and reproductive peculiarities of aphids, it is clear that the evolution of their archaic groups: adelgids, phylloxeras, eriosomatids, and hormaphidids is fully or partially associated with life in closed galls formed on gymnosperms or angiosperms. Living in closed galls fundamentally distinguishes these aphids from other related groups of hemipteroid gall-forming insects: scale-insects, psyllids and some true bugs; among these groups, there are no examples of the formation of closed galls nor examples of cyclical parthenogenesis, although other (non-cyclical) variants of parthenogenesis are quite common (especially in scale-insects) (Gavrilov 2007; Kuznetsova et al. 2021). Closed galls, known for a few thrips species, usually crack before sexual maturation of the first gall generation (Kranz et al. 2002) and, thus, there are no problems with free exit of insects to mate with individuals from other galls. Cyclical parthenogenesis in thrips is not known, although the group as a whole is characterized by arrhenotokic parthenogenesis and haplodiploidy (Kuznetsova et al. 2021).

In this article, it is not possible or necessary to consider the remaining numerous groups of gall-forming animals, but it can be noted that among terrestrial animals, regular cyclical parthenogenesis has been proven only for some gall wasps (Hymenoptera: Cynipidae) living in closed galls (Stone et al. 2002; Csóka et al. 2005). Unlike hemipteroid insects, gall wasps, like most other gall-forming animals, are characterized by gnawing mouthparts. For this reason, their larvae and/or adults can theoretically gnaw through plant tissue and ensure their escape at any time. However, in reality, the emergence of wasps of the same species from galls in the same area is usually significantly extended over time. In a number of species this occurs only after wintering in a dead gall. Thus, the synchrony of the appearance of adult individuals is greatly disrupted. For example, in experiments with *Andricus quercuslanigera* (Ashmead, 1881) in Texas, emergence of the parthenogenetic generation from oak galls occurred from September 9 to February 24 (Hood et al. 2018). As a result, just like aphids, gall wasps

exhibit a wide variety of life cycle options, which can include regular obligate alternation of parthenogenetic and bisexual generations, or be limited to only parthenogenetic generations or only bisexual, ending within one year or stretching over two years, be combined with a change of host plants or not, etc. (Stone et al. 2002).

More or less regular heterogony is also known in a number of groups of primary aquatic animals, for example, in some trematodes, rotifers and crustaceans (White 1973) and, quite obviously, arose in these groups for some other reasons that differ from those described above for aphids.

The frequent reference in the review literature (see, for example, White 1973; Gokhman and Kuznetsova 2017) to the presence of cyclical parthenogenesis in gall midges (Diptera: Cecidomyiidae) — for example, in subfamilies Porricondylinae and Lestremiinae — actually refers to the facultative appearance of bisexual generations, which does not have a regular character. The point is that representatives of some genera of gall midges reproduce primarily by paedogenesis, but a small part of their larvae can undergo complete metamorphosis and become capable of bisexual reproduction (Ivanova-Kazas 1981).

Just as often and erroneously, the life cycle of the beetle *Micromalthus debilis* LeConte, 1878 is cited as an example of cyclical parthenogenesis. However, the reproduction of this species is carried out exclusively by parthenogenesis (Perotti et al. 2016).

Summarizing the results of the discussion, we can highlight the following main theses characterizing the evolution of the reproductive characteristics of aphids.

1. Cyclical parthenogenesis of aphids is a special variant of heterogony (alternation of parthenogenetic and bisexual generations), strictly associated with the change of seasons in temperate climates and caused by the obligate birth of thelytokous females from fertilized eggs.
2. The origin of such a life cycle can be explained by the long (millions of years) evolution of the most archaic group of recent aphids — adelgids on their main host plants (*Picea* spp. or the ancestral plant taxa), starting from the Triassic or Jurassic periods. Feeding of the spring generation of adelgids on developing spruce shoots always causes the formation of closed strobiloid-like galls, the opening of which is extremely extended in time and prevents panmixia in populations.
3. Non-synchronous opening of galls disrupts the initial synchrony of development of individuals in the population and leads to the simultaneous existence of all stages of ontogenesis during the summer period. Subsequent secondary synchronization of ontogeneses is possible only in the second half of summer on secondary or primary host plants under conditions of cessation of plant shoot growth.
4. The evolution of other archaic groups of aphids: phylloxeras, eriosomatids, and hormaphidids is also fully or partially associated with life in closed galls, but on angiosperms. Such galls, unlike galls on spruce trees, have a much larger internal cavity, which allows several parthenogenetic generations to develop inside them.
5. The loss of the ovipositor in phylloxeras (and the aphidoid aphids hypothetically descended from them) can be explained precisely by the original life in galls, where egg laying does not require special adaptations.

6. The evolutionary transition from oviparity of parthenogenetic generations to viviparity probably occurred in the ancestors of modern Eriosomatidae, as evidenced by the plesiomorphic features of the reproductive biology of the latter.

7. The appearance of placental viviparity and telescopic embryonization, which occurred probably among gall-forming eriosomatids, made it possible to significantly accelerate the change of generations, and, consequently, increase the number of descendants, regardless of the time of opening of the galls.

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ORCID

Ilya A. Gavrilov-Zimin https://orcid.org/0000-0003-1993-5984

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IN MEMORIAM

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In Memoriam: Cytogeneticist Dr. Sc. Ninel A. Petrova (1940–2024) — life and scientific heritage

Valentina Kuznetsova[1], Paraskeva Michailova[2], Andrey Przhiboro[3], Natalia Khabazova[1]

1 *Department of Karyosystematics, Zoological Institute, Russian Academy of Sciences, Universitetskaya emb. 1, 199034 St. Petersburg, Russia* **2** *Research Group of Cytotaxonomy and Evolution, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Tsar Osvoboditel blvd. 1, 1000 Sofia, Bulgaria* **3** *Laboratory of Freshwater and Experimental Hydrobiology, Zoological Institute, Russian Academy of Sciences, Universitetskaya emb. 1, 199034 St. Petersburg, Russia*

Corresponding authors: Valentina Kuznetsova (valentina.kuznetsova@zin.ru); Paraskeva Michailova (pmichailova@yahoo.com)

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Abstract

The article is dedicated to the memory of Dr. Sc. Ninel A. Petrova, who passed away on 18 April 2024, and to her scientific heritage. Ninel was born on 28 March 1940 in Leningrad, the former Soviet Union. From 1959 to 1964, she studied at the Faculty of Biology and Soil Science of the Leningrad State University. After graduating from the university, Ninel joined the Zoological Institute of the Russian Academy of Sciences, where she worked for almost 60 years, rising from a research assistant to a leading researcher. Her scientific research focused on the structure and evolution of polytene chromosomes of two families of Diptera, black flies (Simuliidae) and non-biting midges (Chironomidae). Ninel has published nearly 200 scientific articles, three monographs and one monograph chapter and has become one of the leading experts in her field. Along with scientific activities, Ninel worked for 25 years as the Scientific Secretary of the Specialized Scientific Council for the Defense of Dissertations at the Zoological Institute.

