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Comparative cytogenetics of two species of *Dermanura* (Chiroptera, Phyllostomidae) in Midwestern Brazil

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Abstract

Dermanura Gervais, 1856 is represented by small frugivorous bats of the Stenodermatinae subfamily. The taxonomy of this group presents controversies and has been subject to changes, especially since the morphological characters evaluated have left gaps that are difficult to fill regarding good species characterization. Previous studies performed in *Dermanura cinerea* Gervais, 1856 found that the karyotype of this species has a diploid number of chromosomes equal to 30 and 56 autosomal arms. The objective of the present study was to describe, for the first time, the karyotypes of the species *Dermanura anderseni* (Osgood, 1916) and *Dermanura gnoma* (Handley, 1987) based on classical cytogenetic markers. For both species, the diploid number found was 2n = 30 and NFa = 56. Two pairs of chromosomes showed markings of the nucleolus organizing regions (AgNORs) in the species *D. anderseni* and only one pair in *D. gnoma*, differing from what has already been described for *D. cinerea*. The two species analyzed here also showed differences in the sex chromosome system, with *D. gnoma* showing a neo-XY type system while in *D. anderseni* the classic XY sexual system was observed. In both species, visualization of the constitutive heterochromatin occurred in the pericentromeric region of all chromosomes, as well as in the short arms of the subtelocentric chromosomes. The present work represents an important expansion of karyotypic information for the subfamily Stenodermatinae, bringing chromosomal features that are possible to use in the taxonomic implications of the group.

Keywords

Bats, Stenodermatinae, *Dermanura gnoma*, *Dermanura anderseni*, karyotype

Introduction

Among bat families, Phyllostomidae Gray, 1825 is the most morphologically and ecologically diverse, with more than 200 species arranged in 60 genera and 11 subfamilies (Solari and Martinez-Arias 2014; Baker et al. 2016). The subfamily Stenodermatinae Gervais, 1856 is represented in Brazil by 13 genera and 35 species (Nogueira et al. 2014; Pereira et al. 2017). Some genera of this subfamily are complex regarding their phylogenetic relationship, as is the case of *Artibeus* Leach, 1821, for which several questions have been raised (Van-Den-Bussche et al. 1993; Hoofer et al. 2008). Owen (1991) divided this genus into three taxa based on morphological characters: *Artibeus* Leach, 1821 (species with large body), *Dermanura* Gervais, 1856 (species with small body) and *Koopmania* Owen, 1991 (with one representative: *Artibeus concolor* Peters, 1865, with intermediate body size). Through analyses of the mitochondrial gene cytochrome-b and satellite DNA sequences, Van-Den-Bussche et al. (1998) suggested that *Artibeus* and *Dermanura* are sister groups.

The genus *Dermanura* has 11 described species (Hoofer et al. 2008; Solari et al. 2009). Of these, four occur in Brazil: *Dermanura anderseni* (Osgood, 1916), *Dermanura bogotensis* (Andersen, 1906), *Dermanura cinerea* (Gervais, 1856) and *Dermanura gnoma* (Handley, 1987) (Nogueira et al. 2014). The taxonomy of this group presents controversies and has been subject to alterations based on the character set evaluated (Redondo et al. 2008). In general, species identification has been based on morphological and metric characteristics (skull, body size, forearm and weight measurements), which makes taxonomic identification and the establishment of phylogenetic relationships difficult (Sotero-Caio et al. 2017a).

With the advancement in molecular techniques, and cytogenetic approaches, the integrative analysis becomes important, since more robust proposals are provided to identify the group's diversification and evolution patterns (Sotero-Caio et al. 2015; Tavares et al. 2015; Bassantes et al. 2020). Comparative karyotypic studies in bats, especially within Phyllostomidae, have indicated great examples of species with a high level of chromosomal reorganization, diverging from the ancestral species (Pieczarka et al. 2013; Sotero-Caio et al. 2017a, b).

However, knowledge about karyotype data is still scarce for some bat species, and little data has been published for species from South America. Recent studies have been complementing data on chromosomal banding for some species (Calixto et al. 2014; Corrêa and Bonvicino 2016; Sousa et al. 2017; Souza et al. 2017; Côrrea et al. 2017), with karyotype descriptions for species that do not present chromosomal data in the literature (Santos et al. 2002; Garcia and Pessôa 2010; Almeida et al. 2016, Gomes et al. 2016).

Even for many species of Chiroptera, which currently have a reasonable amount of karyotype data, some important gaps can be detected through a detailed data survey. These gaps highlight the importance of expanding chromosomal studies with bats in order to make the information available for the order more robust, especially regarding knowledge about Chiroptera's chromosomal evolution (Marchesin and Morielle-Versute 2004; Garcia and Pessôa 2010; Sotero-Caio et al. 2017a).

In *Dermanura*, the only chromosome set ever described is for *D. cinerea* whose analyses revealed a diploid number of chromosomes equal to 30 and 56 autosomal arms (Santos and Souza 1998b; Noronha 2010). The present work aims to describe the karyotypes of the species *Dermanura anderseni* and *Dermanura gnoma* based on classic cytogenetic markers (conventional staining with *Giemsa*, C-banding and silver nitrate impregnation) in order to identify chromosomal morphology of these species and contribute to the cytogenetic knowledge of bat species.

Material and methods

Specimens were collected in the municipalities of Nova Xavantina (14°42'28.8"S, 52°21'03.9"W) and Chapada dos Guimarães (15°18'25.57"S, 55°49'06.33"W), both in the state of Mato Grosso – Brazil (Fig. 1), which presented two Cerrado Biome phytophysiognomies: (I) Cerradão, a forest type in the Central Brazilian Plateau, with close treetops and a plant community that presents a closed appearance, but with spacing between trees that allows sun to penetrate through and tree heights ranging from 10 to 15 m (Rizzini 1997). (II) Cerrado *stricto sensu*, which is composed of more spaced out, smaller, and twisted trees, providing the appearance of grasses and subshrubs with low vegetation prevailing (Rizzini 1997). Trees in these areas are usually 3 to 6 m high and can reach up to 10 m (Ribeiro and Walter 2008). Five specimens of *Dermanura anderseni* Osgood, 1916 were analyzed, three (2 females and 1 male) captured in the municipality of Nova Xavantina and two (1 male and 1 female) captured in Chapada dos Guimarães. For *Dermanura gnoma* Handley, 1987, two male specimens captured in Nova Xavantina were analyzed. Bats were captured with mist nets (9 × 3 m, with 16 mm mesh) set up from 18:00h to 00:00h in possible bat routes, which were inspected every 30 minutes. The animals were recorded and identified to species level following specialized bibliography (Vizotto and Taddei 1973; Gardner 2008; Reis et al. 2013; Nogueira et al. 2014; Reis et al. 2017). Both species have very evident white facial stripes, however, among other differences, they have different sizes (with forearm 38 to 40 mm in *D. anderseni* and 34 to 38 mm in *D. gnoma*) and different dental formulas (I2 / 2, C1 / 1, P2 / 2, M2 / 2 = 32 for *D. anderseni* and I2 / 2, C1 / 1, P2 / 2, M2 / 3 = 30 for *D. gnoma*). Specimens captured in Nova Xavantina were deposited in the scientific collection of the Laboratório de Genética at the Universidade do Estado de Mato Grosso Nova Xavantina campus under the following collection numbers: RM 216, 234, 302 (*D. anderseni*) and 257, 333 (*D. gnoma*) (License No. 18276-1 – IBAMA/SISBIO/MT) and specimens captured in Chapada dos Guimarães were deposited in the zoological collection of the Instituto de Biociências at the Universidade Federal de Mato Grosso, Cuiabá campus, under license no. 46359-1 – ICMBio/SISBIO/MT (UFMT-PNCG 863 and 866, *D. anderseni*).

Chromosomal preparations were obtained through direct bone marrow extraction, according to the procedure described in Morielle-Versute et al. (1996), with minor adjustments during routine work in the laboratory. To observe the metaphases, conventional staining with Giemsa was performed. The technique for visualizing constitu-

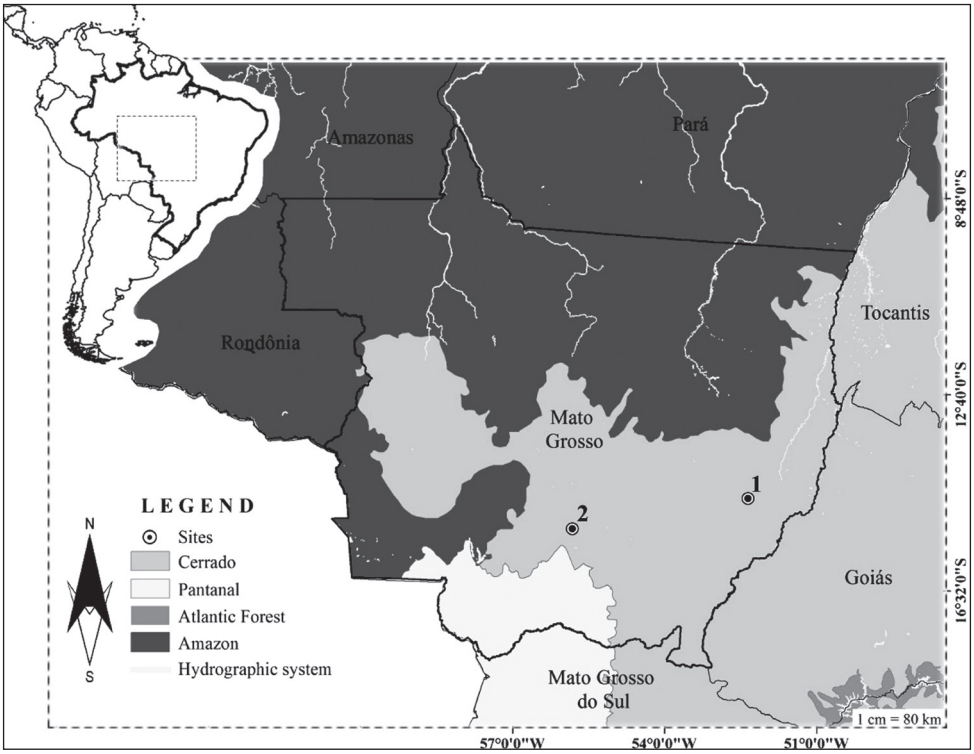

Figure 1. Capture locations of the species *Dermanura anderseni* and *Dermanura gnoma* **1** Nova Xavantina, in Cerradão and Cerrado *stricto sensu* areas and **2** Chapada dos Guimarães in a Cerrado *stricto sensu* area, Mato Grosso, Brazil.

tive heterochromatin (C-banding) was performed according to the protocol proposed by Sumner (1972); staining with propidium iodide and silver nitrate impregnation (AgNORs) followed Howell and Black (1980).

The slides with chromosomal preparations were analyzed using optical microscopy. Slides that showed good quality metaphases were photographed under an Olympus BX51 microscope (Tokyo, Japan). The free edition of Adobe Photoshop Cs6 portable program was used to assemble karyotypes. Afterwards, chromosomes were measured and classified according to the position of the centromere, following Levan et al. (1964).

Results

The diploid number found for *Dermanura gnoma* Handley, 1987 was 2n = 30 and the number of autosomal chromosome arms was NFa = 56. The karyotypes are composed of ten pairs of meta-submetacentric chromosomes (1, 2, 3, 4, 8, 9, 11, 12, 13 and 14) and four subtelocentric chromosome pairs (5, 6, 7 and 10) (Fig. 2A). The X chromo-

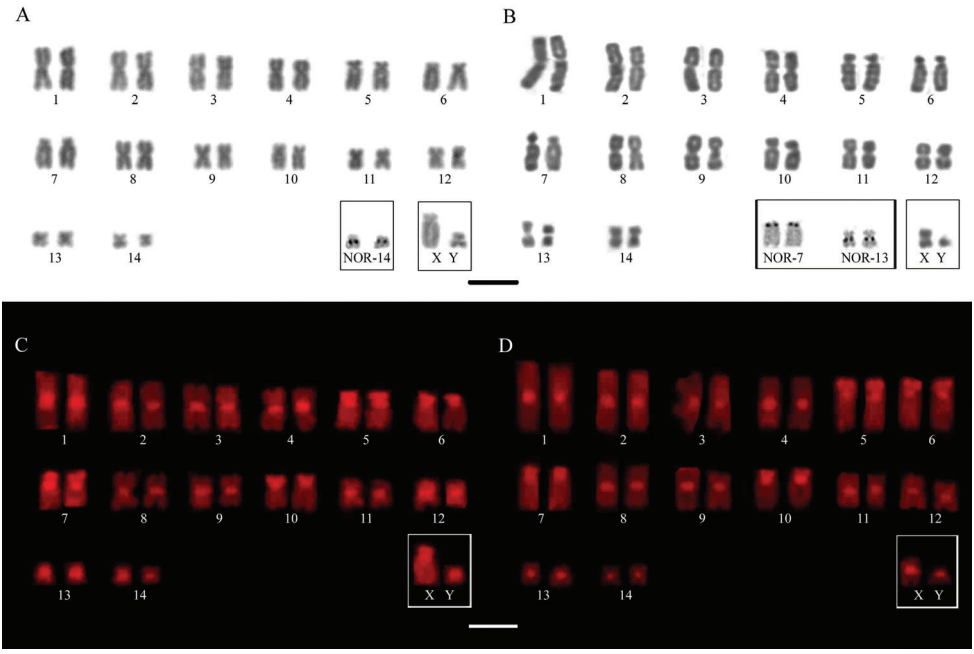

Figure 2. A, B karyotypes of male individuals stained with *Giemsa*, highlighted in the boxes the Ag-NORs marks and the pair of sex chromosomes of **A** *Dermanura gnoma* (2n = 30 and NFa= 56) and **B** *Dermanura anderseni* (2n = 30 and NFa = 56) **C, D** karyotypes showing the distribution of constitutive heterochromatin for both species studied **C** *D. gnoma* and **D** *D. anderseni*. Scale bar: 5 μm.

some is a large subtelocentric and the Y chromosome is a small metacentric. With silver nitrate staining, the nucleolus organizing regions (AgNORs) were observed only in the number 14 pair, in the interstitial position of the long arm (Fig. 2A). In this species, C-banding showed constitutive heterochromatin markings in the pericentromeric regions of all autosomal chromosomes and in X and Y sex chromosomes. Small blocks of heterochromatin were also observed in interstitial regions in the short arms of pairs 5, 6, 7, 10 and on chromosome X (Fig. 2C).

The karyotype of the species *Dermanura anderseni* Osgood, 1916 also presented the diploid number 2n = 30 and the number of autosomal chromosome arms NFa = 56. Similar to *D. gnoma*, the karyotype of this species also presents ten pairs of metacentric chromosomes (1, 2, 3, 4, 8, 9, 11, 12, 13 and 14) and four pairs of subtelocentric chromosomes (5, 6, 7 and 10). The X chromosome is a medium metacentric and the Y chromosome is a small acrocentric. AgNORs were observed in two chromosome pairs: in the short arm of subtelocentric pair no. 7 and in the interstitial position of the long arm of chromosome 13 (Fig. 2B). The constitutive heterochromatin regions presented pericentromeric markings on all autosomal chromosomes and on the X and Y sex chromosomes, in addition to small blocks on the short arms of the subtelocentric pairs 5, 6, 7 and 10 (Fig. 2D).

Discussion

The cytogenetic analyses presented here indicated that for both species, *D. anderseni* and *D. gnoma*, the diploid number found was 2n = 30 and NFa = 56. Among the four species of *Dermanura* that occur in Brazil, only the *D. cinerea* karyotype was previously known, with karyotype form also described as 2n = 30 and NFa = 56 (Tucker 1986; Souza and Araujo 1990; Santos and Souza 1998b; Santos et al. 2002; Calixto et al. 2014; Sotero-Caio et al. 2017b). However, the analysis of the band patterns showed relevant differences on the C-banding, AgNORs patterns, and on chromosome morphologies of the sexual pair between these species.

Our results verified that the sexual system described as neo-XY was found in *D. gnoma*, while in *D. anderseni* the classic XY sexual system was observed, where X is a medium metacentric and Y is a small acrocentric. The classic XY sexual system is the most common for bat species studied to date (Baker 1970; Varella-Garcia et al. 1989; Pieczarka et al. 2005; Calixto et al. 2014). According to Tucker's proposal (1986), the neo-XY sexual system had its origin when the short arm of the X chromosome underwent fission and there was fusion with the Y chromosome, forming a small metacentric Y and the X chromosome is represented by a large subtelocentric. This system was also observed by Souza and Araújo (1990) in *D. cinerea*, suggesting that this condition is apomorphic in relation to the condition observed in *D. anderseni*.

Regarding the location of the ribosomal sites, Santos et al. (2002) described multiple AgNORs for *D. cinerea* involving pairs 10 (medium metacentric) and 13 (small metacentric). In the present study, multiple AgNORs were observed for *D. anderseni*, however, involving pairs 7 (medium subtelocentric) and 13 (small metacentric). In *D. gnoma* the AgNORs are localized in a single and different pair of chromosomes (pair 14 – small metacentric). Thus, this marker is relatively well informative for this group of bats and can, if used with caution, be a good species-specific indicator.

Observations of the nucleolus organizing regions can be performed through FISH (fluorescence in situ hybridization) using probes for rDNA 5S, 18S and 45S (Calixto et al. 2014). In the genus *Dermanura*, FISH for rDNA 18S were only observed in *D. cinerea* in pairs 9, 10 and 13 (Santos et al. 2002), confirming the occurrence of a multiple system of rDNA sites. However, this technique has not yet been applied to other species within the genus.

Studies on the distribution pattern of constitutive heterochromatins (C+ bands) agreed with what is generally observed among bats; that is, the presence of blocks of C+ bands in the pericentromeric regions of all chromosomes and in the terminal regions of the subtelocentric chromosome pairs (Souza and Araújo 1990; Santos and Souza 1998b; Rodrigues et al. 2003; Silva et al. 2005; Garcia and Pessôa 2010; Lemos-Pinto et al. 2012; Calixto et al. 2014).

For the family Phyllostomidae, the C+ bands are located in the pericentromeric regions of the chromosomes (Varella-Garcia et al. 1989; Rodrigues et al. 2000; Barros et al. 2009; Sbragia et al. 2010); however, additional conspicuous heterochromatic blocks have been found in interstitial and telomeric regions in several species, such as

Carollia perspicillata (Linnaeus, 1758), *Choeroniscus minor* (Peters, 1868), *Glossophaga soricina* (Pallas, 1766), *Artibeus lituratus* (Olfers, 1818), *A. planirostris* (Spix, 1823), *Dermanura cinerea* (Gervais, 1856), *Sturnira lilium* (É. Geoffroy, 1810), *Platyrrhinus lineatus* (É. Geoffroy, 1810), *Uroderma magnirostrum* Davis, 1968, *U. bilobatum* Peters, 1866, *Diaemus youngi* (Jentnik, 1893), *Desmodus rotundus* (É. Geoffroy, 1810) and *Diphylla ecaudata* (Spix, 1823) (Varella-Garcia et al. 1989; Souza and Araújo 1990; Santos and Souza 1998a, b; Neves et al. 2001; Santos et al. 2001; Silva et al. 2005). The constitutive heterochromatin observed in the chromosomes of *D. gnoma* and *D. anderseni* follows a distribution pattern similar to that found in bats of the Phyllostomidae family.

Studies conducted with chromosomal mapping of different bat families have revealed that this group is characterized by karyotypic conservation, caused by slow chromosomal evolution (Baker et al. 1980; Varella-Garcia et al. 1989; Souza and Araújo 1990; Morielle-Versute et al. 1996; Pieczarka et al. 2005). However, in a general analysis of the chromosomal data available for the different species already studied, it seems that this conservatism is not a rule. In Phyllostomidae, several subfamilies seem to have relatively divergent karyotypes, at least in their microstructure. In addition, there is a diversity of karyotypic formulas that are quite interesting when comparing different subfamily groups, which supports karyotype studies as an important study area (Pieczarka et al. 2013; Ribas et al. 2015; Sotero-Caio et al. 2015, 2017a).

Overall, the data presented herein expands the cytogenetic knowledge of bats in the Stenodermatinae subfamily and is the first work to analyze the karyotypes of *Dermanura gnoma* and *D. anderseni*. The comparative analysis of the species of this subfamily reveals high conservation for this group and reinforces its position as a well-established phylogenetic unit within the order Chiroptera.

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Cloning and physical localization of male-biased repetitive DNA sequences in *Spinacia oleracea* (Amaranthaceae)

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Abstract

Spinach (*Spinacia oleracea* Linnaeus, 1753) is an ideal material for studying molecular mechanisms of early-stage sex chromosome evolution in dioecious plants. Degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) technique facilitates the retrotransposon-relevant studies by enriching specific repetitive DNA sequences from a micro-dissected single chromosome. We conducted genomic subtractive hybridization to screen sex-biased DNA sequences by using the DOP-PCR amplification products of micro-dissected spinach Y chromosome. The screening yielded 55 male-biased DNA sequences with 30 576 bp in length, of which, 32 DNA sequences (12 049 bp) contained repeat DNA sequences, including *LTR/Copia*, *LTR/Gypsy*, simple repeats, and DNA/CMC-EnSpm. Among these repetitive DNA sequences, four DNA sequences that contained a fragment of *Ty3-gypsy* retrotransposons (SP73, SP75, SP76, and SP77) were selected as fluorescence probes to hybridization on male and female spinach karyotypes. Fluorescence *in situ* hybridization (FISH) signals of SP73 and SP75 were captured mostly on the centromeres and their surrounding area for each homolog. Hybridization signals primarily appeared near the putative centromeres for each homologous chromosome pair by using SP76 and SP77 probes for FISH, and sporadic signals existed on the long arms. Results can be served as a basis to study the function of repetitive DNA sequences in sex chromosome evolution in spinach.

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Keywords
FISH, Genomic subtraction hybridization, Retrotransposon, Sex chromosome evolution, Spinach

Introduction

Sex chromosomes evolved from autosomes by stages; the key event in the evolution of sex chromosomes includes the emergence of sex-determining genes, recombination suppression, accumulation of repetitive sequences, degeneration of Y chromosome, and dosage compensation effect of X chromosome (Abbott et al. 2017). Sex chromosomes in mammals are mostly ancient, but sex chromosomes in plants, insects and some fishes have recently evolved. In all evolutionary steps, recombinant suppression is a key step in the evolution of sex chromosomes. The ceasing of recombination avoids the occurrence of progeny sterilization or hermaphroditism among progenies. In early-stage sex chromosome evolution, the recombination of male-specific regions on the Y chromosome is reduced or restricted (Akagi et al. 2014). The size of the recombination suppression region and differences of size between X/Y or Z/W chromosomes generally reflect the stage of sex chromosome evolution. Homomorphic chromosomes are generally considered to be relatively young sex chromosomes, and their non-recombination regions are commonly very small. For example, asparagus (*Asparagus officinalis* Linnaeus, 1753) has a very young pair of sex chromosomes and very small male-specific regions (Harkess et al. 2017). Papaya (*Carica papaya* Linnaeus, 1753) also has homomorphic sex chromosomes with larger sex-specific regions than asparagus. Analysis of high-density linkage map of papaya revealed that 225 out of the 347 markers co-segregated with sex phenotype. This finding revealed the severe recombination suppression around the sex-determining site (Ma et al. 2004). Spinach (*S. oleracea* L., 2n = 12) is a diploid dioecious leafy vegetable with a pair of homomorphic sex chromosomes (Arumuganathan et al. 1991; Xu et al. 2017). In spinach, a Y-chromosomal region around the male-determining locus does not recombine with the counterpart region on the X chromosome (Takahata et al. 2016; Kudoh et al. 2018).

Repetitive DNA sequences, primarily transposons, retrotransposons (RTs), and tandem repeats (satellite DNA, small satellite DNA, and microsatellite DNA sequences), make up the majority of all the nuclear DNA in most eukaryotic genomes (Biscotti et al. 2015). These sequences used to be called garbage sequences, but their substantial roles in a variety of biological processes, including gene expression, transcriptional regulation, chromosome structure construction, have been recently discovered. Their functions and evolution are popular research topics (Mehrotra et al. 2014; Cioffi et al. 2015). Accumulation of repetitive sequences is among the most common features of the sex chromosomes of dioecious species. A great portion of repetitive sequences has been identified from the sex chromosomes of humans (Erlandsson et al. 2000), mammals (Dechaud et al. 2019), fish (Faber-Hammond et al. 2015), birds (Zhou et al. 2014), and insects (Bachtrog et al. 2003). In flowering plants, highly repetitive regions were distributed on the sex chromosomes of dioecious plants with heteromorphic sex chromosomes, such

as *Silene latifolia* Poire, 1789 and *Rumex acetosa* (Linnaeus, 1753) (Hobza et al. 2017), similar to some dioecious plants with younger sex chromosomes. Papaya is a model species for studying early-stage sex chromosomes (Ming et al. 2008). The Y chromosome contains a small male-specific (MSY, 8.1 Mb, only 13% of the entire Y chromosome) (Vanburen et al. 2015). Studies have shown the enriched repetitive DNA sequences in this region (Wang et al. 2012). Further studies showed that repetitive DNA sequences accounted for approximately 77% of hermaphrodite-specific region in the Y chromosome (HSY), 79.2% of MSY, and 67.2% on the X-counterpart, the values of which were significantly higher than the ratio of repeat sequences in the entire genome (51%) (Yu et al. 2007; Na et al. 2014). The types of transposons in the HSY region, including *Ty1-Copia* and *Ty3-Gypsy*, are primarily RTs. The expansion of the sex-determining region was proposed to be related to the accumulation of *Ty3-gypsy* RTs (Na et al. 2014). BAC sequencing revealed that the Y-chromosome region around the male-determining locus in spinach contains a large amount of repetitive elements, most of which are novel *Ty1-copia-like* and its derivative elements (Kudoh et al. 2018). However, the BAC clone sequences account for only a part of the sex-linked non-recombining region. Further experiments are needed to determine the size of the sex-linked region in spinach.

Genomic subtraction is used for isolating DNA that is absent in deletion mutants. The method removes the sequences present in the wild-type (tester DNA) and the deletion mutant genomes (driver DNA) from wild-type DNA featured by simple, rapid, sensitive, and economic means. This technique is widely applied in the separation and identification of gene rearrangement and in the preparation of polymorphism loci probe (Straus et al. 1990; Hou et al. 1995; Asalone et al. 2019). However, whether the technique can be used to screen sex chromosome-specific DNA sequences has not been reported.

In this study, the X and Y chromosome of spinach were successfully isolated and amplified by degenerated-oligonucleotide-primed polymerase chain reaction (DOP-PCR) (Deng et al. 2013). Single chromosome DOP-PCR amplified products tend to enrich chromosome-specific DNA repeat sequences (Zhou et al. 2007). Then, using X chromosome DOP-PCR amplified products as the driver DNA and Y chromosome DOP-PCR amplified products as the tester DNA, the enriched repetitive DNA sequences on the Y chromosome were obtained by genomic subtraction hybridization. Our study provided a new approach for exploring enriched DNA repetitive sequences from spinach Y chromosome.

Material and methods

Plant materials

The seeds of spinach (*S. oleracea* Linnaeus, 1753, cv. Japan) were planted in the garden field of Henan Normal University under natural conditions. Genomic DNA from each male and female spinach was extracted from young leaves using the traditional cetyltrimethylammonium bromide method (Rogers et al. 1989).

Microdissection of X and Y chromosome in spinach

The X/Y chromosome is the largest submetacentric chromosome (Ellis et al. 1960; Deng et al. 2013). The microdissection of the largest chromosome in spinach was carried out according to the procedures described by Deng et al. (2013). Initially, the largest chromosome in spinach was identified based on its size and was microdissected using a glass needle that was fixed to the arm of a Leitz micro-operation instrument on an inverted phase-contrast microscope (Olympus 1 M, Japan). Ten chromosomes were isolated. The microdissected chromosome was collected into an Eppendorf tube (0.2 mL) and separately digested with proteinase K buffer at 0.5 mg/mL (Roche, Indianapolis) in 1× *Taq* polymerase buffer (Promega, Madison). The isolated chromosomal DNA was incubated in a proteinase K solution at 37 °C for 2 h. Proteinase K was then inactivated at 90 °C for 10 min. Then, the chromosome DNA was amplified by DOP-PCR in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA). To obtain high-concentration DNA products for genomic subtraction library construction, the DOP-PCR products were amplified from X and Y chromosome by two rounds of recursive enrichment based on previous studies (primer sequence: CCGACTCGAGNNNNNNATGTGG) (Deng et al. 2013).

Construction of Y chromosome genomic subtraction library

The modified DOP-PCR primer that contains *Bam*H I digestion site (modified primer sequence: CGGAGGATCCNNNNNNATGTGG), was used to amplify the products from the second round spinach Y chromosome DOP-PCR amplification. DOP-PCR amplification was performed in 50 μL reaction volume containing 1 × PCR buffer, 1.5 mmol/L dNTP Mixture (Transgene, Beijing, China), 2.5 U *Taq* polymerase (Takara, Bejing, China), 100 ng template DNA, and 0.2 μM primer. The amplification was performed by initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing temperature 55 °C for 90 s, extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min. Then, the concentration of the amplified DOP-PCR products was quantified, followed by enzyme digestion in 25.0 μL volume containing 2 × K buffer and 20 000 U *Bam*H I (Takara, Beijing, China) at 37 °C for 3 h in a metal bath for further quantification and quality control.

As described from Y chromosome amplification, the modified DOP-PCR primer was used to amplify the second round DOP-PCR amplification products of spinach X chromosome along with the quality control and quantification of the products. Finally, the amplified products from both X and Y chromosomes were purified using Takara MiniBEST DNA Fragment Purification Kit Ver.4.0 (Takara, Beijing, China).

The DOP-PCR amplified products from the libraries of X (Driver DNA) and Y (Tester DNA) chromosomes were mixed in a 100:1 ratio for subsequent hybridizations. The mixed DOP-PCR amplified products were treated by water bath at 99 °C for 10 min, mixed with 4 mL of PERT (8% phenol, 1.25 M sodium perchlorate, and 0.12 M disodium hydrogen phosphate dissolved in 1000 mL of distilled water) for

72 h, annealed at 25 °C and at 100 rpm on the shaking table for 72 h (shaking for 8 h and stopping for 8 h), and then placed on the shaking table overnight. After 72 h of annealing, the hybridization solution was purified by suction filtration with a syringe and an organic filter. It was extracted twice with chloroform:isoamyl alcohol at 24:1, centrifuged at 12 000 rpm for 5 min, precipitated by 1% volume of sodium acetate and 2.5 volumes of absolute ethanol at -20 °C overnight, centrifuged at 10 000 rpm for 10 min, washed twice with 70% absolute ethanol, dissolved in 800 μL sterile ddH_2O, and transferred into a 1.5 mL centrifuge tube. Then, the mass of the hybridization solution was quantified by microspectrophotometer for further steps.

Enzyme digestion, purification, dephosphorylation, and re-purification of the vector were conducted.

A mixture of 1.0 μL of PUC119, 2.0 μL of 10 × K buffer, 2.0 μL of *BamH* I, and 15 μL ddH_2O were quantified into 20.0 μL for 3 h digestion in a 30 °C water bath. Then, purification was performed according to Takara MiniBEST DNA Fragment Purification Kit Ver. 4.0 (Takara, Beijing, China). The reaction mixture was placed in a 0.2 mL centrifuge tube containing 40.0 μL (1–20 pmol) vector DNA, 5.0 μL 10 × K alkaline phosphatase buffer, and 1.0 μL CIAP, and adjusted to 50 μL. The reaction was conducted in a metal bath at 37 °C for 15 min, and then at 50 °C for 15 min for dephosphorylation, purification, and mass quantification.

Cloning of DNA subtraction library

Gradient design was carried out according to the ratio between the hybrid liquid and the vector, after which the optimized reaction mixture was placed in the microcentrifuge tube containing 1.5 μL PUC119 (Takara Code: 3319), 0.1 μL T4 DNA ligase (Takara Code: 2011A), and 2.0 μL 10 × buffer at contents up to 20 μL. The reaction was conducted in the metal bath at 16 °C for 5 h. The ligation products were transformed into competent cells, screened according to blue and white spots, and amplified by colony PCR using universal primer M13 (CGCCAGGGTTTTCCCAGTCACGAC).

Screening and identification of DNA subtractive library

The selected recombinant plasmids were identified using the spinach female and male genomic DNAs as probes labeled with DIG (Roche: 11277065910) by dot hybridization method. Basically, the subtractive DNA libraries with male-specific DNA sequences were hybridized and formed colonies on films. These colonies were selected for further Sanger sequencing.

Screening of repetitive DNA sequences

On the basis of the results of dot-blot hybridization, the male-hybridized colonies (PCR-amplified products derived from bacterial solution with more than 250 bp) were selected for Sanger sequencing at Shanghai Invitrogen Biotechnology Co., Ltd. The

sequencing results were analyzed by BLASTn and RepeatMasker (http://www.repeatmasker.org/). Initially, sequencing products were blasted against the spinach reference genome (http://www.spinachbase.org/cgi-bin/spinach/index.cgi) with a cutoff of 90% similarity and E-value 1e-10 to prevent the contamination of the DNA from other organisms. Sequences with no hits were deleted. Then, the DNA sequences were aligned to RepeatMask libraries to classify the type of repeats. Ultimately, the DNA sequences were annotated using BLASTn against the NCBI nucleotide database. Based on the sequencing results, primers for each group of repetitive DNA sequences were designed by Oligo7 for PCR amplification (Suppl. material 1: Table S1). Amplification of those repetitive DNA was performed in a 20 μL reaction setting, which included 1 × PCR buffer, 0.75 mM dNTP Mixture, 1 U *Taq* polymerase (Takara, Bejing, China), 100 ng template DNA, and 0.1 μM primer. The reaction was carried out using the following cycle conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing temperature 55 °C for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products with a single amplified band were purified for fluorescent probe labeling.

Chromosome localization of repeat DNA sequences

The spinach seeds were initially soaked in a moisturized and low-temperature (4 °C) environment overnight. Then, the seeds were placed in a constant temperature incubator at 25 °C in the dark. The seeds with approximate 1 cm root length were placed in a 1.5 mL centrifugal tube for nitrous oxide pretreatment. Subsequently, the roots were fixed in 90% glacial acetic acid for 10 min and finally stored in the refrigerator at -20 °C in 70% ethanol. Each selected tissue was rinsed by distilled water for 10 min, after which it underwent dissection and digestion using a solution containing 1% pectolyase Y23 (Yakult Pharmaceutical, Tokyo) and 2% cellulose Onozuka R-10 (Yakult Pharmaceutical, Tokyo) for 1.5 h at 37 °C (one section per tube with 20 μL of the enzyme solution). The abovementioned treated root sections were carefully split into individual cells by using needles and by intensive vortexing at room temperature along with soaking in 100% ethanol. Furthermore, the cells were collected from the bottom of the tube by centrifugation and re-suspended in an acetic acid ethanol solution (9:1 dilution). Finally, the cell suspension was dropped onto glass slides in a box lined with wet paper towels for observation.

Then, 45S rDNA (the probe was donated by Fangpu Han, a researcher from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) was labeled using Alexa fluor-488-dUTP (green), and the male-specific bands were labeled using Texas-red-dCTP (red) with the nick translation method based on previous protocols (Birchler et al. 2008). The labeled probes were placed in a refrigerator with light prevention at -20 °C.

Fluorescence *in situ* hybridization (FISH) between spinach chromosomes at metaphase and DNA probes derived from each repetitive sequence was performed according to the method described in previous studies (Gao et al. 2011). Selected chromosome

plates during the metaphase-stage for hybridization were placed into the ultraviolet crosslinker for 2 min with 0.125 J. A probe solution containing in 2 × SSC and 1 × TE was added to the slides. After denaturation in boiling water for 5 min, the slides with probe were incubated at 55 °C in a humid chamber for 8–12 h. After hybridization, the slides were washed in 2 × SSC and mounted on Vectashield mounting medium containing 1.5 μg/ml 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, USA). The FISH images were captured with an ANDOR CCD under an Olympus BX63 fluorescence microscope. The images were processed by Adobe Photoshop 7.0.

Results

Preparation of Tester DNA and Driver DNA

The amplified products from the X/Y chromosome were identified using a male-specific marker T11A (Onodera et al. 2011). Two X chromosomes and three Y chromosomes were successfully microdissected and amplified (Suppl. material 1: Fig. S1). The major Y chromosome DNA products amplified from DOP-PCR ranged from 200 bp to 1500 bp, and the DNA products of around 500 bp were mostly enriched (Fig. 1A). The majority of the X-chromosome DNA products amplified from DOP-PCR were enriched from 200 bp to 1500 bp. DNA products exhibited an even distribution among different sizes (Fig. 1B).

Cloning and screening DNA genomic subtractive library

From the subtractive library, 2700 single colonies were obtained, among which 480 single colonies with lengths between 250 and 1500 bp were randomly selected for PCR amplification (Suppl. material 1: Fig. S2). To identify Y-specific DNA, the hybridization of spinach female/male genomic DNA with DNA sequences from subtractive library was performed. Unique hybridization between male spinach and DNA could be potentially from Y chromosome. Fifty-five Y chromosome-specific recombinant plasmids in spinach were identified by dot blot hybridization (Suppl. material 1: Fig. S3).

Screening male-biased repetitive DNA sequences

Sanger sequencing of the 55 selected DNA sequences yielded a total of 30, 576 bp of product, ranging from 248 bp to 1, 354 bp in length (MN830920–MN830942, MN810356–MN810387). A total of 12, 049 bp of DNA products were identified as repeat sequences using RepeatMasker softerware (http://www.repeatmasker.org/), which accounted for 39.4% of the total sequences. Thirty-two of the 55 DNA sequences contained repeat DNA sequences, including *LTR/Copia*, *LTR/Gypsy*, simple repeats and DNA/CMC-EnSpm (Suppl. material 1: Table S2). The sequence alignment of these 55 DNA sequences against the NCBI database (nr database) through

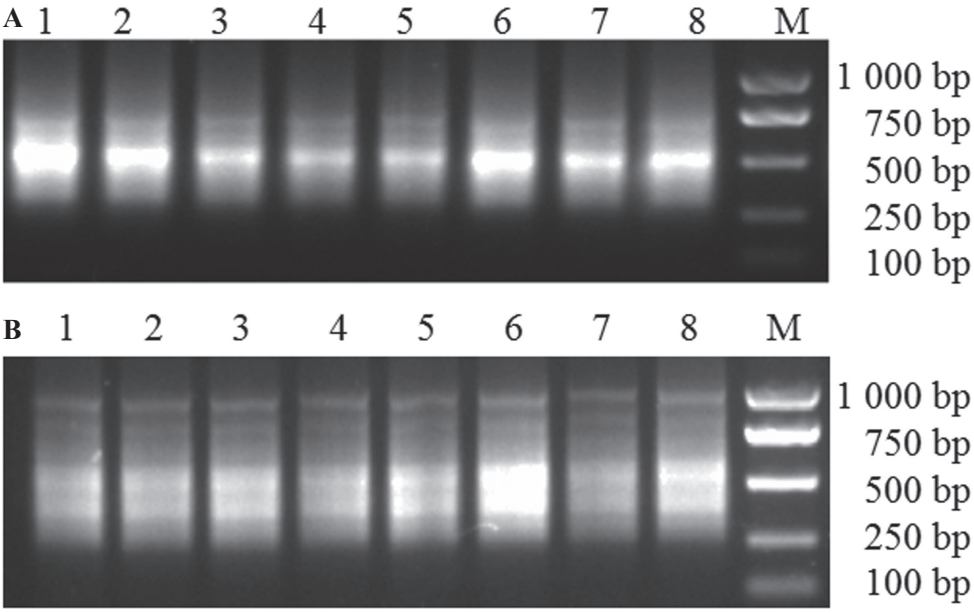

Figure 1. Gel electrophoresis results of spinach Y (**A**) /X (**B**) chromosome DOP-PCR products using modified primer Lane M: Trans 2K plus DNA Marker; Lane 1–8(**A**): spinach Y chromosome second round DOP-PCR products as template using modified primer; Lane 1–8(**B**): spinach X chromosome second round DOP-PCR products as template using modified primer

BLASTn with default settings generated 55 significant hits. Those hits included spinach BAC clones, uncharacterized mRNA, and the transcription factor MYB80 (LOC110782202) of spinach from previous annotations and BAC libraries (*S. oleracea* L.) (Table 1).

Chromosome localization of repetitive DNA sequences

Using the 32 DNA sequences that contained repeat DNA sequences as probes, we tried to identify the distribution of fluorescence signals on the Y chromosome. However, no fluorescence signals were found on the chromosomes using four simple repeats (SP5-1, SP55-1, SP55-3 and SP55-4) and two DNA/CMC-EnSpm DNA sequences (SP55-3 and SP55-4). When four *LTR/Copia* DNA sequences (SP3-4, SP3-8, SP17-1 and SP1-86) were selected to be used as probes, the signals showed a dispersed distribution in all chromosomes (Suppl. material 1: Figure S4). Four DNA sequences (SP73, SP75, SP76, and SP77) containing *Ty3-gypsy* family RTs were selected as probes for FISH (Suppl. material 1: Table S2).

Four pairs of primers were generated according to the DNA sequences SP73, SP75, SP76, and SP77 (Suppl. material 1: Table S1). PCR and gel electrophoresis assays generated one band for each DNA sample ranging from 1, 000 bp to 2, 000 bp (Suppl. material 1: Fig. S5). Interestingly, one 1318 bp sequence of SP73 shared high

Table 1. BLAST search for spinach male-biased DNA fragments.