Keywords

Bibliography, biography, Chironomidae, chromosomal rearrangements, Diptera, evolution, polytene chromosomes, research highlights, Simuliidae, systematics

Introduction

Dr. Sc. Ninel A. Petrova (Figs 1, 2A–E, 3, 4) passed away on 18 April 2024 after nearly 60 years of work devoted to the cytogenetic and cytotaxonomic studies of insects of the dipteran families Chironomidae (non-biting midges) and Simuliidae (black flies). Her contributions to the understanding of the structure and evolution of polytene chromosomes of Diptera cannot be overestimated. There can be very few scientists working on the comparative cytogenetics and karyosystematics of these groups who have not cited the publications of Ninel Petrova.

Figure 1. Dr. Ninel A. Petrova (photo by P. Michailova).

Life and times

Ninel Petrova was born in the town of Petrodvorets near Leningrad (now Peterhof in St. Petersburg) on 28 March 1940. She never saw her father, who died in the 1939–1940 Soviet-Finnish War. Ninel was just over a year old when the Great Patriotic War (1941–1945) began, and she and her mother were evacuated from besieged Leningrad to the Ryazan Province, to the town of Pronsk. Life in evacuation, hungry and unsettled, was a traumatic experience. Ninel's memories of this period, based largely on her mother's accounts, can be found in the book "War's Tragic Memory: The Great Patriotic War in the Memoirs of the Staff of the Zoological Institute of the Russian Academy of Sciences" (2021).

After the war, the family returned to Leningrad. Ninel's mother remarried, and Ninel had a stepfather. According to her stories, he was a very kind man and did everything possible for her upbringing. In 1957, Ninel graduated from school. She was interested in biology and, after working for two years in different places, she entered the Leningrad State University (now St. Petersburg State University), the Faculty of Biology and Soil Science where she specialized in plant genetics, specifically rye, at the Department of Genetics and Breeding. Her scientific supervisor was Prof. Vasily S. Fedorov (1903–1976), a famous geneticist and breeder, a remarkable scientist, the creator of the genetic collection of rye and the first domestic variety of tetraploid rye "Leningradskaya Tetra". During the dark period of 'Lysenkoism' in the former Soviet Union, Prof. Fedorov introduced students to the basics of genetics in lectures formally devoted to the criticism of classical genetics. He was a wonderful supervisor who played an important role in the shaping of Ninel Petrova as a scientist and whom she remembered with warmth and gratitude all her life. After graduating and defending her diploma project, Ninel joined the Zoological Institute of the Russian Academy of Sciences in Leningrad as a research assistant in the newly established Laboratory of Karyosystematics and Population Genetics and remained there for the whole of her working career. Ninel was very lucky with her teachers. The head of this laboratory was Prof. Lidia A. Chubareva, one of the founders of the cytotaxonomic approach to insect research in the former Soviet Union. During her years of work at the Institute, Ninel rose from a laboratory assistant to a leading researcher. She not only participated in the scientific life of the laboratory, but also for 25 years held the position of the Scientific Secretary of the Specialized Scientific Council for the Defense of Candidate (Ph. D.) and Doctoral (Dr. Sc.) dissertations at the Zoological Institute in the specialties of "Entomology" and "Parasitology". In this position, she contributed to the formation of the scientific team and the maintenance of the scientific community of the institute. Many colleagues who defended their dissertations in those years were very grateful for the help and advice she gave them, especially regarding dissertation documentation and preparation for defense.

Research highlights

Dr. Sc. Ninel Petrova was a scientist who made great and very valuable contributions to the cytotaxonomy and cytogenetics of the large insect order Diptera. Her research focused on two large and practically important families, Simuliidae (black flies) and Chironomidae (non-biting midges), and on their giant polytene chromosomes. These chromosomes are found in the interphase nuclei of the salivary glands of larvae and consist of thousands of DNA strands resulting from multiple replication cycles without separation of sister chromatids. As Ninel has shown in numerous publications, rearrangements detected in polytene chromosomes can be successfully used to solve the problems of systematics and phylogenetics of these insects at various taxonomic levels, from the separation of closely related species to the analysis of phylogenetic relationships between higher taxa.

Under the supervision of Prof. Lidia Chubareva, who was one of the world leaders in cytotaxonomic studies of black flies, Ninel worked on comparative cytogenetics of this highly important group of insects. These studies resulted in a series of publications, first jointly with her supervisor (e.g., Chubareva and Petrova 1968, 1969) and then independently (Petrova 1972, and others) or with other colleagues (see List of main publications at the end of the article and Supplementary file 1). Many of her taxonomic publications have successfully combined karyological and morphological approaches. On the example of several taxa of black flies (the genera *Cnephia* Enderlein, 1921, *Helodon* Enderlein, 1921, *Schoenbaueria* Enderlein, 1921, *Austrosimulium* Tonnoir, 1925, and others), Ninel Petrova demonstrated how karyological characteristics can be used to clarify the status and position of taxa as well as the phylogenetic relationships between them. In one of her earlier publications (Petrova et al. 1971), devoted to a large and widespread genus *Simulium* Latreille, 1802 and published in co-authorship with Prof. Ivan A. Rubtsov, a well-known Russian expert in the systematics of Simuliidae, the prospect of using chromosomal characters to distinguish closely related species differing only slightly in external morphology was clearly demonstrated. In 1975, Ninel successfully defended her Candidate (Ph. D.) dissertation "Comparative karyological study of bloodsucking black flies of the genera *Cnephia* End., *Metacnephia* Crossk. and *Sulcicnephia* Rubz. (Diptera, Simuliidae)".

After nearly a decade devoted to the study of Simuliidae, in the late 1970s Ninel began to apply karyotaxonomic methods and approaches to another large and diverse dipteran family, Chironomidae, without leaving her first object, Simuliidae. In 1992, she successfully defended her Doctoral dissertation entitled "Polytene chromosomes of chironomids and simuliids, their use in the study of the systematics and evolution of these insect groups (Diptera: Chironomidae, Simuliidae)" and was awarded a well-deserved scientific degree "Doctor of Sciences" (D. Sc.). Ninel has made a great contribution to the knowledge of structural and functional organization of dipteran polytene chromosomes and to the cytotaxonomy of Simuliidae and Chironomidae. Ongoing research on the former led to the publication (in co-authorship with Prof. Chubareva) of the world's first review of karyotypes of black flies (Chubareva and Petrova 2008).

This book (with the subtitle 'Atlas') contains the detailed cytophotomaps of polytene chromosomes of 124 species in 32 genera of black flies of Russia and neighboring countries (descriptions and drawings of taxonomically significant morphological characters of the studied species were also provided). This monograph has become a desktop book for all specialists dealing with comparative cytogenetics of black flies.

Since then, Ninel has focused her efforts mostly on the study of polytene chromosomes and karyosystematics of the family Chironomidae (although she has also published important papers on black flies during these years), which is of both theoretical and practical interest. Chironomid larvae are widely distributed and abundant in aquatic ecosystems, where they play an important role. Ninel carefully studied the morphological characteristics of polytene chromosomes of species from different phylogenetic lineages and subfamilies within the family Chironomidae. Her original chromosome maps of chironomid species from five subfamilies are not only of great taxonomic importance, but have also been successfully used to trace species relatedness at the cytogenetic level (Petrova et al. 2000).

Ninel studied the structural and functional organization of polytene chromosomes of chironomids originating from different regions of Russia and from other countries, including Mongolia, Lithuania, Italy, Bulgaria, Belarus, Ukraine and others (Petrova 1990; Petrova et al. 1986, 1992, 2000, 2004, 2013, 2014a, b). During her life, she participated in many scientific expeditions to collect the material, e.g. to the European North, the Caucasus, Tajikistan and Kyrgyzstan (Fig. 2A, B, D).

Ninel's studies on the variability of polytene chromosomes are also very important because they take into account both seasonal and geographical variability in their band structure (Petrova 1990; Petrova et al. 2004, 2014a, b). She has made a remarkable contribution to the use of polytene chromosomes of model chironomid species to trace metal pollution in various aquatic ecosystems. Because of her authority as a brilliant expert on chironomid cytogenetics, she was invited to participate in two NATO projects named, respectively, "Polytene chromosomes as a model for heavy metal-induced genome instability" (2004–2006) and "Pollution of water resources assessed by genome alterations in midges (2008–2010)". As part of these projects carried out jointly with Bulgarian and Italian colleagues (Fig. 3), Ninel studied the influence of heavy metals on the structural and functional organization of chromosomes of model species of chironomids collected in Russia, Bulgaria and Italy. She has been actively involved in the development of these projects and has established the presence of both multiple heritable and a number of somatic chromosomal rearrangements (Michailova et al. 1996, 1998, 2012; Petrova and Michailova 2002; Petrova 2013; Michailova and Petrova 2015). During these years, Ninel conducted classes and lectures for students from Turin and Milan. Using her knowledge and skills, the research team conducted a number of laboratory experiments with heavy metal ions, which have led to practically important conclusions about the genotoxic effect of some of them (Michailova et al. 2001a, b). This study confirmed that the chironomid genome is a very sensitive structure and responds much more strongly to toxicants than the external morphology of larvae.

Figure 2. Photos of Ninel A. Petrova **A** with L.A. Chubareva (at left), expedition to Azerbaijan, 1969 **B** with E.A. Kachvoryan (at right), 1990s (?) **C** with N.V. Golub (at left), N.V. Khabazova and V.G. Kuznetsova (at right), at Zoological Institute (St. Petersburg), September 1997 **D** expedition to Tajikistan (?), 1975 **E** at Zoological Institute (St. Petersburg), 27 January 2014. (**A–D** photos from personal archive of N.A. Petrova; **E** photo by A.A. Przhiboro).

Figure 3. Photo of Ninel A. Petrova with Bulgarian and Italian colleagues, participants of the NATO project (Turin, 2010). P. Michailova (at left), L. Ramella, S. Bovero and G. Sella (at right).

The extraordinary self-sacrifice of N. Petrova in the name of science deserves special mention. She conducted herself in the field like a true scientist, collecting the scientific material. Without a moment's hesitation, she went to Chernobyl (Ukraine) after the accident at the nuclear power station (26 April 1986) and collected chironomids to study the influence of radiation on the structural and functional organization of their polytene chromosomes. This activity resulted in her research on the chromosomal variability of chironomid species from Chernobyl (Michailova and Petrova 1994; Petrova and Michailova 1996a, b).

During her long life in science, Ninel published about 200 scientific articles, three monographs (Chubareva and Petrova 2008; Petrova 2013; Petrova and Zhirov 2022) and a chapter (Belyanina et al. 1983) in an important collective monograph. Using combined analysis of chromosomes and morphological characters, she discovered and described five new species and two genera of Simuliidae (Petrova 1977, 1983; Chubareva and Petrova 1981; Petrova et al. 1995; see the list of taxa below). Also, as part of an international research team, she described a new chironomid species *Polypedilum pembai* Cornette et al. from Malawi, southeastern Africa (Cornette et al. 2017). This species is notable for the ability of its larvae to survive the dry season in a completely dehydrated ametabolic state known as anhydrobiosis, similar to that in its widely known congener, *P. vanderplankii* Hinton, 1951. Although *P. pembai* has been described based on a suite of features including adult, larval and pupal morphology and

DNA sequencing results, its independence was initially inferred based on the chromosomal data obtained by Ninel (Petrova et al. 2015). One species of Simuliidae and one genus of Chironomidae were named in honor of Ninel Petrova (see below).

An important result of Dr. Petrova's research on chironomids was her last monograph "Structure of polytene chromosomes and morphology of chironomid larvae (Diptera, Chironomidae)" (Petrova and Zhirov 2022). In this book, Ninel together with her former Ph. D. student, the late Sergey V. Zhirov (Kuznetsova et al. 2019), reviewed the data on the polytene chromosomes in larvae belonging to five subfamilies of Chironomidae. This book is mostly based on the authors' original materials and contains original microphotographs of polytene chromosomes. In addition to the chromosomal data, it includes the data on larval morphology. This monograph summarized valuable comparative information on polytene chromosome morphology and chromosomal polymorphism in different populations and species of Chironomidae.