ID	Accession number	Size	Description	Query Cover	E Value	Per. Ident
SP1-3	MN830920	661	PREDICTED: *Spinacia oleracea* uncharacterized LOC110790287 (LOC110790287), transcript variant X4, ncRNA	96%	0	94.25%
SP1-71	MN830921	326	Select seq AP017640.1 *Spinacia oleracea* DNA, BAC clone: 009-126-13E-1, strain: 03-009, complete sequence	96%	3.00E-162	100.00%
SP1-86	MN810356	293	Select seq AP017641.1 *Spinacia oleracea* DNA, BAC clone: 009-160-1L-1, strain: 03-009, complete sequence	93%	2.00E-103	92.03%
SP1-89	MN810357	294	Select seq AP017637.1 *Spinacia oleracea* DNA, BAC clone: 009-26-14K-1, strain: 03-009, complete sequence	93%	2.00E-137	99.28%
SP2-26	MN810358	248	PREDICTED: *Spinacia oleracea* uncharacterized LOC110800978 (LOC110800978), mRNA	94%	1.00E-109	97.87%
SP3-4	MN810359	270	Select seq XM_021992625.1 PREDICTED: *Spinacia oleracea* uncharacterized LOC110787992 (LOC110787992), mRNA	98%	1.00E-79	87.41%
SP3-8	MN810360	261	*Spinacia oleracea* mitochondrion, complete genome	96%	3.00E-116	97.23%
SP3-36	MN830922	334	PREDICTED: *Spinacia oleracea* uncharacterized LOC110799754 (LOC110799754), mRNA	96%	1.00E-160	99.07%
SP3-88	MN830923	293	*Spinacia oleracea* DNA, BAC clone: 009-26-14K-1, strain: 03-009, complete sequence	68%	3.00E-67	91.04%
SP4-1	MN810361	433	Select seq XM_022007996.1 PREDICTED: *Spinacia oleracea* uncharacterized LOC110802566 (LOC110802566), mRNA	99%	6.00E-168	91.88%
SP4-2	MN810362	432	PREDICTED: *Spinacia oleracea* uncharacterized LOC110791229 (LOC110791229), mRNA	99%	0	95.82%
SP4-3	MN810363	423	PREDICTED: *Spinacia oleracea* uncharacterized LOC110802566 (LOC110802566), mRNA	96%	5.00E-179	94.51%
SP4-4	MN810364	426	Select seq XM_022001236.1 PREDICTED: *Spinacia oleracea* uncharacterized LOC110796204 (LOC110796204), mRNA	98%	0	94.41%
SP4-7	MN810365	432	PREDICTED: *Spinacia oleracea* uncharacterized LOC110802566 (LOC110802566), mRNA	98%	0	96.49%
SP4-8	MN810366	433	PREDICTED: *Spinacia oleracea* uncharacterized LOC110802566 (LOC110802566), mRNA	98%	0	96.73%
SP4-10	MN810367	432	Select seq XM_022007996.1 PREDICTED: *Spinacia oleracea* uncharacterized LOC110802566 (LOC110802566), mRNA	98%	0	96.02%
SP4-11	MN810368	423	PREDICTED: *Spinacia oleracea* uncharacterized LOC110802566 (LOC110802566), mRNA	98%	8.00E-177	93.68%
SP4-38	MN810369	293	*Spinacia oleracea* DNA, BAC clone: 009-123-11N-1, strain: 03-009, complete sequence	97%	1.00E-135	97.90%
SP4-48	MN830924	575	Select seq AP017641.1 *Spinacia oleracea* DNA, BAC clone: 009-160-1L-1, strain: 03-009, complete sequence	36%	2.00E-61	88.78%
SP4-53	MN830925	262	PREDICTED: *Spinacia oleracea* probable methyltransferase PMT15 (LOC110782476), mRNA	31%	2.00E-29	97.59%
SP5-1	MN810370	410	Select seq AP017639.1 *Spinacia oleracea* DNA, BAC clone: 009-123-11N-1, strain: 03-009, complete sequence	49%	2.00E-87	96.53%
SP5-2	MN830926	365	Select seq AP017641.1 *Spinacia oleracea* DNA, BAC clone: 009-160-1L-1, strain: 03-009, complete sequence	56%	1.00E-94	97.61%
SP5-3	MN830927	365	*Spinacia oleracea* DNA, BAC clone: 009-160-1L-1, strain: 03-009, complete sequence	57%	2.00E-93	97.14%
SP5-4	MN830928	356	Select seq AP017639.1 *Spinacia oleracea* DNA, BAC clone: 009-123-11N-1, strain: 03-009, complete sequence	56%	4.00E-55	87.13%
SP5-5	MN830929	534	*Spinacia oleracea* DNA, BAC clone: 009-126-13E-1, strain: 03-009, complete sequence	37%	7.00E-88	97.46%
SP5-9	MN830930	430	Select seq XM_022005381.1 PREDICTED: *Spinacia oleracea* uncharacterized LOC110800092 (LOC110800092), mRNA	51%	9.00E-92	95.05%
SP5-10	MN810371	409	*Spinacia oleracea* DNA, BAC clone: 009-123-11N-1, strain: 03-009, complete sequence	50%	3.00E-86	95.63%
SP5-11	MN810372	485	*Spinacia oleracea* DNA, BAC clone: 009-126-13E-1, strain: 03-009, complete sequence	42%	4.00E-90	97.97%
SP5-12	MN830931	326	Select seq AP017640.1 *Spinacia oleracea* DNA, BAC clone: 009-126-13E-1, strain: 03-009, complete sequence	96%	1.00E-161	100.00%

ID	Accession number	Size	Description	Query Cover	E Value	Per. Ident
SP5-48	MN830932	277	Select seq AP017638.1 *Spinacia oleracea* DNA, BAC clone: 009-41-10L-1, strain: 03-009, complete sequence	94%	1.00E-75	87.17%
SP6-20	MN810373	573	Chain A, Cryo-EM structure of the spinach chloroplast ribosome reveals the location of plastid-specific ribosomal proteins and extensions	92%	6.00E-171	87.52%
SP7-3	MN810374	549	Select seq XM_021994667.1 PREDICTED: *Spinacia oleracea* uncharacterized LOC110789945 (LOC110789945), mRNA	79%	6.00E-133	86.73%
SP7-4	MN830933	504	PREDICTED: *Spinacia oleracea* transcription factor MYB80 (LOC110782202), mRNA	95%	0	98.96%
SP7-5	MN830934	587	*Spinacia oleracea* mitochondrion, complete genome	94%	0	98.74%
SP7-7	MN810375	536	PREDICTED: *Spinacia oleracea* uncharacterized LOC110777888 (LOC110777888), mRNA	53%	3.00E-116	93.73%
SP7-9	MN830935	504	Select seq XM_021986329.1 PREDICTED: *Spinacia oleracea* transcription factor MYB80 (LOC110782202), mRNA	93%	0	98.94%
SP7-10	MN810376	536	PREDICTED: *Spinacia oleracea* uncharacterized LOC110783205 (LOC110783205), mRNA	53%	9.00E-107	91.64%
SP7-11	MN830936	563	Select seq XM_021982478.1 PREDICTED: *Spinacia oleracea* pentatricopeptide repeat-containing protein At5g02860 (LOC110777897), mRNA	94%	0	99.06%
SP10-9	MN810377	790	Select seq XM_022003128.1 PREDICTED: *Spinacia oleracea* uncharacterized LOC110797998 (LOC110797998), mRNA	98%	0	84.22%
SP13-1	MN810378	267	Select seq XM_021992625.1 PREDICTED: *Spinacia oleracea* uncharacterized LOC110787992 (LOC110787992), mRNA	97%	2.00E-83	88.97%
SP13-2	MN810379	267	PREDICTED: *Spinacia oleracea* uncharacterized LOC110787992 (LOC110787992), mRNA	97%	2.00E-73	86.69%
SP17-1	MN810380	715	PREDICTED: *Spinacia oleracea* uncharacterized LOC110799950 (LOC110799950), mRNA	91%	0	91.10%
SP17-2	MN830937	1128	Select seq AP017639.1 *Spinacia oleracea* DNA, BAC clone: 009-123-11N-1, strain: 03-009, complete sequence	98%	0	93.99%
SP51-1	MN830938	758	PREDICTED: *Spinacia oleracea* tudor domain-containing protein 3 (LOC110774971), transcript variant X2, mRNA	51%	6.00E-178	99.71%
SP51-2	MN830939	766	PREDICTED: *Spinacia oleracea* tudor domain-containing protein 3 (LOC110774971), transcript variant X2, mRNA	45%	1.00E-179	100.00%
SP51-3	MN830940	758	PREDICTED: *Spinacia oleracea* tudor domain-containing protein 3 (LOC110774971), transcript variant X2, mRNA	51%	1.00E-179	100.00%
SP52-1	MN830941	644	Select seq AP017637.1 *Spinacia oleracea* DNA, BAC clone: 009-26-14K-1, strain: 03-009, complete sequence	42%	5.00E-79	87.73%
SP52-3	MN830942	638	*Spinacia oleracea* DNA, BAC clone: 009-26-14K-1, strain: 03-009, complete sequence	42%	5.00E-69	85.87%
SP55-1	MN810381	1001	Select seq AP017640.1 *Spinacia oleracea* DNA, BAC clone: 009-126-13E-1, strain: 03-009, complete sequence	93%	0	84.31%
SP55-3	MN810382	1001	PREDICTED: *Spinacia oleracea* uncharacterized LOC110802605 (LOC110802605), mRNA	50%	0	99.28%
SP55-4	MN810383	1001	PREDICTED: *Spinacia oleracea* uncharacterized LOC110802605 (LOC110802605), mRNA	53%	0	99.78%
SP73	MN810384	1318	Select seq AP017638.1 *Spinacia oleracea* DNA, BAC clone: 009-41-10L-1, strain: 03-009, complete sequence	100%	0	99.85%
SP75	MN810385	1354	*Spinacia oleracea* DNA, BAC clone: 009-41-10L-1, strain: 03-009, complete sequence	100%	0	99.93%
SP76	MN810386	1163	PREDICTED: *Spinacia oleracea* uncharacterized LOC110779482 (LOC110779482), partial mRNA	45%	3.00E-152	85.66%
sp77	MN810387	1154	PREDICTED: *Spinacia oleracea* uncharacterized LOC110779482 (LOC110779482), partial mRNA	45%	3.00E-147	85.23%

percentage identity with Y-specific BAC clone 009-41-10L-1 from previous studies (AP017638.1) (99%) (Suppl. material 1: Fig. S6). Another target band of a 1354 bp-SP75 DNA sequence (Suppl. material 1: Fig. S5) containing 907 bp Gypsy/DIRS1

RT sequence also presented a high-percentage homologous DNA with Y -specific BAC clone 009-41-10L-1 (AP017638.1) (99%) (Suppl. material 1: Fig. S7) and with a male-specific SCAR marker of spinach (FJ169475.1) (99%). SP76 was 1, 163 bp (Suppl. material 1: Fig. S5) and contained 939 bp Gypsy/DIRS1 RT DNA sequence. SP77 was 1, 154 bp (Suppl. material 1: Fig. S5) and contained 940 bp Gypsy/DIRS1 RT DNA sequence. PCR products with specific amplified bands were purified to construct fluorescent probes.

For chromosomal localization, 45S rDNA was used as a probe to distinguish each chromosome, the prominent fluorescent signals of which were observed on chromosomes 2, 5, and 6 (Deng et al. 2012). Initially, the homologous sex chromosome pair was identified by 45S rDNA probes based on the weak signals from X and Y chromosomes (Deng et al. 2012). This was the largest homologous pair among the six pairs of chromosomes. FISH performed using SP73 and SP75 as DNA probes detected an even distribution of fluorescence signal on each pair of homologous chromosomes. Those signals were concentrated near the putative centromere and pericentromeric regions. Stronger signals observed on the sex chromosomes compared to that on the A chromosomes. Nevertheless, no significant difference of fluorescence signals was found between X and Y chromosomes (Figs 2, 3). In addition, hybridization signals primarily appeared near the putative centromeres for each homologous chromosome pair by using SP76 and SP77 probes for FISH, and sporadic signals existed on the long arms. X and Y chromosomes were not distinguished based on signals (Figs 4, 5).

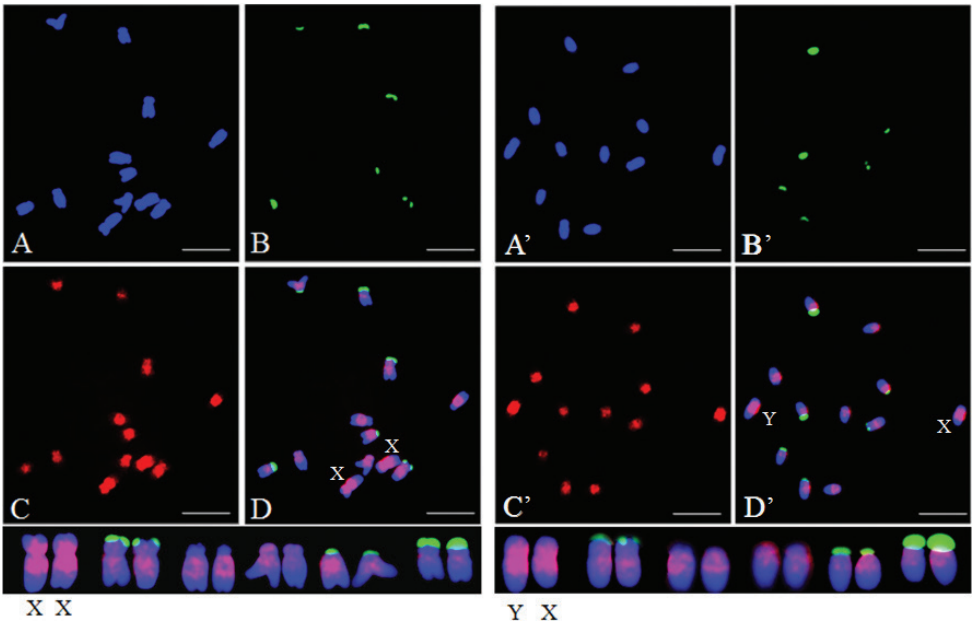

Figure 2. Distribution patterns of hybridization signals from female and male spinach using 45S rDNA (green) and SP73 (red) as probes **A** (**A'**), DAPI **B** (**B'**), 45S rDNA (green) as probe **C** (**C'**), SP73 (red) as probe **D** (**D'**), The merged figure of **A** (**A'**), **B** (**B'**) and **C** (**C'**). Scale bas: 10 μm.

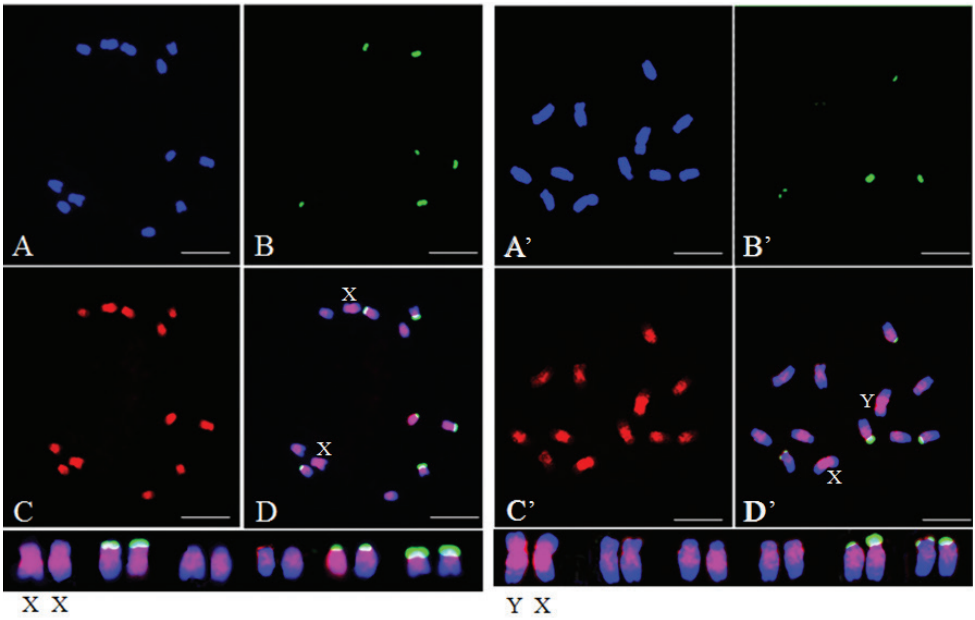

Figure 3. Distribution patterns of hybridization signals from female and male spinach using 45S rDNA (green) and SP75 (red) as probes **A** (**A'**), DAPI **B** (**B'**), 45S rDNA (green) as probe **C** (**C'**), SP75 (red) as probe **D** (**D'**), The merged figure of **A** (**A'**), **B** (**B'**) and **C** (**C'**). Scale bars: 10 μm.

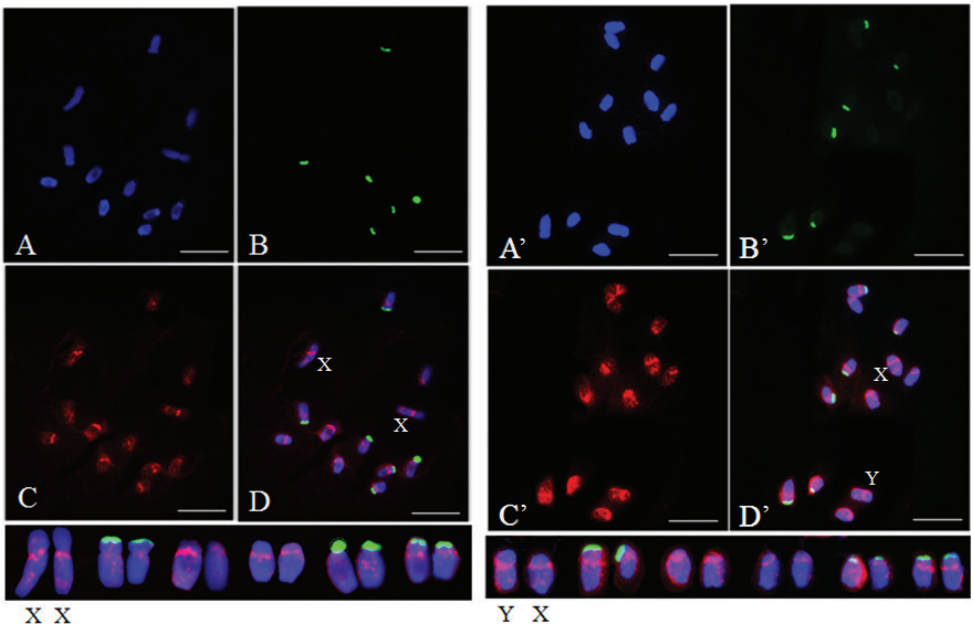

Figure 4. Distribution patterns of hybridization signals from female and male spinach using 45S rDNA (green) and SP76 (red) as probes **A** (**A'**), DAPI **B** (**B'**), 45S rDNA (green) as probe **C** (**C'**), SP76 (red) as probe **D** (**D'**), The merged figure of **A** (**A'**), **B** (**B'**) and **C** (**C'**). Scale bars: 10 μm.

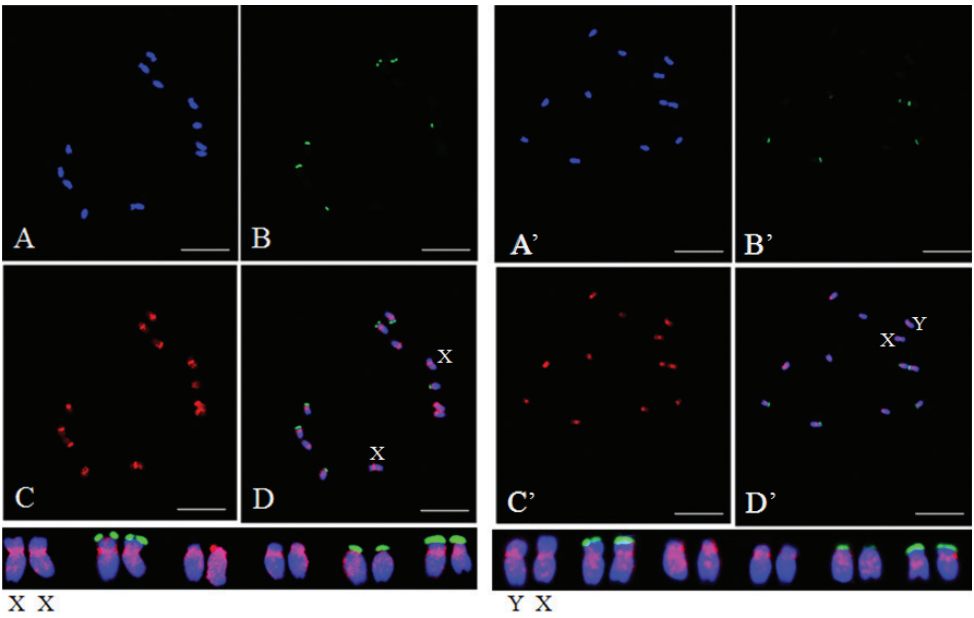

Figure 5. Distribution patterns of hybridization signals from female and male spinach using 45S rDNA (green) and SP77 (red) as probes **A** (**A'**) DAPI **B** (**B'**) 45S rDNA (green) as probe **C** (**C'**) SP77 (red) as probe **D** (**D'**). The merged figure of **A** (**A'**), **B** (**B'**) and **C** (**C'**). Scale bars: 10 μm.