Ninel was a researcher who collaborated productively with colleagues in Russia and some other countries such as Bulgaria, Italy and Armenia. She enjoyed passing on her knowledge and skills to younger colleagues who came to her laboratory and teaching them working with polytene chromosomes. The Zoological Institute and the Department of Karyosystematics, where she worked, have lost a remarkable scientist devoted to science in general.

Scientific presentations

Ninel Petrova has repeatedly made excellent presentations at national (Soviet and All-Russian) and international conferences and congresses. We will list just a few of them, such as all five International Conferences on Karyosystematics of Invertebrate Animals (in 1979 and 2006 in Leningrad/St. Petersburg, in 1991 in Cheboksary, in 1997 in Moscow, in 2010 in Novosibirsk and in 2016 in Saratov), International Symposia on Chironomidae (e.g., in 1997 in Freiburg, Germany), Congresses of the All-Russian Entomological Society (e.g., in 2002 and 2012 (Fig. 4) in St. Petersburg, in 2007 in Krasnodar, and in 2017 in Novosibirsk), Congresses of the Russian Society of Geneticists and Breeders (e.g., in 2014 in Rostov-on-Don and in 2019 in St. Petersburg), All-Russian Dipterological Symposia (e.g., in 2016 in Krasnodar and in 2020 in Voronezh), the X International Balbiani Ring Workshop (in 2021 in Varna, Bulgaria) and many others. Her reports have always been received with great interest.

The taxa described by Ninel Petrova

In Chironomidae

Polypedilum pembai Cornette, N. Yamamoto, M. Yamamoto, Kobayashi, Petrova, Gusev, Shimura, Kikawada, Pemba et Okuda, 2017

In Simuliidae

Levitinia Chubareva et Petrova, 1981
Levitinia tacobi Chubareva et Petrova, 1981
Metacnephia pamiriensis Petrova, 1977
Metacnephia paraskevae Petrova, Chubareva et Kachvoryan, 1995
Prosimulium pamiricum Chubareva et Petrova, 1983
Rubzovia Petrova, 1983 [described as a genus; currently recognized as a subgenus of *Simulium*]
Rubzovia vantshi Petrova, 1983 [current name: *Simulium* (*Rubzovia*) *vantshi* (Petrova, 1983)]

The taxa named in honor of Ninel Petrova

In Chironomidae

Ninelia E. Makarchenko et M. Makarchenko, 2004 [described with the wording: 'described in honor of … N.A. Petrova, a kind and sympathetic person who devoted most of her life to chironomid karyology, moral (and not only moral) support of young (and not so young) Russian chironomidologists'].

Figure 4. Photo of Ninel A. Petrova with colleagues, participants of the XIV All-Russian Entomological Congress, Section of karyosystematics (2012, St. Petersburg). N.A. Petrova, N.V. Golub, V.G. Kuznetsova, S.V. Zhirov, N.V. Durnova, V.E. Gokhman, S.M. Grozeva, and V.A. Lukhtanov (right to left).

In Simuliidae

Sulcicnephia petrovae Rubtsov, 1976 [described with the wording: 'named after Ninel Alekseevna Petrova, who collected the material in difficult conditions of the high-mountain country'].

Note: All listed taxonomic names are currently valid.

Awards

Ninel Petrova was repeatedly awarded prizes and diplomas for her scientific achievements. One of the most significant awards for her was the medal "For the Rescue of the Perished", which she received in the framework of the Decree of the President of the Russian Federation № 419 of 21.04.2006 "On awarding state awards of the Russian Federation to active participants of liquidation of consequences of the accident at the Chernobyl Nuclear Power Plant".

Personal characteristics

Ninel was a sweet, friendly and very creative person. Her interactions with colleagues and friends were always very kind and even-keeled. She was passionate about music, classical Russian literature and painting, and enjoyed attending concerts and exhibitions in her beautiful city of St. Petersburg. Ninel passed on her hobbies to her daughter Olga, who teaches literature and Russian language at a school in St. Petersburg.

Conclusions

Ninel passed away at the age of 84 years old. Throughout her life she carried a sincere interest in science, working in the same laboratory for almost 60 years and becoming one of the leading specialists in her field. Ninel's colleagues and friends will always remember her as a wonderful professional and a very kind person. We express our deepest grief on the death of our dear friend and colleague. She will always live in our hearts and memories.

List of main publications by Ninel Petrova

The publications mentioned in the above text are asterisked. A more complete list of publications by N.A. Petrova with comments (compiled by A. Przhiboro) is given as Suppl. material 1.

1968

*Chubareva LA, Petrova NA (1968) Homologous lines of chromosome polymorphism in the natural populations of blackflies (Diptera, Simuliidae). Tsitologiya 10(10): 1248–1256. [In Russian with English summary].

1969

*Chubareva LA, Petrova NA (1969) Karyological peculiarities of *Helodon ferrugineus* Wahlb. in relation to some questions of systematics. Tsitologiya 11(2): 234–241. [In Russian with English summary].

1971

Chubareva LA, Petrova NA, Petrukhina TE (1971) The structural peculiarities of the polytene chromosomes and the taxonomy of the black flies (Diptera, Simuliidae). Tsitologiya 13(6): 784–789. [In Russian with English summary].

*Petrova NA, Rubtsov IA, Chubareva LA (1971) On the position of *Simulium* (*Schönbaueria*) *gigantea* Rubz. in the system of classification of simuliids (Morphological and karyological characters in the systematics). Parazitologiya 5(1): 40–50. [In Russian with English translation in Parasitology, 1972, 1(1): 45–57].

1972

*Petrova NA (1972) Karyological features of the Karelian black flies [sic!] of the genus *Cnephia* End. Tsitologiya 14(6): 769–773, 2 plates. [In Russian with English summary].

1973

Petrova NA (1973) A comparative karyological analysis of three genera of the family Simuliidae (Diptera). Tsitologiya 15(8): 1055–1059. [In Russian with English summary].

Petrova NA (1973) A comparative karyological study of 3 species of black-flies of the genus *Metacnephia* Crosskey (Diptera, Simuliidae) from Armenia. Tsitologiya 15(4): 439–445, 3 plates. [In Russian with English summary].

1974

Petrova NA (1974) Inversional polymorphism in natural populations of two species of black flies (Diptera, Simuliidae). Genetika 10(1): 80–89. [In Russian with English summary].

1975

Chubareva LA, Petrova NA (1975) Karyotype of the plesiomorphic New Zealand species *Austrosimulium tillyardi* and its genetical relations with some other genera of the family Simuliidae (Diptera). Zoologicheskii Zhurnal 54(4): 552–558. [In Russian with English summary].

*Petrova NA (1975) Comparative karyological study of blood-sucking black flies of the genera *Cnephia* End., *Metacnephia* Crossk. and *Sulcicnephia* Rubz. (Diptera, Simuliidae). Candidate of Biological Sciences Dissertation, Leningrad: Zoological Institute, Academy of Sciences of the USSR, 242 pp. [In Russian].

1976

Chubareva LA, Rubtsov IA, Petrova NA (1976) Morphological and karyological similarities and differences in Palearctic and Neotropical species of the genus *Hemicnetha* End. (Diptera, Simuliidae). Entomologicheskoe Obozrenie 55(2): 452–457. [In Russian with English translation in Entomological Review, 1976, 55(2): 137–142].

1977

*Petrova NA (1977) A new species of black flies; *Metacnephia pamiriensis* sp. n. (Simuliidae) from Pamir. Parazitologiya 11(3): 210–212, 1 plate. [In Russian with English summary].

Petrova NA, Chubareva LA, Kuzmenko KN (1977) The karyotypes of five species of Chironomidae (Diptera). Tsitologiya 19(8): 900–905. [In Russian with English summary].

Rubtsov IA, Petrova NA (1977) Blackflies of the tribe Cnephiini (Diptera, Simuliidae) and diagnoses of the genera *Cnephia* Enderlein and *Astega* Enderlein. Entomologicheskoe Obozrenie 56(3): 691–697. [In Russian with English translation in Entomological Review, 1977, 56(3): 145–149].

1978

Maksimova FL, Petrova NA (1978) Geographical variability of karyotype in *Chironomus plumosus* (Diptera, Chironomidae). Zoologicheskii Zhurnal 57(12): 1816–1826. [In Russian with English summary].

Petrova NA, Chubareva LA (1978) The peculiarities of the karyotype of *Prodiamesa olivacea* Meig. (Diptera, Chironomidae, Orthocladiinae). Tsitologiya 20(10): 1208–1211. [In Russian with English summary].

Petrova NA, Maksimova FL (1978) The role of chromosome rearrangements in the speciation of chironomids (Diptera, Chironomidae). Genetika 14(7): 1201–1207. [In Russian with English translation in Soviet Genetics, 1978, 14: 849–853].

1979

Chubareva LA, Petrova NA (1979) Main characters of karyotypes of blackflies (Diptera, Simuliidae) of the world. In: Chubareva LA (Ed.) Karyosystematics of the Invertebrate Animals. Leningrad: Zoological Institute, Academy of Sciences of the USSR, pp. 58–95. [In Russian with English summary].

Petrova NA (1979) Chromosomal rearrangements distinguish species of the genus *Metacnephia* (Diptera, Simuliidae). In: Nartshuk EP (Ed.) Ecological and Morphological Principles of Diptera Systematics (Insecta). Materials of Symposium (13–15 September 1978, Voronezh), Leningrad: Zoological Institute AN SSSR, pp. 79–81. [In Russian with English translation in Skarlato OA (Ed.), 1979, Systematics of Diptera (Insecta): Ecological and Morphological Principles. New Delhi, India: Amerind Publishing Co. Pvt. Ltd., pp. 125–128].

1980

Chubareva LA, Petrova NA (1980) A method for making of cytological preparations for caryological study of Diptera. In: Chubareva LA (Ed.) New Data on Karyosystematics of Dipterous Insects. Trudy Zoologicheskogo Instituta AN SSSR, 95: 73–80. [In Russian with English summary; signed for press on 29 December 1980 and possibly therefore actually published in 1981]

Maksimova FL, Petrova NA (1980) On the problem of sympatric species evolution in chironomids (Diptera, Chironomidae). In: Lukina EV (Ed.) Problems of Population Cytogenetics of Plants and Animals, pp. 70–76. [In Russian].

Pankratova VYa, Chubareva LA, Petrova NA (1980) On the systematics of some *Chironomus* species (Chironomidae) from the Lake Sevan. In: Chubareva LA (Ed.) New Data on Karyosystematics of Dipterous Insects. Trudy Zoologicheskogo Instituta AN SSSR, 95: 50–54. [In Russian with English summary].

Petrova NA (1980) Cytological sex determination of blackflies (Simuliidae). In: Chubareva LA (Ed.) New Data on Karyosystematics of Dipterous Insects. Trudy Zoologicheskogo Instituta AN SSSR, 95: 55–58. [In Russian with English summary].

Petrova NA (1980) Karyological research of Orthocladiinae (Diptera, Chironomidae). Genetica 52–53: 275–279. https://doi.org/10.1007/BF00121837

1981

*Chubareva LA, Petrova NA (1981) A new genus of black flies (Diptera, Simuliidae) from Tadzhikistan. Entomologicheskoe Obozrenie 60(4): 898–900. [In Russian with English translation in Entomological Review, 1982, 60(4): 140–144].

Petrova NA (1981) Morphological peculiarities of polytene chromosomes in two black fly species (Diptera, Simuliidae). Tsitologiya 23(11): 1317–1320. [In Russian with English summary].

Petrova NA, Chubareva LA, Zolotaryova LV, Kaliberdo TA (1981) The karyotypes of chironomids from the Bratsk Reservoir (Diptera). Tsitologiya 23(10): 1180–1187. [In Russian with English summary].

1982

Chubareva LA, Petrova NA (1982) Cytogenetic method of chromosome analysis in chironomids. In: Akhrorov F (Ed.) Methodical Guide to Chironomid Research. Dushanbe: Donish, pp. 64–73. [In Russian].

Petrova NA, Zolotareva LV (1982) Morphology and karyotype of a larva of *Micropsectra* sp. (Diptera, Chironomidae) from the East Pamir. Zoologicheskii Zhurnal 61(10): 1605–1607. [In Russian with English summary].

1983

*Belyanina SI, Maksimova FL, Bukhteeva NM, Il'inskaya NB, Petrova NA, Chubareva LA (1983) Chapter 2. Systematics and morphology. Division 2. Karyotype. In: Sokolova NJu (Ed.) *Chironomus plumosus* L. (Diptera, Chironomidae). Systematics, Morphology, Ecology, Production. Moscow: Nauka Publishers, pp. 61–96. [In Russian].