Discussion

The establishment of single chromosome subtractive hybridization technique in spinach

Chromosome microdissection technology has the advantage of being able to isolate specific DNA products from a single chromosome. Moreover, the isolated products of the target sequences can be enriched through PCR (Zhou et al. 2007). In this study, we combined the conventional genomic subtraction hybridization with single chromosome microdissection to rapidly clone male-biased DNA sequences from spinach sex chromosomes. Twenty-one of 55 cloned DNA sequences were partially overlapped to BAC clone 009-126-13E-1, BAC clone 009-160-1L-1, BAC clone 009-26-14K-1, BAC clone 009-123-11N-1, and BAC clone 009-41-10L-1 located on the male-determining region of the spinach Y chromosome (Kudoh et al. 2018). Specifically, repetitive DNA sequence SP73 and SP75 shared more than 1000 bp highly-homologous sequences compared with the reported BAC clone 009-41-10L-1. The technique proposed above can be used to screen sex-biased DNA fragments of X and Y chromosomes in spinach.

FISH localization of male-biased repetitive DNA sequences in spinach

Sex reversal from hermaphroditism to dioecy in flowering plants requires two mutants, namely, one male-sterile mutant (generally for the first time) and one female-sterile

mutant. These mutant sites are used to stabilize sex on a pair of chromosomes (Charlesworth and Charlesworth 1978, Charlesworth 2013). Subsequently, suppression recombination occurs and plays a substantial role in maintaining dioecy by preventing the segregation of hermaphrodite or sterile progenies through crossing over. Repetitive sequence insertion plays a major role in ceasing recombination and leads to the formation of sex determination region and divergence of homologous sex chromosomes. A large number of transposon DNA sequences were enriched in the sex-determining region of asparagus (Jamsari et al. 2004). In *S. latifolia*, the Y chromosome is the largest and has accumulated a large number of repeated DNA sequences. Further analysis showed that transposons are inserted into the Y chromosome more frequently than into the other parts of the genome. FISH results showed that *Gypsy* RTs on Y chromosome of *S. latifolia* were 2.7 times more than those of *Copia* RTs (Cermak et al. 2008). Kudoh et al. (2018) reported that a higher amount of repetitive DNA sequences has accumulated near the Y linked region in spinach. However, very few direct cytological studies have explored the repetitive DNA sequences around the sex-determining locus. The BAC clone sequences are only a part of the sex-linked non-recombining region (Kudoh et al. 2018). Thus, other strategies should be used to explore the DNA sequences on a non-recombining region. On the basis of the DOP-PCR products of X and Y chromosomes from previous studies (Deng et al. 2013), genomic subtractive hybridization and dot-blot hybridization identified the specific repetitive DNA sequences on spinach sex chromosomes. In this study, the obtained Y chromosome-specific repetitive DNA sequences are mostly *Ty3-gypsy* family RTs. However, Kudoh et al. (2018) reported that most of the repeats found in the Y chromosome region around the male-determining locus are novel *Ty1-copia-like* RTs. The main reason for this could be the fact that the BAC clone sequences is only part of the sex-linked non-recombining region. Moreover, FISH localization identified the distribution pattern of repetitive DNA sequences on the sex chromosomes and autosome of spinach. The fluorescence signals of the DNA sequences containing *LTR/Copia* were distributed in a dispersed manner on all chromosomes (Suppl. material 1: Fig. S4). Although the localization of SP73, SP75, SP76 and SP77 did not differ between sex and autosomes, it did differ on the concentration of signals. It is evident that sex chromosomes have an accumulation of such repeats (SP73 and SP75). However, no significant difference was found in fluorescence signal intensity between X and Y chromosomes (Figs 2–5). The limited discrepancy of repetitive DNA detected between X and Y chromosome could be related to the early stages of sex chromosomes in spinach. Another reason for this result maybe is that dot blotting tends to produce false positives. In *Spinacia*, except for the cultivated spinach accession, *S. turkestanica* (Iljin, 1936) and *S. tetrandra* (Steven ex M. Bieb., 1808) are two extra species. Heteromorphic sex chromosomes were found in some accessions from *S. tetrandra* besides the common homomorphic sex chromosomes (Fujito et al. 2015). Thus, male-biased repetitive DNA sequences obtained in this study can be used as probes to explore their distribution in the homomorphic and heteromorphic sex chromosomes and to elucidate their possible role in the evolution of spinach sex chromosome.

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JZ, SW, and CD designed the experiments. JZ and SW conducted the study, processed the data and wrote the manuscript. LY, JZ, NL, SL, YZ, RQ, WG, and CD discussed the results and revised the manuscript. All authors have read and approved the final manuscript.

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Supplementary material 1

Figures S1–S6, Table S1

Authors: Jian Zhou, Shaojing Wang, Li'ang Yu, Ning Li, Shufen Li, Yulan Zhang, Ruiyun Qin, Wujun Gao, Chuanliang Deng

Data type: Figure/Table

Explanation note: **Figure S1.** Procedure of isolation of biggest chromosome in spinach by micromanipulator. **Figure S2.** Partial PCR products of recombinant clones using M13R and M13F as primers. **Figure S3.** 3 Dot blot hybridization results of partial subtractive hybridization clones. **Figure S4.** Distribution patterns of hybridization signals from female and male spinach using 45S rDNA (green) and SP1-86 (red) as probes. **Figure S5.** PCR amplification result of SP73, SP75, SP76 and SP77 marker. **Figure S6.** Pair-wise alignment between SP73 and BAC clone 009-41-10L-1. **Figure S7.** Pair-wise alignment between SP75 and BAC clone 009-41-10L-1. **Table S1.** Primer sequences for repetitive DNA sequences. **Table S2.** Summary of repetitive elements in 55 DNA Sequences.

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Comparative study of four *Mystus* species (Bagridae, Siluriformes) from Thailand: insights into their karyotypic diversity

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Abstract

Karyotypes of four catfishes of the genus *Mystus* Scopoli, 1777 (family Bagridae), *M. atrifasciatus* Fowler, 1937, *M. mysticetus* Roberts, 1992, *M. singaringan* (Bleeker, 1846) and *M. wolffii* (Bleeker, 1851), were analysed by conventional and Ag-NOR banding as well as fluorescence in situ hybridization (FISH) techniques. Microsatellite $d(GC)_{15}$, $d(CAA)_{10}$, $d(CAT)_{10}$ and $d(GAA)_{10}$ repeat probes were applied in FISH. The obtained data revealed that the four studied species have different chromosome complements. The diploid chromosome numbers (2n) and the fundamental numbers (NF) range between 52 and 102,

54 and 104, 56 and 98, or 58 and 108 in *M. mysticetus*, *M. atrifasciatus*, *M. singaringan* or *M. wolffii*, respectively. Karyotype formulae of *M. mysticetus*, *M. atrifasciatus*, *M. singaringan* and *M. wolffii* are 24m+26sm+4a, 26m+24sm+2a, 24m+18sm+14a and 30m+22sm+6a, respectively. A single pair of NORs was identified adjacent to the telomeres of the short arm of chromosome pairs 3 (metacentric) in *M. atrifasciatus*, 20 (submetacentric) in *M. mysticetus*, 15 (submetacentric) in *M. singaringan*, and 5 (metacentric) in *M. wolffii*. The $d(GC)_{15}$, $d(CAA)_{10}$, $d(CAT)_{10}$ and $d(GAA)_{10}$ repeats were abundantly distributed in species-specific patterns. Overall, we present a comparison of cytogenetic and molecular cytogenetic patterns of four species from genus *Mystus* providing insights into their karyotype diversity in the genus.

Keywords
Chromosomes, fluorescence in situ hybridization (FISH), karyotype, *Mystus*

Introduction

Bagridae are the largest family of Thai catfishes, with six genera (*Bagrichthys* Bleeker, 1857, *Batasio* Blyth, 1860, *Hemibagrus* Bleeker, 1862, *Mystus* Scopoli, 1777, *Pseudomystus* Jayaram, 1968, and *Sperata* Holly, 1939) and 28 species in Thailand. They play an important role in the national economic value of the country, as they are kept in aquaria and contribute heavily to the aquaculture industry. Most species of the genus *Mystus* are booming in aquaculture, with some of them being kept in aquaria (Vidthayanon 2005). However, several species in this family are rather morphologically similar especially during the juvenile stage that may pose difficulties for their identification. *Mystus* is a poorly diagnosed group, and they are morphologically similar and diagnostic characteristics are usually subtle (Ng 2003; Ferdous 2013).

Cytogenetic studies on Thai bagrids are quite scarce; as yet only conventional cytogenetics have been applied to determine chromosome numbers and karyotype complements. Therefore, their chromosomal evolution is not clear, even though from family Bagridae up to 45 species have been karyotyped so far. The diploid chromosome number (2n) varies between 2*n* = 44 [*Coreobagrus brevicorpus* Mori, 1936)] and 2n = 80 [*Batasio fluviatilis* (Day, 1888)]. The fundamental number (number of chromosome arms, NF) varies between 64 [for *M. tengara* (Hamilton, 1822) and *M. vittatus* (Bloch, 1794)] and 116 [for *Horabagrus brachysoma* (Günther, 1864) and *H. nigricollaris* Pethiyagoda et Kottelat, 1994] (Arai 2011).

Focusing on the genus *Mystus*, chromosomal diversity and chromosomal variations among populations can be found. The so far reported 2n for diploid chromosome numbers varies between 50 and 58 chromosomes and for NF from 64 to 110 (Table 1). Intra-specific variations of 2n were reported in *M. mysticetus* Roberts, 1992 (2n = 50, 52) (Donsakul 2002; Supiwong et al. 2014a, b) and *M. vittatus* (Bloch, 1794) (2n = 50, 54, 58) (Das and Srivastava 1973; Manna and Prasad 1974; Tripathi and Das 1980; Rishi 1981; Sharma and Tripathi 1986; Khuda-Bukhsh and Barat 1987; John et al. 1992; Choudhury et al. 1993; Ramasamy et al. 2010). The cytogenetic characterization of a species could be applied to other fields such as systematics,

but also economic interests, as breeding practices of organisms by using chromosome set management (Na-Nakhon et al. 1980), strain improvement (Sofy et al. 2008) and brood stock selection (Mengampan et al. 2004).

Conventional cytogenetics may be sufficient to identify intra- and interspecific variations and is an inexpensive approach. However, it has restrictions, and accordingly the use of molecular cytogenetic analyses plays an increasing role for more precise characterization of the structure of genomes, including that of fishes. Especially, fluorescence in situ hybridization (FISH) for mapping of repetitive DNA sequences provided important contributions to the characterization of biodiversity and evolution in divergent fish groups (Cioffi and Bertollo 2012), especially as some microsatellite repeats are species-specific (Cioffi et al. 2015). To date, there are only three studies within Bagridae using such FISH techniques, all performed by our group (Supiwong et al. 2013a, 2014a, b).

In the present study, chromosomal structures and genetic markers for Thai populations of *M. atrifasciatus* Fowler, 1937, *M. mysticetus*, *M. singaringan* (Bleeker, 1846) and *M. wolffii* (Bleeker, 1851) (Fig. 1A–D) were for the first time analysed by cytogenetics and molecular cytogenetics.

Material and methods

Ten males and ten females of each species were collected from the Chi (Maha Sarakham Province), Songkhram (Bueng Kan Province), Chao Phraya (Sing Buri Province) and Pak Phanang Basins (Nakhon Sri Thammarat Province), Thailand from 2016–2018. The procedures followed ethical protocols as approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand ACUC-KKU-15/2559. Preparation of fish chromosomes from kidney cells was done as previously reported (Supiwong et al. 2012; Pinthong et al. 2015). The chromosomes were stained with Giemsa solution for 10 minutes. Ag-NOR banding was performed by applying two drops of 2% gelatin to the chromosomes, followed by four drops of 50% silver nitrate (Howell and Black 1980). Metaphases were evaluated according to the chromosome classification of Levan et al. (1964). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a). Fundamental number, NF (number of chromosome arm) was obtained by assigning a value of two to metacentric and submetacentric chromosomes and one to subtelocentric and acrocentric chromosomes. The chromosome sizes were calculated applying the method of Tanomtong (2011).

Microsatellites d$(GC)_{15}$, d$(CAA)_{10}$, d$(CAT)_{10}$ and d$(GAA)_{10}$ repeat probes (Kubat et al. 2008) were directly labeled by Cy3 at 5′ ends during synthesis (Sigma, St. Louis, MO, USA). FISH under high stringency conditions on mitotic chromosome spreads (Pinkel et al. 1986) was performed as previously reported (Supiwong et al. 2017b; Yano et al. 2017). The evaluation was done on an epifluorescence microscope Olympus BX50 (Olympus Corporation, Ishikawa, Japan).

Results

Diploid number, fundamental number and karyotype of *Mystus atrifasciatus*, *M. mysticetus*, *M. singaringan* and *M. wolffii*

The four studied *Mystus* species have different diploid chromosome numbers (2n) and fundamental numbers (NF) as follows: the 2n (NF) were 52 (102), 54 (104), 56 (98) and 58 (108) in *M. mysticetus*, *M. atrifasciatus*, *M. singaringan* and *M. wolffii*, respectively. The karyotypes of *M. atrifasciatus* (24m+26sm+4a), *M. mysticetus* (26m+24sm+2a), *M. singaringan* (24m+18sm+14a) and *M. wolffii* (30m+22sm+6a) were species-specific (Fig. 1E–H; Table 1). Differentiated sex chromosomes between male and female specimens could not be identified in all analyzed species.

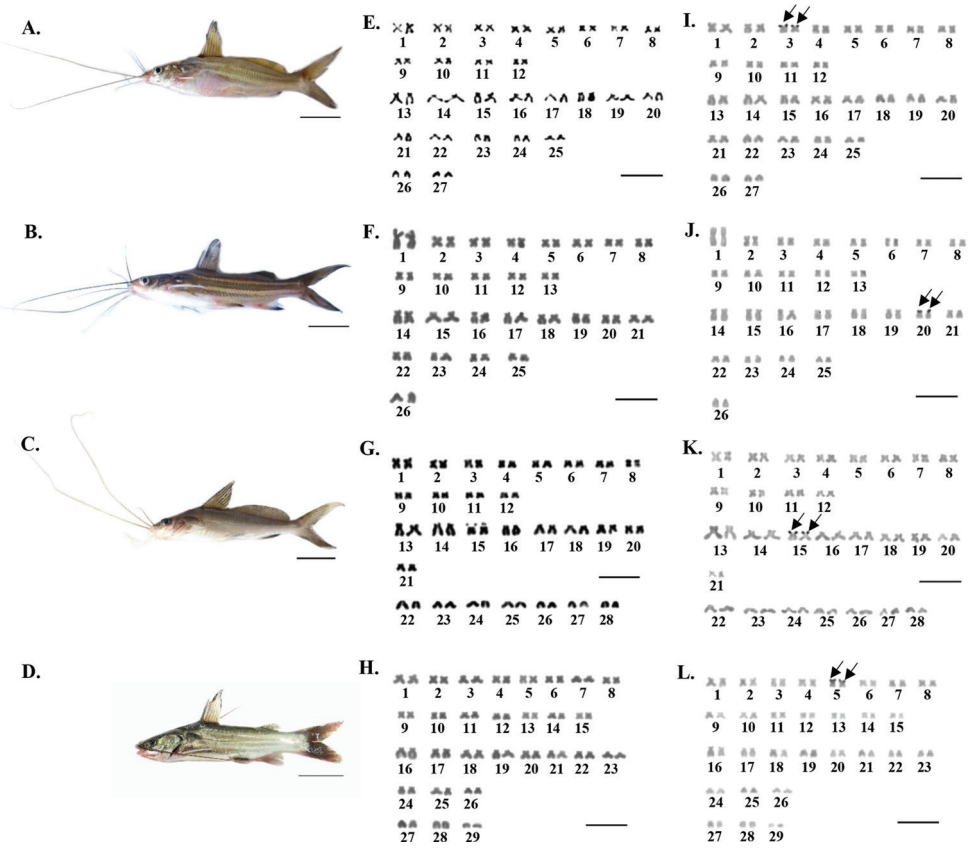

Figure 1. Specimens and karyotypes from conventional staining and Ag-NOR banding techniques of *Mystus atrifasciatus* (**A, E, I**), *M. mysticetus* (**B, F, J**), *M. singaringan* (**C, G, K**) and *M. wolffii* (**D, H, L**); arrows indicate NOR carrying chromosomes. Scale bars: 2 cm (**A–D**); 5 μm (**E–L**).

Table 1. Comparative cytogenetics of *Mystus* genus (2n = diploid chromosome number, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, t = telocentric, NOR = nucleolar organizer regions, NF = fundamental number, and – = not available).

Species	2n	NF	Karyotype	NOR	Locality	Reference
Mystus albolineatus Roberts, 1994	56	108	28m+6sm+12st+10a	-	Thailand (Ayutthaya)	Donsakul (2000)
M. atrifasciatus Fowler, 1937	54	92	30m+8sm+16a	-	Thailand (Nakhon Phanom)	Magtoon and Donsakul (2009)
	54	96	24m+18sm+12st/a		Thailand (Bueng Kan)	Supiwong et al. (2014 a, b)
	54	**104**	**24m+26sm +4a/t**	**2**	**Thailand (Maha Sarakham)**	**Present study**
M. bleekeri (Day, 1877)	56	90	20m+14sm+10st+12a	-	India (Jammu)	Sharma and Tripathi (1986)
	56	102	32m+14sm+10a	-	India	Chanda (1989)
M. bocourti (Bleeker, 1864)	56	104	24m+18sm+6st+8a	-	Thailand (Nong Khai)	Donsakul (2000)
	56	100	22m+22sm+12st/a	-	Thailand (Sing Buri)	Supiwong et al. (2013a, 2014a, b)
M. cavasius (Hamilton, 1822)	58	102	18m+16sm+10st+14a	2	India (Jammu)	Sharma and Tripathi (1986); Rishi et al. (1994)
	58	108	18m+22sm+8t	-	India (Orissa)	Tripathi and Das (1980)
	58	102	14m+26sm+4st+14a	-	India (Bihar)	Khuda-Bukhsh et al. (1980)
M. gulio (Hamilton, 1822)	58	102	30m+12sm+2st+14a	2	India (West Bengal)	Das and Khuda-Bukhsh (2007b)
Female	58	108	12m+34sm+4st+8t	-	India (Orissa)	Choudhury et al. (1993)
Male	58	110	13m+33sm+4st+8t	-	India (Orissa)	Choudhury et al. (1993)
M. multiradiatus Roberts, 1992	54	98	30m+10sm+4st+10a	-	Thailand (Kanchana-buri)	Magtoon and Donsakul (2009)
	54	96	18m+24sm+12st/a	-	Thailand (Maha Sarakham)	Supiwong et al. (2014 a, b)
M. mysticetus Roberts, 1992	50	92	28m+14sm+8a	-	Thailand (Ayutthaya)	Donsakul (2002)
	52	100	26m+22sm+4st/a	-	Thailand (Maha Sarakham)	Supiwong et al. (2014a, b)
	52	**102**	**26m+24sm+2a**	**2**	**Thailand (Bueng Kan)**	**Present study**
M. ngasep Darshan et al., 2011	56	90	12m+22sm+8st+14t	-	India (Manipur)	Sing et al. (2013)
M. singaringan (Bleeker, 1846)	56	94	24m+14sm+10st+8a	-	Thailand (Nakhonsawan)	Donsakul (2001)
	56	**98**	**24m+18sm+14a**	**2**	**Thailand (Sing Buri)**	**Present study**
M. tengara (Hamilton, 1822)	54	64	10m+44a	-	India (Haryana)	Nayyar (1966)
Female	54	101	9m+38sm+7a	-	India	Rishi (1973)
Male	54	102	10m+38sm+6a	-	(Haryana)	Rishi (1973)
Female	54	97	25m+18sm +11a	-	India	Rishi and Rishi (1981)
Male	54	98	26m+18sm +10a	-	(Haryana)	Rishi and Rishi (1981)
M. vittatus (Bloch, 1794)	54	108	22m+26sm+6st	-	India (Orissa)	Tripathi and Das (1980)
	54	108	22m+20sm+12st	-	India (Jammu)	Sharma and Tripathi (1986)
	58	110	10m+30sm+12st+6t	-	India (Orissa)	Choudhury et al. (1993)
	54	108	20m+24sm+10st	-	India	Rishi (1981)
	50	64	14m+36a	-	India	Das and Srivastava (1973)
	58	104	16m+10sm+20st+12a	-	India (West Bengal)	Manna and Prasad (1974)
	54	106	28m+22sm+2st+2a	2	India (Orissa)	Khuda-Bukhsh and Barat (1987); John et al. (1992)
	54	78	6m+18sm+30a	-	India (Tamilnadu)	Ramasamy et al. (2010)
M. wolffii (Bleeker, 1851)	58	100	26m+10sm+6st+16a	-	Thailand (Tak)	Donsakul (2000)
	58	**108**	**30m+22sm+6a**	**2**	**Thailand (Nakhon Sri Thammarat)**	**Present study**