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1984

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1986

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*Petrova N, Kiknadze I, Michailova P (1986) Integration of species in the plumosus-group of Chironomidae. In: Aukshtikal'nene AM, Permyakova LV (Eds) A System of Species Integration. Vilnius, pp. 138–161. [In Russian with English summary].

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1987

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1988

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1989

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1990

*Petrova N (1990) Characteristics of chironomid karyotypes (Diptera, Chironomidae) of the world fauna II. Subfamily Chironominae. Entomologicheskoe Obozrenie 69(1): 193–214. [In Russian].

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1991

Michailova P, Petrova N (1991) Chromosome polymorphism in geographically isolated populations of *Chironomus plumosus* L. (Chironomidae, Diptera). Cytobios 67: 161–175.

Petrova NA (1991) Chromosomal rearrangements in three species of chironomids (Diptera, Chironomidae) from the Chernobyl zone. Genetika 27(5): 836–848. [In Russian with English summary].

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ORCID

Valentina Kuznetsova https://orcid.org/0000-0001-8386-5453
Paraskeva Michailova https://orcid.org/0000-0002-7036-9714
Andrey Przhiboro https://orcid.org/0000-0001-6665-8778

Supplementary material 1

A list of publications by Ninel Petrova (compiled by A. Przhiboro)
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IN MEMORIAM

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

Wilhelm Hofmeister (1824–1877) and the ideas of evolutionary embryonization*

Ilya A. Gavrilov-Zimin[1,2]

1 *S.I. Vavilov Institute for the History of Science and Technology, St. Petersburg Branch, Russian Academy of Sciences, Universitetskaya nab. 5, St. Petersburg, Russia* **2** *Zoological Institute, Russian Academy of Sciences, Universitetskaya nab. 1, St. Petersburg, Russia*

Corresponding author: Ilya A. Gavrilov-Zimin (coccids@gmail.com)

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This year marks the 200th anniversary of the birth of Friedrich Wilhelm Benedikt Hofmeister, an outstanding German amateur biologist and one of the forerunners of genetics (Fig. 1). Details of his biography have been analyzed in a number of publications (e.g., Goebel 1905; Kaplan and Cooke 1996; Martin 2017, etc.), so there is no need to reproduce them in the present memorial article. In both the educational and scientific literature (see the works cited above), Hofmeister is known primarily as the discoverer of the alternation of generations (gametophyte and sporophyte) in the life cycle of plants. However, this view is not entirely accurate, as formal priority in this matter still belongs to another amateur, the Polish Count Michal Leszczyc-Suminski (1848) (see some details below).

It is also believed (e.g., Kaplan and Cooke 1996: 1650) that Hofmeister's research strongly influenced another prominent German amateur, Gregor Mendel (1822–1884), and inspired him to conduct the famous experiments in plant hybridization, that laid the foundation for the new biological discipline, genetics.

In the anniversary year, I would like to draw the readers' attention to the importance of Hofmeister's work for the development of ideas about the evolutionary embryonization of ontogenesis, which had not previously attracted much attention.

* "Comparative Cytogenetics" to 200-year jubilee of one of the forerunners of genetics.

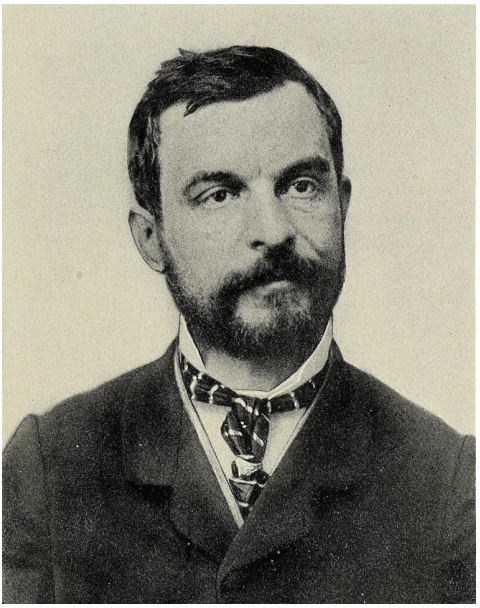

Figure 1. Wilhelm Hofmeister (1824–1877), after Goebel (1905).

The concepts of evolutionary (or phylogenetic) embryonization (and vs. disembryonization) are now widely used to reconstruct the phylogeny of various groups of plants and animals (see review: Gavrilov-Zimin, 2024). Often, these concepts are so tightly woven into descriptions of the course of evolution of specific taxa that they are not even considered controversial, but are accepted as indisputable facts. But like many other paradigms of modern natural science, these ideas have come a long way from very simple, even naive views that existed long before Darwin's evolutionary concept, to an increasingly complex and comprehensive understanding of the nature of phenomena. On this long path, the elucidation of the embryonic development of plants has constantly and very much lagged behind embryological studies of animals, despite the fact that objectively plant organisms are much simpler than animals.

If animal embryology and corresponding ideas about embryonization of ontogenesis go back to Aristotle (about 2400 years ago — see modern translations: Aristotle 1940, 1996) and his ideas about the ontogenesis of holometabolous insects, we can speak about plant embryology only since the works of Marcello Malpighi (1628–1694), Nehemiah Grew (1641–1712) and Rudolf Camerer (1665–1721) at the end of the XVII century. Along with the zoologists Antoni Van Leeuwenhoek (1632–1723), Robert Hooke (1635–1703), and Jan Swammerdam (1637–1680), these were the first microscopists, and with their names is associated a major turning point in the history of biology, the boundary between Renaissance biology and the "new" biology. It was then that a huge array of fundamentally new information was discovered, absolutely unknown and not even suspected by biologists of Antiquity and the Renaissance. In Malpighi' (see, for example, the compilation of his works: Opera omnia, 1687) one can see, in fact, the first scientific botanical illustrations in the history of science, i.e. not only the external appearance of plants, which was depicted by numerous artists of different epochs, but also the subtle internal structure being studied, including the organs of the flower with developing embryos. In the same period of time, the essence of the sexual process in flowering plants, the role of stamens and pistil in the fertilization of the egg cell and formation of the plant embryo was first understood. Apparently, the first to verify this experimentally was the English gardener and amateur botanist Jacob Bobart (1599–1680) and after him the corresponding ideas were developed by the English professional botanist Nehemiah Grew in his book "The Anatomy of Plants" (Grew

1682). Thus, botanical embryology is a child of the early modern era of human history, and the gap between it and animal embryology, which began as early as Aristotle, is as much as 2,000 years. In general, the entire eighteenth century in plant embryology was passed under the sign of constant disputes about preformism and the possibility of sexual reproduction in plants, without any fundamental progress compared to the works of Malpighi and Grew (Baranov 1955). Such progress was only in the 19th century and was associated with the next stage of improvement of microscopic equipment and dissection methods. These innovations made it possible to bring embryological research to a previously unattainable level and to begin a detailed study of the reproductive organs and developing embryos of a wide range of plants, both the most primitive and highly developed. In this regard, we can recall the names of many botanist-embryologists, such as Carl Nägeli (1817–1891), Matthias Schleiden (1804–1881), Eduard Strasburger (1844–1912), Sergei Navashin (1857–1930), and many others.