Chromosome markers in *Mystus atrifasciatus*, *M. mysticetus*, *M. singaringan* and *M. wolffii*

One single pair with NOR-bearing chromosomes was present in all four species analyzed. NOR positions were observed at regions adjacent to the telomere of the short arm of the chromosome pairs 3 (metacentric), 20 (submetacentric), 15 (submetacentric), and 5 (metacentric) in *M. atrifasciatus*, *M. mysticetus*, *M. singaringan* and *M. wolffii*, respectively (Figs 1I–L, 2). The typical diversity of chromosome shapes

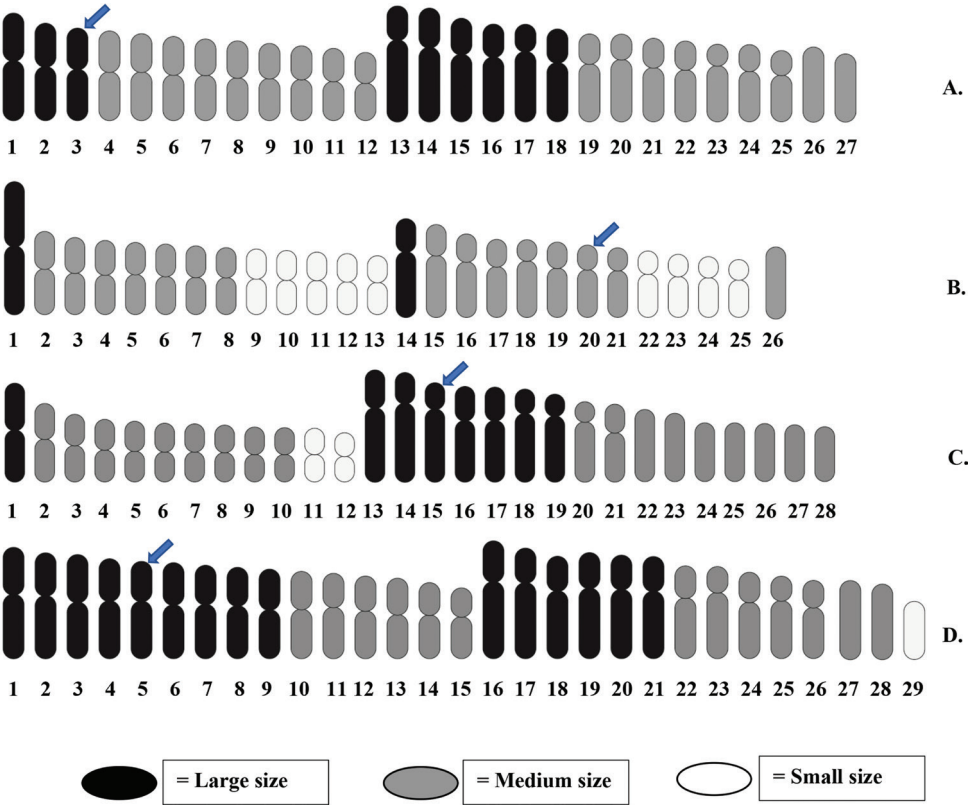

Figure 2. Idiograms representing shapes and sizes of chromosomes (haploid set) of **A** *Mystus atrifasciatus* **B** *M. mysticetus* **C** *M. singaringan* and **D** *M. wolffii*; arrows indicate NOR carrying chromosomes.

Table 2. Cytogenetic and FISH studies on four *Mystus* fishes in Thailand (2n = diploid chromosome number, NF = fundamental number or number of chromosome arm, m = metacentric, sm = submetacentric, a = acrocentric, NOR = nucleolar organizer region, I = interstitial site, T = telomere, W = whole chromosome).

Mystus Species	2n	NF	Chromosome type			Ag-NOR pair (type)	Microsatellite patterns			
			m	sm	a		$(GC)_{15}$	$(CAA)_{10}$	$(CAT)_{10}$	$(GAA)_{10}$
M. atrifasciatus Fowler, 1937	54	104	24	26	4	3 (m)	T&I	T&I	T&I	I
M. mysticetus Roberts,1992	52	102	26	24	2	20 (sm)	T&I	T&I	T&I	T&I
M. singaringan (Bleeker, 1846)	56	98	24	18	14	15 (sm)	T&I	T&I	T	T&I
M. wolffii (Bleeker, 1851)	58	108	30	22	6	5 (m)	W&T	T	W&T	T&I

and sizes among the four analyzed species is shown in Fig. 2 and Table 2. Karyotypic complements comprise most bi-armed and few mono-armed chromosomes revealed in *M. atrifasciatus*, *M. mysticetus* and *M. wolffii* whereas in *M. singaringan*, there are several pairs of both bi-armed and mono-armed chromosomes. Chromosome sizes are classified as large (L), medium (M) and small (S) in each species as follows: 18L+36M in *M. atrifasciatus*, 4L+30M+18S in *M. mysticetus*, 16L+36M+4S in *M. singaringan*, and 30L+26M+2S in *M. wolffii*.

Patterns of microsatellite repeats in the genomes of *Mystus atrifasciatus*, *M. mysticetus*, *M. singaringan* and *M. wolffii*

The mapping of $d(GC)_{15}$, $d(CAA)_{10}$, $d(CAT)_{10}$ and $d(GAA)_{10}$ microsatellites showed different hybridization signals among the species. The repeats of $d(GC)_{15}$ and $d(CAA)_{10}$ are abundantly distributed in the telomeric regions of several pairs and in interstitial sites of some chromosomes in *M. atrifasciatus*, *M. mysticetus*, *M. singaringan*. In contrast, in *M. wolffii*, $d(GC)_{15}$ repeats are dispersed throughout all chromosomes, while $d(CAA)_{10}$ repeats are accumulated at telomeric positions of some chromosome pairs with more density in only one pair. The $d(CAT)_{10}$ repeats in *M. atrifasciatus* and *M. mysticetus* display high accumulations at the telomeric regions of almost all chromosomes and interstitial sites in some pairs whereas they have high accumulations at only the telomeric regions of almost all chromosomes in *M. singaringan*, and highly distributed in some chromosome pairs in *M. wolffii*. The $d(GAA)_{10}$ repeats are abundantly distributed at interstitial and telomeric regions of several chromosome pairs in *M. mysticetus*, *M. singaringan* and *M. wolffii*, while they are highly accumulated in some chromosome pairs of *M. atrifasciatus* (Fig. 3; Table 2).

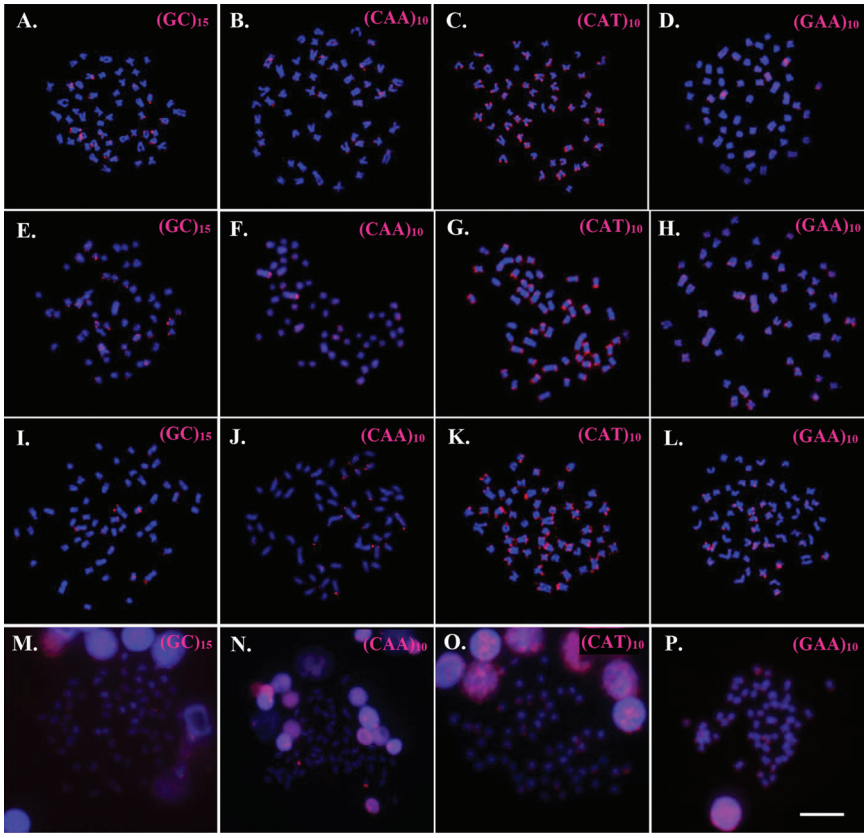

Figure 3. Metaphase chromosome plates showing $d(GC)_{15}$, $d(CAA)_{10}$, $d(CAT)_{10}$ and $d(GAA)_{10}$ microsatellites mapping on chromosomes of *Mystus atrifasciatus* (**A, E, I, M**), *M. mysticetus* (**B, F, J, N**), *M. singaringan* (**C, G, K, O**) and *M. wolffii* (**D, H, L, P**). Scale bars: 5 μm.

Discussion

Diploid chromosome numbers, fundamental numbers and karyotypes of *M. atrifasciatus*, *M. mysticetus*, *M. singaringan* and *M. wolffii*

The diploid chromosome numbers (2n) in all analyzed species confirmed previous cytogenetic studies (Donsakul 2000, 2001; Magtoon and Donsakul 2009; Supiwong et al. 2014a, b), except for *M. mysticetus* with 2n=50 reported in a previous study (Donsakul 2002) and 52 in the present one. In agreement with the literature, 2n in the genus *Mystus* ranges between 50 and 58 chromosomes (Arai 2011; Table 1). The possible mechanisms that promoted intra- and interspecific karyotype diversification are biogeographic barriers, small population, limited gene flow (Galetti Jr et al. 2000). Although all studied species except *M. mysticetus*, had the same 2n as previous studies, the karyotypes were different, probably because of different sampling sites should be considered (Fig. 4). The predominant 2n in this genus is 56 chromosomes (five from 13 species) and may represent an ancestral character in this family (Sharma and Tripathi 1986). This is consistent with the hypothesis of Oliveira and Gosztonyi (2000) that 2n=56 could be a plesiomorphic character in the order Siluriformes. However, NF and karyotypes found in the present study differ from all previous reports (Donsakul 2000, 2001, 2002; Magtoon and Donsakul 2009; Supiwong et al. 2014a, b). These differences may be species-specific variations within populations, and/or misidentification of species, or different species in presumed species complexes. NF in *Mystus* vary from 64 to 110. Ghigliotti et al. (2007) suggested that species with a higher NF value are more advanced in evolutionary terms than such with lower one. That hypothesis can be described that primitive karyotype of fish possesses many acrocentric chromosomes (mono-arm chromosomes). During evolution, the mono-arm chromosomes changed to bi-arm chromosomes. The NF would be unaltered, but the 2n would decrease. Changes in NF appear to be related to the occurrence of pericentric inversions, which play a major role for karyotypic rearrangement in fishes and other vertebrates (King 1993; Galetti Jr et al. 2000; Wang et al. 2010). Accordingly, from comparative analysis among the here studied four *Mystus* species, NF data and analyses of karyotypic complements indicate for that *M. singaringan* has the most primitive karyotype while *M. wolffii* has the most derivative karyotype. As often seen in fishes of this family, no heteromorphic sex chromosomes for males and females could be identified. Nonetheless it must be mentioned, that there are two species, *M. gulio* (Hamilton, 1822) and *M. tengara* (Hamilton, 1822), which have differentiated sex chromosome systems as XX/XY and ZZ/ZW, respectively (Arai 2011). Accordingly, differentiated sex chromosome system in this fish group seems to be a quite rare phenomenon.

Karyotypes of the genus *Mystus* in Thailand showed high diversification (Table 1). Seven species have been cytogenetically studied. The 2n ranged between 50 chromosomes in *M. mysticetus* (Donsakul 2002) and 58 chromosomes in *M. wolffii* (Donsakul 2000; present study). The predominant 2n is 56 chromosomes found in *M. albolineatus* (NF = 108, 28m+6sm+12st+10a) (Donsakul 2000), *M. bocourti*

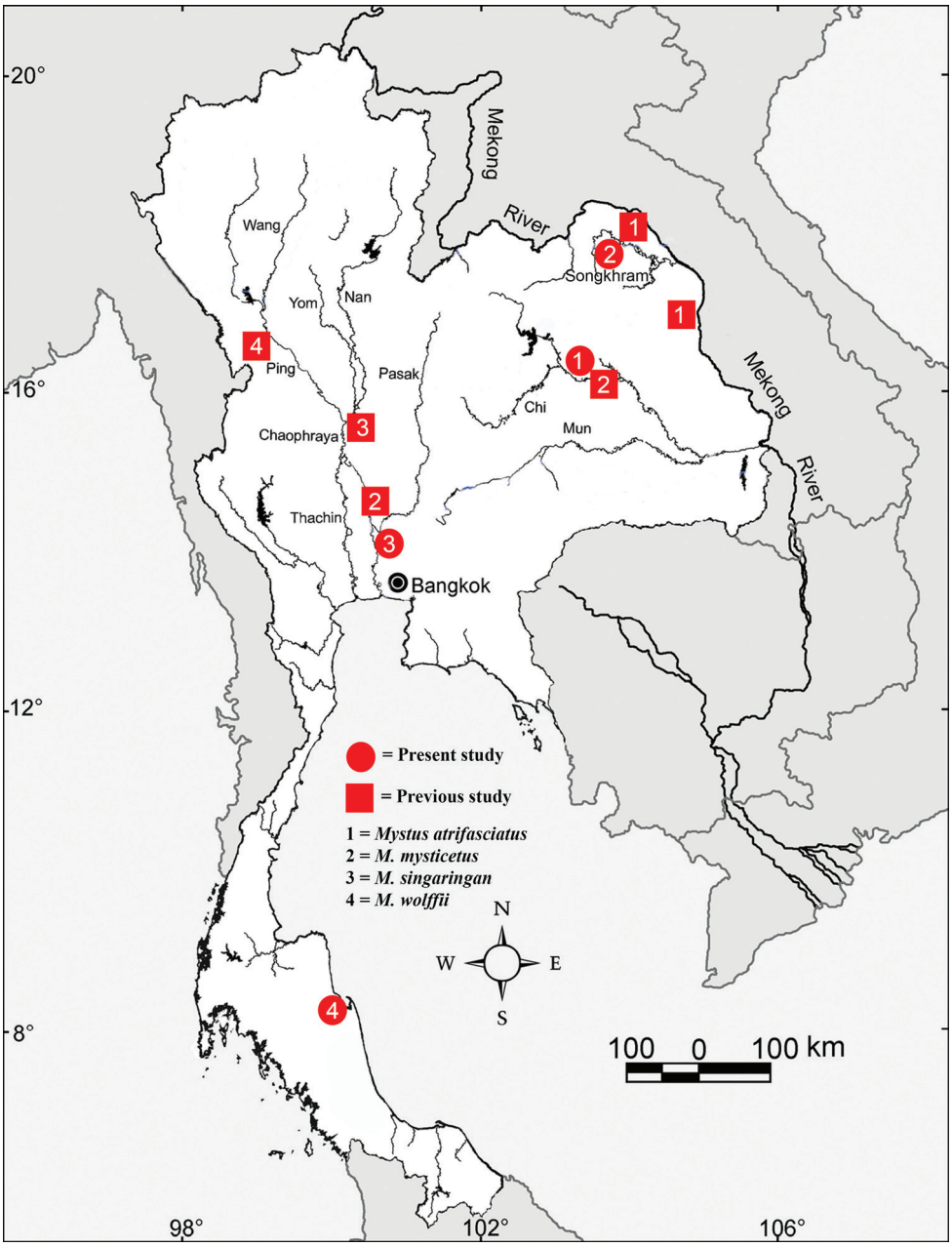

Figure 4. Map showing the comparison of sampling sites among present and previous studies**.**

(NF = 100, 22m+22sm+12st/a; NF = 104, 24m+18sm+6st+8a) (Donsakul 2000; Supiwong et al. 2013a, 2014a, b) and *M. singaringan* (NF = 94, 24m+14sm+10st+8a; NF = 98, 24m+18sm+14a) (Donsakul 2001; present study). Our results showed differences among NFs and karyotypes in the studied species. Interestingly, *M. mysticetus*

had two variants, 2n = 50 chromosomes (NF=92, 28m+14sm+8a), found in Ayutthaya Province, Central Thailand (Donsakul 2002), and 52 chromosomes (NF = 100, 26m+22sm+4st/a; NF=102, 26m+24sm+2a) found in Maha Sarakham and Bueng Kan Provinces, Northeast of Thailand (Supiwong et al. 2014a, b; present study) (Fig. 4). This variation may be caused by a rearrangement of chromosomes by centric fusion and pericentric inversion during chromosomal evolution in groups of populations separated by a geographic barrier.

Chromosome markers for *M. atrifasciatus*, *M. mysticetus*, *M. singaringan* and *M. wolffii*

Nucleolus organizer regions (NORs)

The localization of nucleolus organizer regions (NORs) is a simple method to determine chromosomal marker. NORs are specific positions on the chromosome that consist of tandemly repeated sequences of ribosomal genes (rRNA). In eukaryotes, each unit is composed of three genes coding for 18S, 5.8S and 28S ribosomal RNA (Sharma et al. 2002). Generally, most fishes have one pair of small NORs (single NOR) on chromosomes. However, some species of fishes have more than two NORs which may be caused by the translocation between some part of the chromosome with NORs and another chromosome (Sharma et al. 2002). Interspecific and intraspecific NOR polymorphism in the number of NORs per genome, in the chromosomal location of NOR sites, in the relative sizes of individual NORs, and in the number of active NOR sites per cell are commonly observed in fish, where the rDNA loci have been shown to be highly dynamic (Milhomem et al. 2013). Changes in chromosome number and structure can alter the number and structure of NOR as well. The pattern of NORs may be specific to populations, species and subspecies. Robertsonian translocations may cause losses of NOR. Species, which have limited gene exchange due to geographical isolation, have elevated karyotype numbers and NOR variation. (Yüksel and Gaffaroğlu 2008). The NOR is frequently used to compare variations as well as to identify and explain specifications. Therefore, it can be used as taxonomic and systematic characters in order to infer phylogenetic hypotheses of species relationships (Gold 1984; Amemiya and Gold 1990).

If these loci are active during the interphase before mitosis, they can be detected by silver nitrate staining (Howell and Black 1980). The single NOR-bearing chromosome pairs in the present study is consistent in *M. cavasius* (Hamilton, 1822) (Sharma and Tripathi 1986; Rishi et al. 1994), *M. gulio* (Das and Khuda-Bukhsh 2007b), and *M. vittatus* (Khuda-Bukhsh and Barat 1987; John et al. 1992). This character is a common characteristic found in many species in this family such as *Bagrichthys majusculus* Ng, 2002 (Supiwong et al. 2018), *He. menoda* (Hamilton, 1822) (Barat and Khuda-Bukhsh 1986), *He. wyckii* (Bleeker, 1858) (Supiwong et al. 2017c), *Horabagrus brachysoma* (Günther, 1864) (Nagpure et al. 2003), *Ho. nigricollaris* Pethiyagoda et Kottelat, 1994 (Nagpure et al. 2004), *Pelteobagrus ussuriensis* (Dybowski, 1872) (Kim et al.

1982), *Pseudobagus vachelii* (Ueno 1985), *Pseudomystus siamensis* (Regan, 1913) (Supiwong et al. 2013b), *Rita rita* (Hamilton, 1822) (Khuda-Bukhsh and Barat 1987) and *Sperata seenghala* (Sykes, 1839) (Sharma and Tripathi 1986; Das and Khuda-Bukhsh 2007a). However, only a single species, *Tachysurus fulvidraco* (Richardson, 1846), has two NOR carrying chromosome pairs (Zhang et al. 1992). In fishes, a single NOR carrying chromosome pair is considered as a primitive state (Milhomem et al. 2013). Many families such as Chaetodontidae (Supiwong et al. 2017a), Lutjanidae (Phimphan et al. 2017), Notopteridae (Maneechot et al. 2015), Scaridae (Kaewsri et al. 2014), Serranidae (Pinthong et al. 2013), share this character. Also, for fishes the location of NORs in a terminal position, as seen in the studied species, is also considered as a primitive characteristic (Vitturi et al. 1995).

Patterns of microsatellite repeats on the genomes of *Mystus atrifasciatus*, *M. mysticetus*, *M. singaringan* and *M. wolffii*

Repetitive DNAs like microsatellites can be used to spot genomic evolution as previously been reported for different fish groups (Cioffi et al. 2010; Cioffi and Bertollo 2012; Terencio et al. 2013; Yano et al. 2014; Cioffi et al. 2015; Moraes et al. 2017, 2019; Sassi et al. 2019). It is known from fossil records that there is a major evolutionary diversification in Siluriformes fishes; this has in parts already also been verified at chromosomal level.

Here, four bi- and tri-nucleotide microsatellite sequences were mapped on chromosomes of four *Mystus* species. The patterns of microsatellites $d(GC)_{15}$ and $d(CAA)_{10}$ repeats in three species in the present study (*M. atrifasciatus*, *M. mysticetus*, *M. singaringan*) are similar to those found in *Channa micropeltes* (Cuvier, 1831) (Cioffi et al. 2015). On the other hand, they are differences known for *C. gachua* (Hamilton, 1822), *C. lucius* (Cuvier, 1831), *C. striata* (Bloch, 1793) (Cioffi et al. 2015), *Toxotes chatareus* (Hamilton, 1822) (Supiwong et al. 2017b) and Asian swamp eel, *Monopterus albus* (Zuiew, 1793) (Supiwong et al. 2019). The pattern of microsatellite $d(GC)_{15}$ repeats in *M. wolffii* is similar to that of *C. lucius* (Cioffi et al. 2015) and *T. chatareus* (Supiwong et al. 2017a). Interestingly, the patterns of microsatellite $d(CAT)_{10}$ repeats in *M. atrifasciatus*, *M. mysticetus* and *M. singaringan* are similar to the patterns of the $(CA)_{15}$ repeats on chromosomes of other species in the family Bagridae (Supiwong et al. 2013a, 2014b). Comparative study on four species showed that not only there are differences of 2n, NF and karyotype, but the patterns of microsatellite repeat on chromosomes also have difference among them. Thus, the cytogenetic data may be a tool for classification of fish species that there is similar morphology as the stripe *Mystus* (*M. atrifasciatus* and *M. mysticetus*).

From previous reports, it may be carefully deduced that most heterochromatin in fish genomes consist of microsatellites (Cioffi and Bertollo 2012). However, microsatellites have also been found in non-centromeric regions, many of them were located either near or within genes (Rao et al. 2010; Getlekha et al. 2016). Indeed, GC rich motifs are common in exons of all vertebrates (Chistiakov et al. 2006). Since higher re-

combination rates can be found near the telomeric region (Jensen-Seaman et al. 2004), it is possible that the physical proximity of microsatellite and rDNA repeats could favor the evolutionary spreading of both sequences together, despite the possibility of spreading some errors, too. Repetitive DNA sequences could act as primary driving forces in speciation (Biémont and Vieira 2006). These sequences are closely associated with heterochromatic regions, thus contributing to gene activation and structural maintenance of chromosomes (Dernburg et al. 1996). Therefore, great variations in the amount and position of these sequences could create fertility barriers by fostering the occurrence of chromosomal rearrangements (Cioffi and Bertollo 2012).

Indeed, the distribution of microsatellite motifs in fish genomes could be biased to some specific noncoding regions, as found in the Asian swamp eel, *M. albus* (Li et al. 2017). Finally, closely related fish species involved in recent speciation events could present a differential pattern in the distribution and quantity of microsatellite sequences on chromosomes, as demonstrated for naked catfishes (Supiwong et al. 2014b), channid fishes (Cioffi et al. 2015) and four *Mystus* in the present study.

Conclusions

The present research is the first report on NOR and microsatellites $d(GC)_{15}$, $d(CAA)_{10}$, $d(CAT)_{10}$ and $d(GAA)_{10}$ mapping in *M. atrifasciatus*, *M. mysticetus*, *M. singaringan* and *M. wolffii*. There are differences in the diploid chromosome number, the fundamental numbers, karyotypes, pairs having NORs, and patterns of microsatellite distributions on chromosomes. These results indicated that (molecular) cytogenetic data can be used for classification in related fish species and to explain karyotype diversification.

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Genome size variation and karyotype diversity in eight taxa of *Sorbus* sensu stricto (Rosaceae) from China

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Abstract

Eight taxa of *Sorbus* Linnaeus, 1753 sensu stricto (Rosaceae) from China have been studied karyologically through chromosome counting, chromosomal measurement and karyotype symmetry. Genome size was also estimated by flow cytometry. Six taxa, *S. amabilis* Cheng ex T.T.Yu et K.C.Kuan, 1963, *S. hupehensis* var. *paucijuga* (D.K. Zang et P.C. Huang, 1992) L.T. Lu, 2000, *S. koehneana* C.K. Schneider, 1906, *S. pohuashanensis* (Hance, 1875) Hedlund, 1901, *S. scalaris* Koehne, 1913 and *S. wilsoniana* C.K. Schneider, 1906 are diploids with 2n = 34, whereas two taxa, *S. filipes* Handel-Mazzetti,1933 and *S. ovalis* McAllister, 2005 are tetraploid with 2n = 68. In general, the chromosome size is mainly small, and karyotypes are symmetrical with predominance of metacentric chromosomes. Genome size variation of diploids and tetraploids is 1.401 pg –1.676 pg and 2.674 pg –2.684 pg, respectively. Chromosome numbers of *S. amabilis* and *S. hupehensis* var. *paucijuga*, and karyotype and genome size of eight taxa studied are reported for the first time. This study emphasised the reliability of flow cytometry in genome size determination to infer ploidy levels in Chinese native *Sorbus* species.

Keywords

DNA content, flow cytometry, polyploid, *Sorbus* evolution

Introduction

Sorbus Linnaeus, 1753 (sensu stricto, except as noted hereafter) (Maleae, Rosaceae) is distributed mainly in northern temperate regions with its greatest diversity in the mountains of south-western China and adjacent areas of Upper Burma and the Eastern Himalaya. It comprises about 90 species all over the world, with more than 60 species occurring in China (Phipps et al. 1990; Lu and Spongberg 2003; McAllister 2005). Species of *Sorbus* are valuable ornamental plants due to their pinnately compound leaves, attractive white or red flowers and colourful crimson, scarlet, orange, pink, yellow or pure white fruits. *Sorbus* is one of the most challenging groups in taxonomy and systematic for the widespread interspecific hybridisation and genome multiplication (polyploidy) (McAllister 2005; Robertson et al. 2010; Li et al. 2017). Polyploidy is a very common phenomenon in the genus. Tetraploids account for more than half of the species richness and are distributed mainly in the mountains of south-western China, especially the Qinghai-Tibet Plateau (McAllister 2005). Thus, data of chromosome number and ploidy levels in Chinese native *Sorbus* species are valuable in the taxonomy of the genus and in understanding the species' relationships and origins.

Features of chromosomes play an important role in plant taxonomy to elucidate the origin, speciation and phylogenetic relationships of plants (Stebbins 1971; Peruzzi and Altınordu 2014; Sassone et al. 2017; Winterfeld et al. 2020). Chromosome counts were proven to be most valuable in the taxonomy of *Sorbus* long before the era of molecular phylogenetics because they are helpful in understanding the species' relationships and origins (Liljefors 1934, 1953, 1955; Sennikov and Kurtto 2017). The chromosome base number in *Sorbus* is x = 17 and it is common to all members of Maleae. Chromosome counts have been reported for 43 Chinese native *Sorbus* species. Only two ploidies occur in the genus, i.e. diploid (2n = 34) and tetraploid (2n = 68), although four ploidies have been reported in *Sorbus* sensu lato (McAllister 2005; Bailey et al. 2008; Pellicer et al. 2012). Most species occur at one ploidy level, and two Chinese native species, *S. koehneana* C.K. Schneider, 1906 and *S. vilmorinii* C.K. Schneider, 1906, have been reported to have diploids and tetraploids (McAllister 2005).

Genome size estimation (plant genome C-value) by flow cytometry (FCM) (Greilhuber et al. 2005) is a rapid cytogenetic method that has contributed to our understanding of the evolutionary relationships amongst *Sorbus* species (Hajrudinović et al. 2015a, b). FCM profiles revealed the presence of two ploidy levels (cytotypes) in the genus, 2n = 2x (*S. cibagouensis* H. Peng et Z. J. Yin, 2017: 1.480 ± 0.039 pg, *S. hypoglauca* (Cardot, 1918) Handel- Mazzetti, 1933: 1.513 ± 0.041 pg) and 2n = 4x (*S. vilmorinii*: 2.675 ± 0.065 pg) (Xi et al. 2020), consistent with the results of chromosome counts (Pellicer et al. 2012).

The present study aims to (1) determine the chromosome number, karyotype, idiogram and other chromosome morphology and genome size of eight taxa in *Sorbus*; and (2) evaluate the reliability of flow cytometry in genome size determination to infer ploidy levels in Chinese *Sorbus* species.

Materials and methods

Plant material

Eight taxa from two subgenera in *Sorbus*, *S. filipes* Handel-Mazzetti, 1933, *S. hupehensis* var. *paucijuga* (D.K. Zang et P.C. Huang, 1992) L.T. Lu, 2000, *S. koehneana*, *S. ovalis* McAllister, 2005 from subgenus *Albocarmesinae* McAllister, 2005 and *S. amabilis* Cheng ex T.T.Yu et K.C.Kuan, 1963, *S. pohuashanensis* (Hance, 1875) Hedlund, 1901, *S. scalaris* Koehne, 1913, *S. wilsoniana* C.K. Schneider, 1906 from subgenus *Sorbus*, were collected in China (Figure 1) between 2015 and 2016. Three individuals for each taxon were selected for chromosome numbers counting, karyotype analysis and genome size estimation. Voucher specimens are deposited at the Herbarium of Nanjing Forestry University (**NF**).

Chromosome preparations and karyotype analysis

Mature fruits of each plant were harvested separately, then plump seeds were extracted from fruits and washed with tap water. Seeds were stored in sand for 40–120 days at 0–4 °C until germination. Root tip meristems were pre-treated with a mixed solution of 0.1% colchicine and 0.002 mol/l 8-hydroxyquinoline (1:1) at 0–4 °C for 2 h and then fixed in absolute ethanol: glacial acetic acid (2:1) mixture for 24 h at 0–4 °C. The root tips were hydrolysed in 1 mol/l HCl at 60 °C for 10min and then rinsed with tap water for 2–3 min. The fixed roots were stained in Carbol fuchsin for 3–4 h, ground and placed on glass slides for observation. Five metaphase cells per individual were examined. Photos were taken under an optical microscopic Nikon Eclipse Ci-S. A mean haploid idiogram was drawn using KaryoType 2.0 (http://mnh.scu.edu.cn/soft/blog/karyotype/, Altinordu et al. 2016), based on the length of chromosome.

For the numerical characterisation of the karyotypes, the following parameters were calculated: long arm length (LA) and short arm length (SA) of each chromosome, ratio of the longest/shortest chromosomes(L/S), total haploid (monoploid) length of chromosome set (THL), arm ratio of each chromosome (AR) [LA/SA], centromeric index of each chromosome (CI) [SA/ (LA + SA) × 100] and chromosome length of each chromosome (CL) [LA + SA]. Karyotype asymmetry has been determined using the coefficient of variation of centromeric index (CV_{CI}) [(S_{CI} / X_{CI}) × 100, where S_{CI}: standard deviation; X_{CI}: mean centromeric index] (Paszko 2006), coefficient of variation of chromosome length (CV_{CL}) [(S_{CL} / X_{CL}) × 100, where S_{CL}: standard deviation; X_{CL}: mean chromosome length] (Paszko 2006) and Stebbins' classification (Stebbins 1971). The karyotype formula was determined by chromosome morphology based on centromere position according to Levan et al. (1964): median point (M, AR = 1.00), median region (m, AR = 1.01–1.70), submedian (sm, AR = 1.71–3.00), subterminal (st, AR = 3.01–7.00) and terminal region (t, AR > 7.00). Satellite chromosomes were abbreviated as 'sat' (Levan et al. 1964). In terms

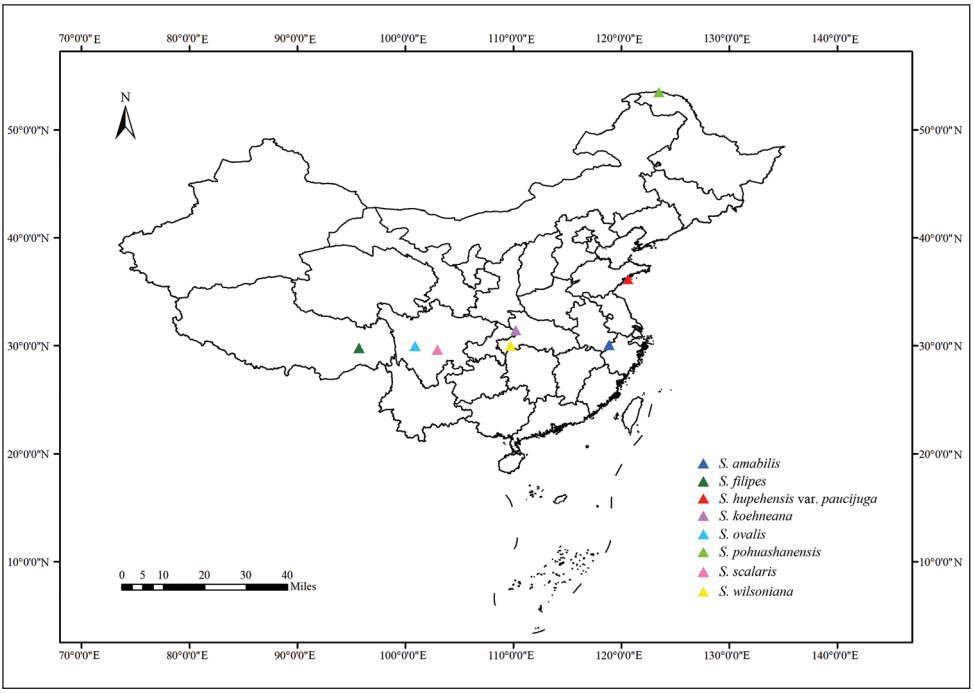

Figure 1. Collection sites of the eight *Sorbus* taxa studied.

of length, chromosomes were classified according to Lima de Faria (1980) as very small (≤ 1 μm), small (> 1 μm and = ≤ 4 μm), intermediate (> 4 μm and = < 12 μm) and large (> 12 μm and ≤ 60 μm).

Genome size estimation

Fully expanded leaf tissue from each sample collected in the field was dried in silica gel. Approximately 1 cm^2 of the sample was chopped along with the internal standard [*Oryza sativa* subsp. *japonica* S. Kato, 1930 'Nipponbare', 2C = 0.91 pg, (Uozu et al. 1997)] using a sharp razor blade in a Petri dish containing 1 ml of 'woody plant buffer' (WPB, Loureiro et al. 2007), following the one-step procedure proposed by Doležel et al. (2007). The nuclear suspension was then filtered through a nylon mesh (400 μm) to remove debris and stained with 50 μl PI. After incubation for 10 min on ice, the relative nuclear DNA content was estimated by recording at least 3000 particles using a BD Influx flow cytometer fitted with a blue laser (488 nm, 200 mW) and analysing three replicates of each individual. The resulting histograms were analysed with the BD FACS software 1.0.0.650. The 2C-value was calculated using the linear relationship between fluorescence signals from stained nuclei of the unknown sample and the internal standard. 1Cx was calculated dividing the 2C-value by the ploidy.

Statistical analysis

Data were analysed with SPSS Statistics 22.0 (IBM, USA). Correlations between chromosome counts and 1Cx, 2C-value were assessed using the Pearson correlation coefficient.

Results and discussion

The chromosome numbers of eight Chinese taxa of *Sorbus* in two subgenera have been determined (Table 1). All taxa have the same base chromosome number (x = 17). Four taxa, *S. amabilis* (Fig. 2A), *S. pohuashanensis* (Fig. 2F), *S. scalaris* (Fig. 2G) and *S. wilsoniana* (Fig. 2H) belonging to subg. *Sorbus*, are all diploids with 2n = 2x = 34. Amongst the taxa studied in subgen. *Albocarmesinae*, two taxa, *S. hupehensis* var. *paucijuga* (Fig. 2C) and *S. koehneana* (Fig. 2D), are diploids, while two other taxa, *S. filipes* (Fig. 2B) and *S. ovalis* (Fig. 2E), are tetraploids with 2n = 4x = 68. Chromosome numbers of two taxa, *S. amabilis* and *S. hupehensis* var. *paucijuga*, are reported for the first time. The chromosome numbers of six other taxa are consistent with the results of previous studies (Lu and Spongberg 2003; McAllister 2005).

Morphometric parameters of chromosomes in eight taxa are also presented in Table 1. The karyotypes differed for the haploid chromosome length, the position of centromeres and satellite, and the karyotype asymmetry. Individual chromosome sizes varied from 0.89 to 4.08 μm. The shortest are observed in *S. koehneana* (0.89–1.79 μm) and *S. wilsoniana* (0.89–1.72 μm) while the longest is observed in *S. pohuashanensis* (2.05–4.08 μm). The total haploid length varies from 20.68 μm (*S. wilsoniana*) to 50.06 μm (*S. pohuashanensis*). Three taxa, *S. filipes*, *S. koehneana* and *S. wilsoniana*, have both very small and small chromosomes. Four taxa, *S. amabilis*, *S. hupehensis* var. *paucijuga*, *S. ovalis* and *S. scalaris*, have only small chromosomes. One taxon, *S. pohuashanensis* has both small and intermediate chromosomes.

With respect to the position of the centromere, the chromosomes of the six taxa are metacentric or submetacentric. *S. amabilis* presents 9 metacentric (5, 8, 10–12, 14–17), 7 submetacentric (1, 3, 4, 6, 7, 9, 13) and 1 subtelocentric (2) chromosome pairs, and *S. ovalis* displays only metacentric chromosome pairs. A pair of satellites was observed in *S. amabilis*, *S. filipes*, *S. hupehensis* var. *paucijuga*, *S. pohuashanensis* and *S. scalaris*, with the satellites being located at the short arms of the fourth, fifth, twelfth, sixth and ninth chromosome pairs, respectively (Fig. 3).

According to the classification of Stebbins (Stebbins 1971), karyotypes of eight taxa are symmetrical and are classified as 1B (*S. koehneana* and *S. ovalis*), 2A (*S. wilsoniana*) or 2B (*S. amabilis*, *S. filipes*, *S. hupehensis* var. *paucijuga*, *S. pohuashanensis* and *S. scalaris*). CV_{CI} and CV_{CL} values of eight taxa ranged from 4.86 to 21.54 and 14.03 to 24.70, respectively (Table 1). CV_{CI} is a parameter indicative of the intrachromosomal symmetry. *S. ovalis* has the most symmetrical karyotype (CV_{CI} = 4.86), whereas

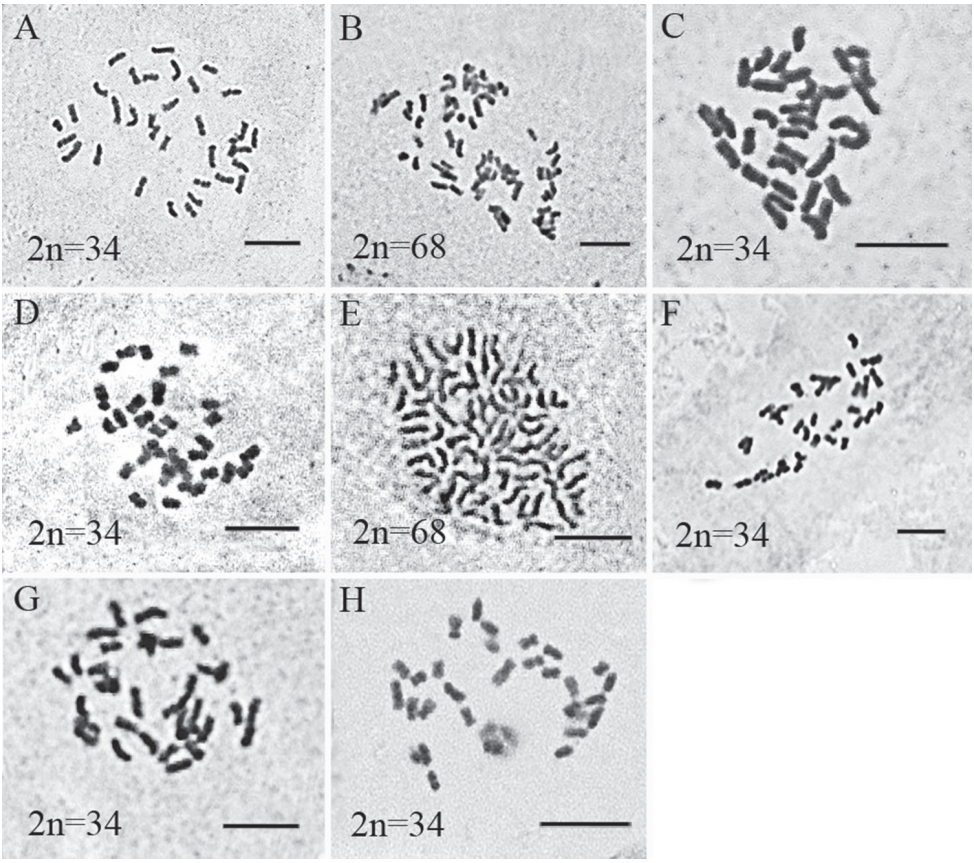

Figure 2. Somatic metaphases of eight *Sorbus* taxa **A** *S. amabilis* **B** *S. filipes* **C** *S. hupehensis* var. *paucijuga* **D** *S. koehneana* **E** *S. ovalis* **F** *S. pohuashanensis* **G** *S. scalaris* **H** *S. wilsoniana*. Scale bar: 5 μm.