In the second half of the 19th century, methodological progress was supplemented by a significant conceptual progress associated with the gradual acceptance by more and more biologists of the ideas of Darwinian evolutionism. In the field of embryology, this led first to the realization of the evolutionary variability of animal ontogenesis, and then, as in previous centuries, gradually migrated from zoological embryology to botanical embryology. However, as in the case of animal embryology, the study of plants has historically proceeded in the direction opposite to the course of evolution, that is embryological features of the most complex animals — vertebrates and the most highly developed plants — angiosperms have been studied first and in great detail. Until the middle of the 19th century, all the few studies on the reproductive biology of higher spore plants – mosses, horsetails, club mosses, ferns, and algae – were carried out in the context of attempts to automatically transfer the already formed ideas about the structure of flower, seed, and fruit to something that had nothing homologous with these structures. For example, the spores of these plants were likened to seeds, then to pollen grains, and the sporangia themselves to a flower. There have been cases of the opposite meaning, quite curiosities, in which the spermatozoa of mosses and algae, observed under the microscope, have been thought to be either infusoria or some kind of "monads".

In 1848, the amateur botanist and artist M. Leszczic-Suminski (1848) was the first to understand and correctly interpret the life cycle of spore plants using the fern as an example, brilliantly illustrating the stages of this cycle with detailed color drawings (Fig. 2). Wilhelm Hofmeister, almost the same age as Suminski and also an amateur botany student without a diploma and salary, a German bookseller, immediately accepted Suminski's discovery and studied in great detail the ontogenesis of various representatives of other groups of higher spore plants (Hofmeister, 1851, 1862) (Fig. 3), generalizing it into a slender system, which to this day is the basis of evolutionary embryology of plants and botany in general. This work made it possible for the first time in the history of biology, which is more than 2000 years, to understand the fundamental difference between the life cycles of plants and the life cycles of animals. The former have two different phases in their cycle – sexual (gametophyte) and asexual (sporophyte), which successively replace each other, while animals have no such

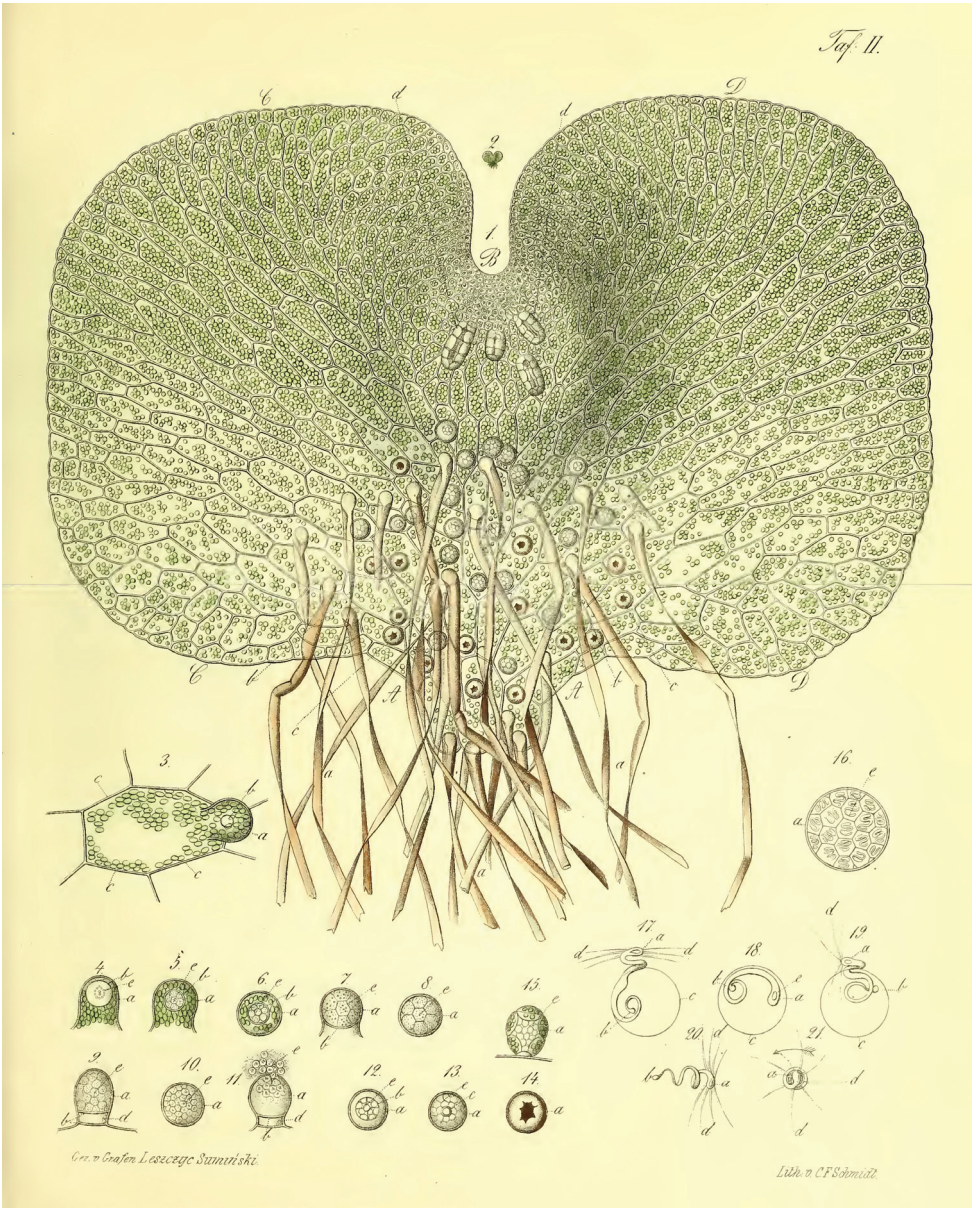

Figure 2. The gametophyte of fern according to Leszczic-Suminski (1848).