S. amabilis has the least symmetrical karyotype (CV_{CI} = 21.54). CV_{CL} revealed that all taxa have little variations in chromosome size of the karyotypes. *S. hupehensis* var. *paucijuga* has the smallest CV_{CL} value (14.03) and *S. amabilis* presents the highest CV_{CL} value (24.70).

Genome size estimates of all the taxa from silica-dried leaves are shown in Table 1 and Figure. 4. The flow cytometric measurements of all taxa and the internal standards exhibit clear and sharp peaks. The coefficients of variation are lower than 5%, supporting the reliability of the flow cytometric assessments. The 2C-values range from 1.401 pg to 1.676 pg for diploid taxa. Two tetraploid taxa, *S. filipes* and *S. ovalis*, have 2C-values of 2.674 pg and 2.684 pg, respectively. 2C-values of tetraploids are approximately twice those of their diploid congeners and the relative DNA content correlate positively with the chromosome number ($r = 0.982$, $P \leq 0.0001$). The 1Cx-values, which indicate the DNA content per genome, range from 0.700 pg to 0.838 pg in diploids and 0.669 pg to 0.671 pg in tetraploids. The correlation between values of 1Cx and chromosome number is negative ($r = –0.687$, $P < 0.05$).

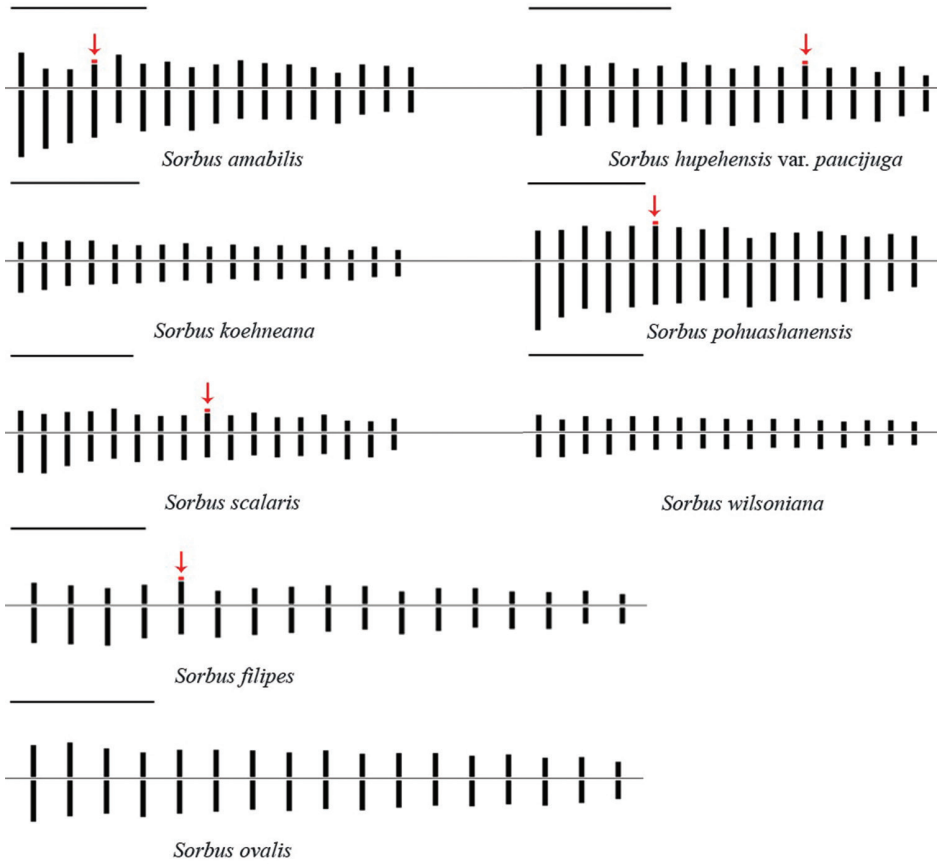

Figure 3. The mean haploid idiogram of the eight *Sorbus* taxa, based on median chromosome values. Arrows indicate secondary constrictions and satellites. Scale bars: 5 μm.

Table 1. Collecting information of materials and cytogenetics data of studied *Sorbus* taxa.

Subgenera	Taxon	2n	L/S	THL (μm)	VCL (μm)	MAR	X_{CI} (%)	CV_{CI}	CV_{CL}	Haploid karyotype formula	Stebbins' classification	2C (pg, mean ± s.d.)	1Cx (pg)
Subgenus *Albocarmesinae*	*S. filipes*	68	2.49	26.63	0.98–2.12	1.63	38.68	12.98	21.11	10m (1sat) + 7sm	2B	2.684 ± 0.042	0.671
	S. hupehensis var. *paucijuga*	34	2.13	31.50	1.15–2.41	1.68	37.61	9.75	14.03	10m (1sat) + 7sm	2B	1.407 ± 0.007	0.704
	S. koehneana	34	2.27	22.18	0.89–1.79	1.33	43.36	9.47	19.20	15m + 2sm	1B	1.571 ± 0.029	0.785
	S. ovalis	68	2.29	31.84	1.19–2.52	1.19	45.85	4.86	18.12	17m	1B	2.674 ± 0.015	0.669
Subgenus *Sorbus*	*S. amabilis*	34	2.49	37.38	1.54–3.73	1.71	38.87	21.54	24.70	9m +7sm (1sat) +1st	2B	1.401 ± 0.026	0.700
	S. pohuashanensis	34	2.08	50.06	2.05–4.08	1.46	41.35	13.23	16.66	13m (1sat) + 4sm	2B	1.664 ± 0.052	0.832
	S. scalaris	34	2.10	29.03	1.14–2.39	1.58	39.47	14.44	19.39	13m (1sat) + 4sm	2B	1.676 ± 0.044	0.838
	S. wilsoniana	34	1.95	20.68	0.89–1.72	1.25	44.84	9.57	18.37	16m + 1sm	2A	1.556 ± 0.089	0.778

L/S: Ratio of the longest/shortest chromosomes; THL: Total haploid (monoploid) length of chromosome set; VCL: Variation in chromosome length; MAR: Mean arm ratio; X_{CI}: Mean centromeric index; CV_{CI}: Coefficient of Variation of Centromeric Index; CV_{CL}: Coefficient of Variation of Chromosome Length; m: metacentric chromosome; sm: submetacentric chromosome; st: subtelocentric chromosome; sat: satellite chromosomes; s.d.: standard deviation.

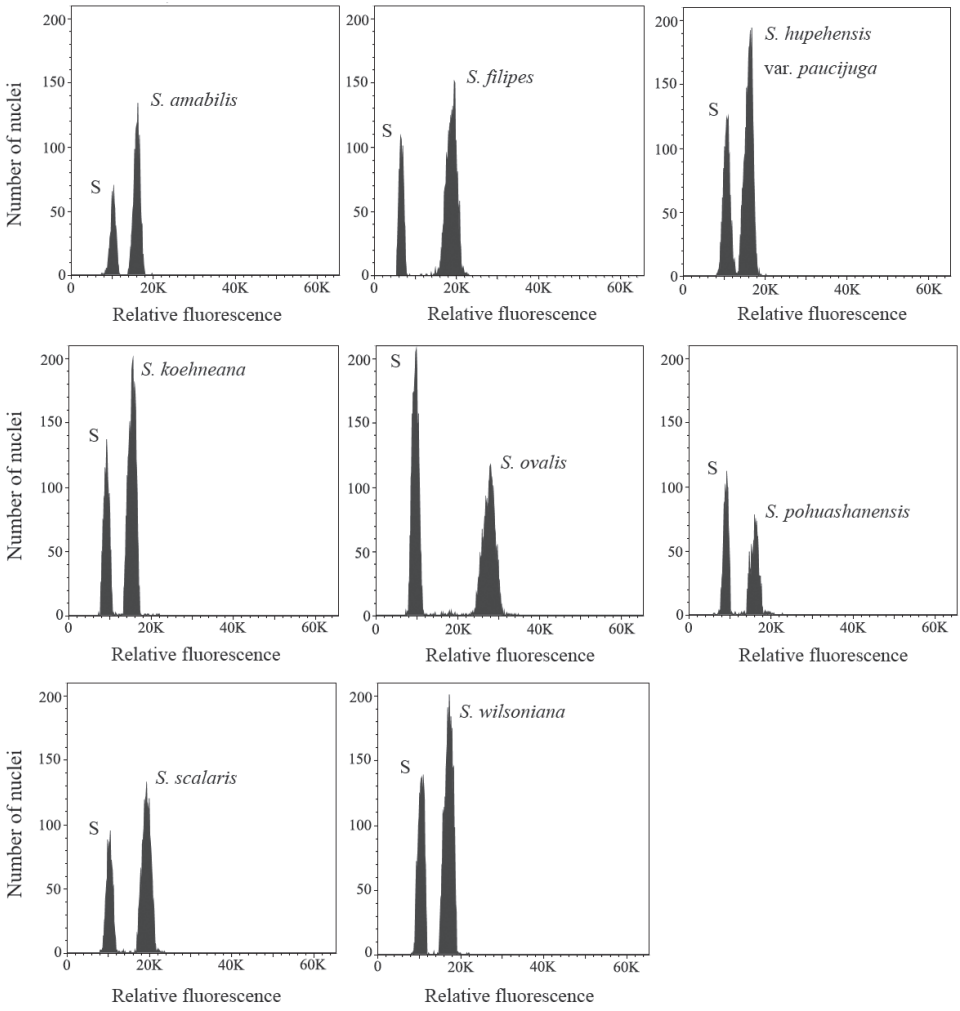

Figure 4. Flow cytometric histograms of each *Sorbus* species analyzed simultaneously with the internal standard *Oryza sativa* subsp. *japonica* 'Nipponbare' (S).

Genome sizes of the eight taxa studied are reported for the first time. Our results are consistent with the chromosome counts and the variation reported for the genus in previous studies (Pellicer et al. 2012; Xi et al. 2020). Combining the results of previous findings with the results of this study, the total range of 2C-value for the genus for diploids and tetraploids is 1.401 pg ~1.631 pg and 2.674 pg ~ 3.226 pg, respectively. In addition, our data reflect that tetraploids (mean of 1Cx = 0.670 pg) have lesser values of monoploid genome size than diploids (mean of 1Cx = 0.773 pg), indicating a genome downsizing trend in the genus. The decrease in monoploid genomes after polyploidization is usually associated with the loss of repetitive DNA, such as retroelements or retrotransposons (Leitch and Bennett 2004; Bennetzen et al. 2005; Simonin and Roddy 2018).

In *Sorbus*, ploidy levels are closely related to the reproductive strategies: diploids are considered to propagate sexually while polyploids to propagate asexually (Jankun 1993; Aldasoro et al. 1998; Dickinson 2018). Although Lu and Spongberg (2003) recorded tetraploids *S. koehneana*, we have not found any polyploid specimen for the taxon in our sampling, so additional individuals of the taxon are required in future studies and the origin for tetraploids recorded should be considered. In Europe, modern taxonomic studies (Rich et al. 2010; Robertson et al. 2010; Sennikov and Kurtto 2017) and descriptions of new species (Lepší et al. 2009; Vít et al. 2012; Németh et al. 2016; Somlyay et al. 2017) are accompanied by counts of chromosome numbers or DNA ploidy levels, based on flow cytometry. New species also have been discovered constantly from China in recent years (Li and Gao 2015; Guo et al. 2016; Yin et al. 2017) and the difficulty in taxonomy of this genus will continue to increase. Thus, diversity in ploidy levels in Chinese native species needs further analysis of additional species and individuals.

Conclusions

In this work, the first karyotype description and data about genome size are reported for eight *Sorbus* taxa. Consistent with previous studies, FCM has been found to be highly effective in estimating the relative DNA content of *Sorbus* species to infer ploidy. Further investigation on karyotype characteristics and ploidy levels of Chinese native *Sorbus* species is needed for a better understanding of the species' relationships and origins.

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RESEARCH ARTICLE

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Karyotype and COI sequences of *Chironomus sokolovae* Istomina, Kiknadze et Siirin, 1999 (Diptera,Chironomidae) from the bay of Orkhon River, Mongolia

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Abstract

Chironomus sokolovae Istomina, Kiknadze et Siirin, 1999 (Diptera, Chironomidae) is recorded from Mongolia for the first time. Eleven banding sequences determined in the Mongolian population were previously known from Altai and Yenisei populations: sokA1, sokB1, sokB2, sokC1, sokC2, sokD1, sokD2, sokE1, sokF1, sokF2 and sokG1. The additional B-chromosomes are absent. DNA-barcoding of COI gene was carried out for this species for the first time. The phylogenetic tree estimated by Bayesian inference showed a high similarity of the studied species with *Ch. acutiventris* Wülker, Ryser et Scholl, 1983 from the *Chironomus obtusidens*-group. The estimated genetic distance K2P between *Ch. sokolovae* and *Ch. acutiventris* is much lower (0.38%) than the commonly accepted threshold of 3% for species of genus *Chironomus* Meigen, 1803. Our results show that the accepted cytogenetic criteria of species level in the genus *Chironomus* are more in accordance with morphological ones of the same level, than with molecular-genetic criteria accepted for species COI genetic distance.

Keywords

Chironomidae, *Chironomus sokolovae*, COI, Diptera, DNA-barcode, karyotype, Mongolia

Introduction

At present time, nine species of *Chironomus* Meigen, 1803 identified by imago (Hayford 2005; Shcherbina and Zelentsov 2008) are recorded from Mongolia. Seven additional species identified by imago were described from the country as new for science (Sasa and Suzuki 1997) but never found after the original description. Macrozoobenthos of the Orkhon (Kharkhorin) Reservoir on the Orkhon River near Kharkhorin city and river sections upstream and downstream of the reservoir, were studied for the first time during the fieldwork of the Joint Russian-Mongolian Complex Biological Expedition in 2017. Further research has shown that the species *Chironomus sokolovae* Istomina et al. 1999 was erroneously recorded by larvae as *Ch. obtusidens* Goethebuer, 1937 (Prokin and Sazhnev 2019) from the reservoir and connected sections of the Orkhon River with a total number of specimens up to 5000 ind/ m2 and the total biomass up to 3,75 g/m2. Larvae of *Ch. commutatus* Keyl, 1960 co-occurred with *Ch. sokolovae* (Prokin and Sazhnev 2019).

The species *Ch. sokolovae* belongs to *Chironomus obtusidens*-group including six species: *Ch. acutiventris* Wülker, Ryser et Scholl, 1983; *Chironomus bavaricus* Wülker, Ryser et Scholl, 1983; *Ch. obtusidens* Goethebuer, 1937; *Ch. arcustylus* Siirin, 2002; *Ch. heterodentatus* Konstantinov, 1956; *Ch. sokolovae* Istomina, Kiknadze et Siirin, 1999 (Siirin et al. 2002; Kiknadze et al. 2016). Chromosomal polymorphism and cytogenetic differentiation in this group are still poorly studied (Siirin et al. 2002; Kiknadze et al. 2007).

The known range of the species includes the Altai Krai, Altai Republic, and the Tyva Republic in Russia (Istomina et al. 2000; Siirin et al. 2002), and Mongolia (this publication). The species was described from the Chemal River (Altai Republic) and recorded from different water bodies in the Altai region and the Tyva Republic, where it co-occurred with *Ch. acutiventris* and *Ch. heterodentatus* (Istomina et al. 2000; Siirin et al. 2002). Numerous populations of *Ch. sokolovae* larvae inhabit silty sand of the Enisey River ripal zone, near the confluence of the Bolshoy and the Maliy Enisey Rivers (environs of Kyzyl city) (Siirin et al. 2002).

The karyotype and DNA barcoding of COI gene of the *Ch. sokolovae* from the Orkhon River (Mongolia) are described in this publication with the aim of clarifying the species position within the *Ch. obtusidens*-group.

Materials and methods

Two larvae were collected from the bay of the Orkhon River upstream of the reservoir: 47°10.734'N,102°47.384'E, in September 2017. Depth 0.5 m, bottom – silty sand. Temperature 20.0 °C, pH 7.2, EC 172 mkSm/sm, TDS = 98 mg/l. For all analyses larvae were fixed in ethanol (95%). Two fourth instar larvae were used for karyotype analysis by the ethanol-orcein technique (Dyomin 1989). A Micromed-6C (LOMO,

St. Petersburg) light microscope equipped with standart (kit) oil objective ×100, and camera ToupCam5.1 (China) were used for microscopy analysis. Cytomaps from Kiknadze et al. (2016), Keyl (1962), Wülker et al. (1983) and Dévai et al. (1989) were used to identify chromosome banding.

One larva which was studied karyologically was taken for the total DNA extraction using «M-sorb-OOM» (Sintol, Moscow) kit with magnet particles according to manufacturer's protocol. For amplification of COI (cytochrome oxidase subunit I) we used primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA -3') (Evrogen, Moscow) (Folmer et al. 1994). Amplification reaction was carried out in 25 μLreaction 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 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 extention at 72 °C (8 min). PCR products were visualized on 1% agarose gels and later purified by 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). The MEGA6 was used to calculate pairwise genetic distances Kimura 2-parameter (K2P) with codon position preferences: 1st, 2nd, 3rd and noncoding sites (Kimura 1980). The Bayesian analysis was performed using the program MrBayes v.3.2.6 (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) with settings suggested by Karmokov (2019), for 1,000,000 iterations and 1000 iterations of burn-in, nst = 6 (GTP + I + G). The phylogenetic trees resulting in Bayesian inference analyses were visualized and edited using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

In addition, the 27 COI sequences of the genus *Chironomus* from "GenBank" and "Barcode of Life Data Systems" (BOLD; http://www.boldsystems.org) were analyzed. Accession numbers of used sequences in GenBank and BOLD: *Chironomus acutiventris* Wülker, Ryser et Scholl 1983 (AF192200.1), *Ch. annularius* Meigen, 1818 (AF192189.1), *Ch. aprilinus* Meigen, 1830 (KC250746.1), *Ch. balatonicus* Devai, Wulker et Scholl, 1983 (JN016826.1), *Ch. bernensis* Wülker & Klötzli, 1973 (AF192188.1), *Ch. borokensis* Kerkis, Filippova, Schobanov, Gunderina et Kiknadze, 1988 (AB740261), *Ch. cingulatus* Meigen, 1830 (AF192191.1), *Ch. commutatus* Keyl, 1960 (AF192187.1), *Ch. curabilis* Belyanina, Sigareva et Loginova, 1990 (JN016810.1), *Ch. dilutus* Shobanov, Kiknadze et Butler, 1999 (KF278335.1), *Ch. entis* Shobanov, 1989 (KM571024.1), *Ch. heterodentatus* Konstantinov, 1956 (AF192199.1), *Ch. heteropilicornis* Wülker, 1996 (MK795772.1), *Ch. luridus* Strenzke, 1959 (AF192203.1), *Ch. maturus* Johannsen, 1908 (DQ648204.1), *Ch. melanescens* Keyl, 1961 (MG145351.1), *Ch. nipponensis* Tokunaga, 1940 (LC096172.1), *Ch. novosibiricus* Kiknadze, Siirin & Kerkis, 1993 (AF192197.1), *Ch. nuditarsis* Keyl, 1961 (KY225345.1), *Ch. obtusidens* Goetghebuer, 1921 (CHMNO207-15*); *Ch. piger* Strenzke, 1959 (AF192202.1), *Ch. pilicornis* Fabricius, 1787 (BSCHI736-17*), *Ch. plumosus* (Linnaeus, 1758) (KF278217.1),

Ch. riparius Meigen, 1804 (KR756187.1), *Ch. tentans* Fabricius, 1805 (AF110157.1), *Ch. tuvanicus* Kiknadze, Siirin et Wülker, 1993 (AF192196.1), *Ch. whitseli* Sublette & Sublette, 1974 (KR683438.1). The COI sequence of *Ptychoptera minuta* Tonnoir, 1919 (KF297888) was used as outgroup in phylogenetic analysis.

Results and discussions

Karyotype of *Ch. sokolovae* from the Orkhon River, Mongolia

The chromosome set of the species is 2n = 8. The cromosome arm combination is AB, CD, EF and G (the *Chironomus* "thummi" cytocomlex). The additional B-chromosomes are absent. The chromosomes AB and CD are metacentric, EF is submetacentric, and G is telocentric. The karyotype of *Ch. sokolovae* is similar to *Ch. acutiventris*, but differs by fixed inversions in arms B, C, D and F (Kiknadze et al. 2016).