phase change. The understanding of these differences can rightly be considered one of the greatest fundamental discoveries in biology. In particular, it was only after these works that it become possible to discuss the phenomena of evolutionary embryonization and disembryonization as applied to plants. Thus, it became clear that in seed plants the gametophyte phase is completely embryonized and hidden inside the spo-

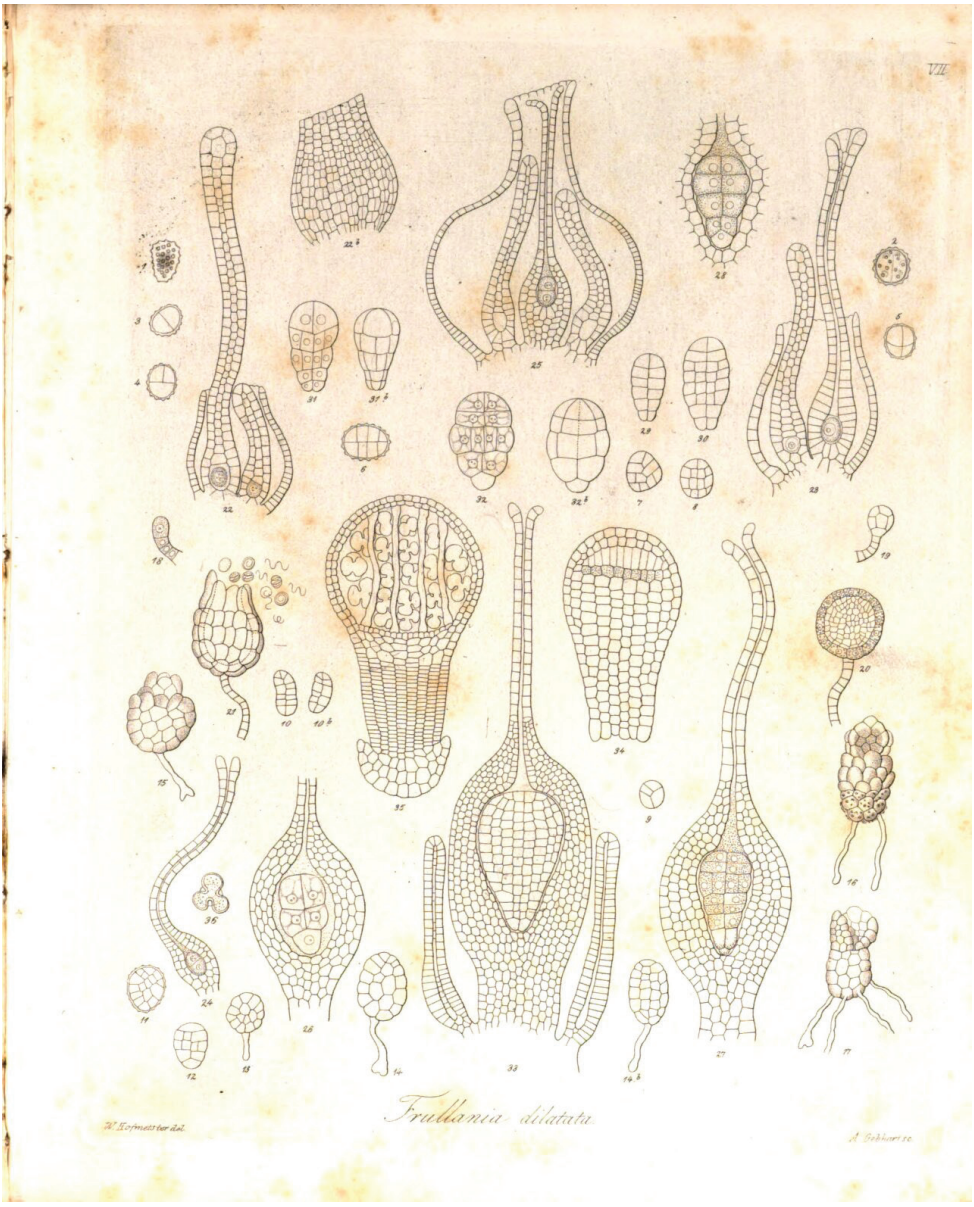

Figure 3. The development of liverwort according to Hofmeister (1851).

rophyte. Here, it is appropriate to quote Hofmeister himself (Hofmeister (1862: 438): "*In more than one respect the formation of the embryo of the Coniferae is intermediate between the higher cryptogams and the phaenogams. Like the primary mother-cell of the spores of the Rhizocarpeae and Selaginellae the embryo-sac is one of the axile cells of the shoot, which in the one case becomes converted into the sporangium, in the other into the*

ovule. In the Coniferae also the embryo-sac soon becomes free from any mechanical connexion with the surrounding cellular tissue. The filling of the embryo-sac by the endosperm may be compared with the production of the prothallium of the Rhizocarpeae and Selaginellae." Moreover, Hofmeister actually constructs an evolutionary series of plants, starting from Charophyta algae and ending with spermatophyte plants: «*The phaenogams therefore form the upper terminal link of a series, the members of which are the Coniferae and Cycadeae, the vascular cryptogams, the Muscineae, and the Characeae. These members exhibit a continually more extensive and more independent vegetative existence in proportion to the gradually descending rank of the generation preceding impregnation, which generation is developed from reproductive cells cast off from the organism itself*».

Based on Hofmeister's works, other botanists in the second half of the 19th century and throughout the 20th century intensively investigated numerous, previously unstudied representatives of higher and lower plants, including those from tropical regions and the southern hemisphere of the planet. Gradually, by the second half of the 20th – early 21st centuries, general theoretical ideas about the regularities of embryonization and disembryonization of organisms in general were formed, mainly in the works of Russian embryologists, evolutionists and theoreticians of biology. Among them are such well-known biologists as I.I. Shmalhausen, A.A. Zakhvatkin and O.M. Ivanova-Kazas and less well-known ones: A.P. Khokhryakov, E.N. Polivanova, A.L. Tikhomirova and some others (see a review of their works in Gavrilov-Zimin, 2024). Among the many names mentioned, Wilhelm Hofmeister's name remains one of the most striking examples of a unique combination of genius, selflessness, brilliant self-education, phenomenal diligence, and unwavering pursuit of truth.

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ORCID

Ilya A. Gavrilov-Zimin https://orcid.org/0000-0003-1993-5984