We found two different karyotypes (genotypic combinations) in both larvae from Mongolia: sokA1.1.B1.1.C1.2.D1.1.E1.1.F1.1.G1.1 and sokA1.1.B1.2.C1.2.D1.2.E 1.1.F1.2.G1.1., which consist of 11 banding sequences out of 18 known for the karyofund of this species (Istomina et al. 1999; Siirin et al. 2002) (Fig. 1).

Arm A. One banding sequence sokA1 1a-e 9a-e 2d-3e 15e-a 3f-i 12c-10a 4a-8g 14i-13a 16a-c 2c-1f 16d-19f C.

Arm B. Two banding sequences: sokB2 was found in homozygous and heterozygous state with sokB1. Both banding sequences are still not mapped.

Arm C. Two banding sequences: sokC1 1a-4g 15e-14c 16e-17a 6hg 11d-12a 11c-8a 4h-6b 12b-14b 16d-a 7d-a 6f-c 17b-22g C, found in heterozygous state with sokC2 1a-4g 15e-c 11c-8a 4h-6b 12b-13f 12a-11d 6gh 17a-16e 14c-15b 14ab 16d-a 7d-a 6f-c 17b-22g C.

Arm D. Two banding sequences: sokD1 1a-2e 23b-21e 8d-10e 4a-8c 21d-16a 13d-15e 13c-11a 3g-2f 23c-24g C found in homozygous and heterozygous state with sokD2 1a-3g 11a-13c 15e-13d 16a-21d 8c-4a 10e-8d 21e-24g C.

Arm E. One banding sequence sokE1 1a-2b 10b-5a 3e-2c 4h-3f 10c-13g C.

Arm F. Two banding sequences: sokF1 1a-i 19b-18a 2a-9f 13dc 11a-13b 10d-a 17d-14a 19c-23f C found in homozygous and heterozygous state with sokF2 1a-i 19b-18a 2a-6e 14a-17d 10a-d 13b-11a 13cd 9f-7a 19c-23f C.

Arm G. One banding sequence sokG1 was found. Not mapped.

All 11 banding sequences found in Mongolian larvae were previously known for both the Enisey and the Altai populations of studied species (Istomina et al. 1999; Siirin et al. 2002). In Mongolian and Enisey populations sokB1 banding sequence was found only in the heterozygous state, whereas in the Altai population it was in the homozygous state (Istomina et al. 1999; Siirin et al. 2002).

DNA-barcoding and phylogenetic analysis

Single nucleotide sequence of *Ch. sokolovae* for the F6.2 gene from the tissue-specific Balbiani rings locus (Alieva et al. 2004) is accessible in GenBank (AF521040), while there is no sequences for barcoding. We obtained the COI barcode for *Ch. sokolovae* with the length of 665 nucleotides (percentage A: 16.9; T: 25.3; G: 11.8; C: 12.5) and deposited it into the GenBank database with accession number – MW471100.

Pairwise genetic distances between *Ch. sokolovae* and the members of the *Ch. obtusidens* group obtained by K2P model (Kimura 1980) shown high variability. Calculated distance between sequences of *Ch. sokolovae* and *Ch. acutiventris* sequences was 0.38%, *Ch. heterodentatus* – 4.60%, *Ch. obtusidens* – 11.56%.

According to Polukonova et al. (2009) and Proulx et al. (2013) *Chironomus* COI interspecific sequence divergences is about 3%. In our study, interspecific divergence between *Ch. sokolovae* and *Ch. acutiventris* is 0.38%, that is much lower than the 3% accepted interspecific threshold. In most cases such low values of distances between species occur due to incorrect species identification when only morphological traits were used. To exclude a possibility of such mistake, we used karyological analysis and confirmed the accuracy of our species identification (Fig. 1).

Phylogenetic analysis using COI sequences showed groups of related species (Fig. 2), which concur with how these species were combined into the groups earlier on the basis of karyological and morphological traits (Kiknadze et al. 2016; Karmokov 2019). Obtained data are highly accurate (~100%) and show that *Ch. sokolovae* belongs to the *Chironomus obtusidens* group and is closest to *Ch. acutiventris*.

Conclusions

The Species *Ch. sokolovae* and *Ch. acutiventris* are similar in their karyotypes but differ in a few fixed inversions in arms B, C, D and F (Kiknadze et al. 2016). The COI sequences of these species are also similar, which could be the effect of a close relationship between the species, indicative of their recent origin (Michailova et al. 2021), or could be the result of interspecific hybridization with fixation of mtDNA in one of the parental species in the population (Guryev and Blinov 2002; Polukonova and Dyomin 2010, 2013). Study by Siirin et al. (2002) mentioned the existence of interspecific hybrids of *Ch. sokolovae* and *Ch. heterodentatus*, which means that hybridization between the *Ch. sokolovae* and *Ch. acutiventris* still occur as a result of living together in the same habitats and co-swarming. At the same time, the mtDNA sequences mostly allows the delimitation between sibling species in such groups of species as *Ch. obtusidens*, *Ch. lacunarius*, *Ch. plumosus* etc. (Fig. 2), that was originally founded based on morphological and cytogenetics traits. For a more detailed analysis of the species position within the group is needed to perform sequencing of mitochondrial and nuclear genomes, coupled with the preliminary cytogenetic and morphological analysis as obligatory.

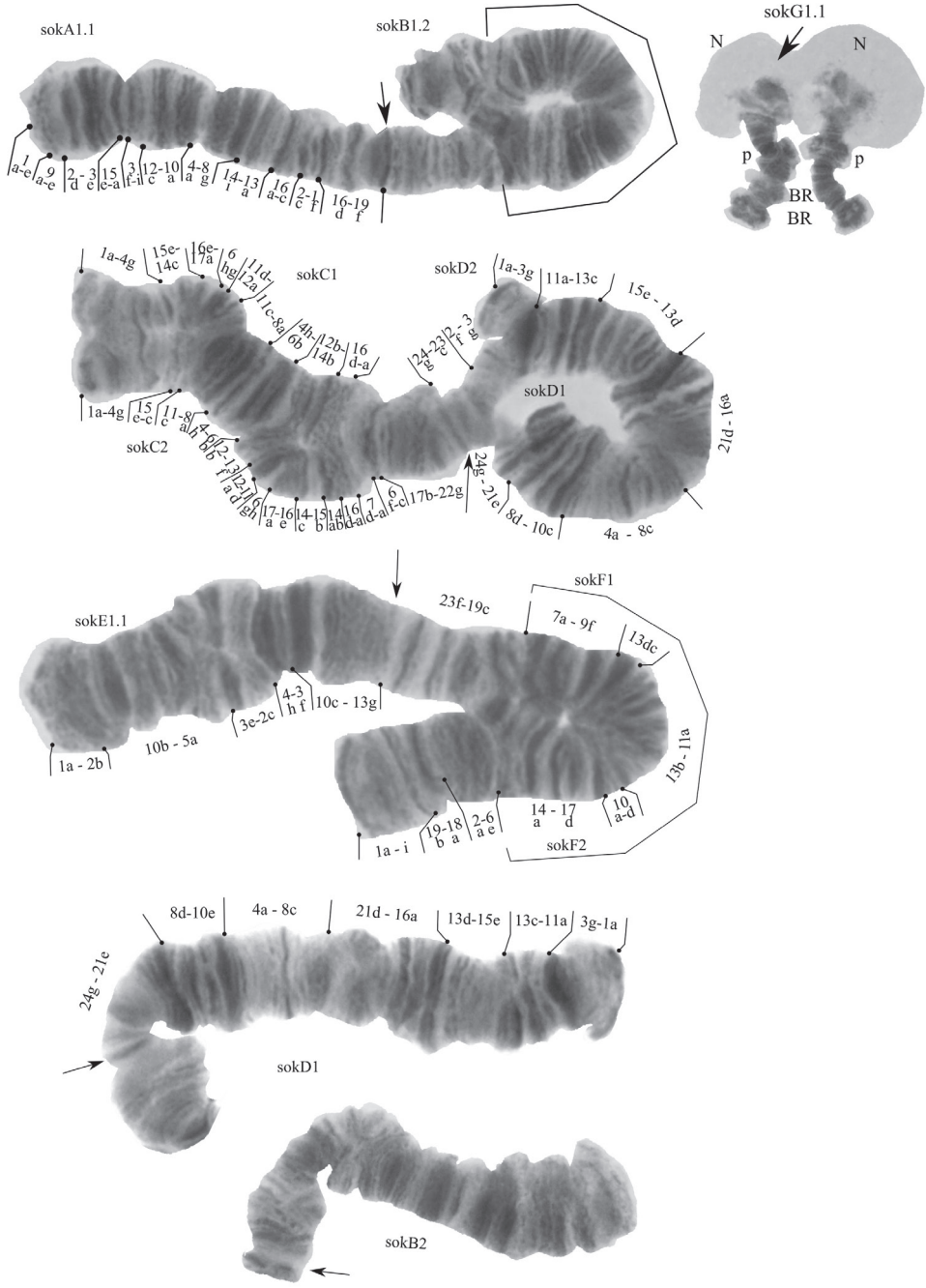

Figure 1. Banding sequences of *Ch. sokolovae* from the Orkhon River, Mongolia. Arrows indicate centromeric band, sokA1, sokB1 and etc. – genotypic combinations of banding sequences in chromosome arms, Br – Balbiani rings, N – nucleous, p – puffs. sokA1.1. – mapped according to picture 2.51.2 by Kiknadze et al. (2016).

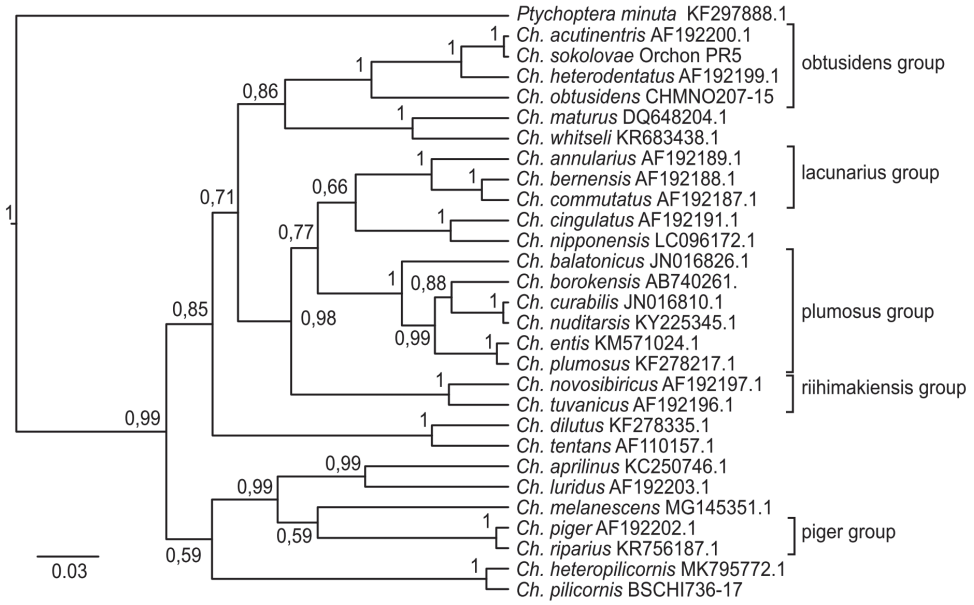

Figure 2. Bayesian tree of the analyzed samples of *Chironomus* spp. inferred from COI sequences. Species name, GenBank accession numbers and group name are shown to the right of the branches. Support values are given if they exceed 0.5. The numbers at the nodes indicate posterior probabilities.

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Unusual chromosomal polymorphism of the common shrew, *Sorex araneus* L., in southern Belarus

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Abstract

Analysis of the frequency of karyotypes and chromosomal rearrangements in the distributional ranges of four metacentric races of *Sorex araneus* Linnaeus, 1758 has revealed features that are not typical for polymorphic populations of this species. The frequency of the acrocentric karyotype and heterozygotes for fusion of acrocentric chromosomes turned out to be significantly higher than expected in case of random crossing. As an explanation for the unusual polymorphism, it has been suggested that metacentric races may hybridize with acrocentric populations that remained from the ancient chromosomal form.

Keywords

Acrocentric morph, chromosomal race, Hardy-Weinberg ratio, dihybrid and trihybrid segregation

Introduction

The polymorphic part of the karyotype of the common shrew, *Sorex araneus* Linnaeus, 1758, is represented by 12 pairs of acrocentric chromosomes (*g*, *h*, *i*, *j*, *k*, *l*, *m*, *n*, *o*, *p*, *q*, and *r*) that have a capacity to form new arm combinations in metacentric chromosomes. The recognition, naming, and systematics of populations with different karyotypes has long been an important issue in the common shrew (Bulatova et al. 2019).

Three races of the common shrew with race-specific metacentrics, West Dvina (*gm*, *hk*, *ip*, *no*, *qr*), Białowieża (*g/r*, *h/n*, *ik*, *m/p*), and Kiev (*g/m*, *hi*, *k/o*) races, have been previously described near the northern, western, and southern borders of Belarus (Fredga and Nawrin 1977; Mishta et al. 1994; Bulatova et al. 2002). An investigation of karyotypes in Belarus showed that some of race-specific metacentrics disappear from the polymorphic populations, and the frequency of the remaining ones decreases from 0.7–1.0 to 0.4–0.5 (Borisov et al. 2010, 2014). To describe this complex polymorphism, it was proposed to distinguish three new chromosomal races that differ in the set of metacentrics from the previously described chromosomal races: new race Borisov (*g/m*, *h/k*, *i*, *n/o*, *p*, *q/r*) from the West Dvina race, new race Oktiabrskiy (*g*, *r*, *h/n*, *i/k*, *m*, *p*, *o*, *q*) from the Białowieża race, and new race Svetlogorsk (*g*, *m*, *h/i*, *k/o*, *n*, *p*, *q*, *r*) from the Kiev race (Fig. 1a) (Borisov et al. 2010, 2017).

Two hypotheses of the origin of chromosomal polymorphism in populations of the common shrew in Belarus were proposed and discussed (Borisov et al. 2014). The first explains chromosomal polymorphism in Belarus by hybridization between metacentric races and ancient acrocentric populations characterized by 10 pairs of acrocentric chromosomes (*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*) that previously existed on the territory of Belarus. The acrocentric karyotypes (morphs) were revealed in some polymorphic populations of the common shrew in southern Belarus (Borisov et al. 2014). However, we have no proof of this hypothesis. Therefore, we didn't rule out the possibility that the origin of the Oktiabrskiy and Svetlogorsk races resulted from hybridization of the Białowieża and Kiev races. In wide hybrid zones, an "acrocentric peak" may occur, "that is a source of selection against the monobrachial hybrids in hybrid zones of chromosomal races with metacentrics and hence results in an increase in frequency of acrocentric morphs" (Searle 1986, p. 278). The Oxford–Hermitage hybrid zone in England and the Drnholec–Łęgucki Młyn hybrid zone in Poland are the best examples of these types of wide hybrid zones (Fedyk et al. 2019).

To investigate the causes of chromosomal polymorphism in the common shrew in southern Belarus, we collected additional samples in peripheral parts of the ranges of four chromosomal races (Fig. 1a). We planned to check whether the frequency of karyotypes in polymorphic populations corresponds to random crossing or deviates from it to obtain evidence for one of the two hypotheses under consideration for the origin of chromosomal polymorphism in southern Belarus.

Material and methods

The study area is a mosaic of forest and meadow biotopes. Animals were captured in 11 sites in the area between the Berezina, Ptich, and Pripyat rivers (Gomel and Mogilev regions) in July–September, 2017–2018 (Fig. 1b). In total, 279 individuals from 11 sites have been analyzed in this survey (Table 1).

Chromosome mounts were prepared from bone marrow and spleen cells after a routine technique with colchicine treatment (Ford and Hamerton 1956). Individual

Table 1. Collection sites, karyotypes of the individual common shrews, and polymorphic chromosome races in the southern Belarus. In the karyotype characteristics, only the variable autosomal arms are included (*g*–*r*). These arms can be presented in a dissociated state as individual acrocentric autosomes (e.g. *h*, *i*, *k*, *o*) or as components of metacentric autosomes (e.g. *hi*, *ko*). The presence of heterozygous karyotypes is indicated by a slash between the two arms, e.g. *h/i*, *k/o* (follows Bulatova et al. 2019). Chromosomal races: Ki – Kiev; Sv – Svetlogorsk; Bi – Białowieża; Ok – Oktiabrskiy, Bs – Borisov, H – hybrids.

No.	Collection site	Latitude, Longitude	Short abbreviation of races	2NA	Karyotype	Number of shrews	
						New data	Borisov et al. (2017)
Polymorphic Svetlogorsk (Sv) race (*h/i*, *k/o*)							
1	Parichi	52°48'04"N, 29°25'58"E	Sv	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	–	3
			Sv	26	*g*, *h/i*, *k/o*, *m*, *n*, *p*, *q*, *r*	–	9
			Sv			–	(12)
2	Zhlobin	52°50'32"N, 29°45'35"E	Sv	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	–	1
			Sv	26	*g*, *h/i*, *k/o*, *m*, *n*, *p*, *q*, *r*	–	8
						–	(9)
Polymorphic Oktiabrskiy (Ok) race (*h/n*, *i/k*)							
3	Lyubonichi	53°15'19N, 29°10'21E	Ok	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*,	2	2
			Ok	25	*g*, *hn*, *i/k*, *m*, *o*, *p*, *q*, *r*	4	2
			Ok	25	*g*, *h/n*, *ik*, *m*, *o*, *p*, *q*, *r*	1	2
			Ok	26	*g*, *h/n*, *i/k*, *m*, *o*, *p*, *q*, *r*	3	5
			Ok	27	*g*, *h/n*, *i*, *k*, *m*, *o*, *p*, *q*, *r*	2	1
			Ok	27	*g*, *h*, *i/k*, *m*, *n*, *o*, *p*, *q*, *r*	3	–
			Ok			(15)	(12)
4	Rozhanov Oktiabrskiy	52°35'51"N, 28°45'08"E	Ok	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	5	3
			Ok	26	*g*, *h/n*, *i/k*, *m*, *o*, *p*, *q*, *r*	19	3
			H	25	*g*, *h/n*, *i*, *ko*, *m*, *p*, *q*, *r*	2	–
						(26)	(6)
5	Zatishie (Oktiabrskiy)	52°34'26"N, 28°44'37"E	Ok	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	2	–
			Ok	26	*g*, *h/n*, *i/k*, *m*, *o*, *p*, *q*, *r*	15	–
			Sv	26	*g*, *hi*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	1	2
						(18)	(2)
6	Luchicy	52°27'16"N, 28°48'35"E	Ok	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	4	2
			Ok	26	*g*, *h/n*, *i/k*, *m*, *o*, *p*, *q*, *r*	8	–
			Bs	27	*g*, *h/k*, *i*, *m*, *n*, *o*, *p*, *q*, *r*	1	1
						(13)	(3)
7	Konkovichi	52°9'22"N, 28°43'30"E	Ok	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	6	4
			Ok	26	*g*, *h/n*, *i/k*, *m*, *o*, *p*, *q*, *r*	21	2
			Ok	25	*g*, *hn*, *i/k*, *m*, *o*, *p*, *q*, *r*	7	–
			Ok	24	*g*, *hn*, *ik*, *m*, *o*, *p*, *q*, *r*	9	–
			Bs	27	*g*, *h/k*, *i*, *m*, *n*, *o*, *p*, *q*, *r*	–	1
						(43)	(7)
8	Borki	52°05'50"N, 27°49'19"E	Ok	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	–	1
			Ok	25	*g*, *r*, *hn*, *i/k*, *m*, *o*, *p*, *q*	–	1
			Ok	27	*g*, *m*, *h/n*, *i*, *k*, *o*, *p*, *q*, *r*	–	1
			Ok	27	*g*, *r*, *h*, *n*, *i/k*, *m*, *o*, *p*, *q*	–	1
						–	(4)
Polymorphic Białowieża (Bi) race ([*gr* or *mp*], *hn*, *ik*)							
9	Turov	52°04'15"N, 27°45'48"E	Bi	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	–	4
			Bi	25	*g*, *h/n*, *i/k*, *m/p*, *o*, *q*, *r*	27	1
			H	26	*g*, *h*, *ik/ko*, *m*, *n*, *p*, *q*, *r*	–	1
			Bs	27	*g*, *h/k*, *i*, *m*, *n*, *o*, *p*, *q*, *r*	2	2
						(30)	(7)
10	Khvoyensk	52°2'11"N, 27°56'40"E	Bi	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	10	–
			Bi	25	*g/r*, *h/n*, *i/k*, *m*, *o*, *p*, *q*	8	10
			Bi	25	*g*, *hn*, *i/k*, *m*, *o*, *p*, *q*, *r*	3	3
			Bi	26	*g*, *h/n*, *i/k*, *m*, *o*, *p*, *q*, *r*	1	3
			Bi	24	*g*, *hn*, *ik*, *m*, *o*, *p*, *q*, *r*	1	2
			H	26	*g*, *h/n*, *k/o*, *m*, *i*, *p*, *q*, *r*	2	–
			Bs	27	*g*, *h/k*, *i*, *m*, *n*, *o*, *p*, *q*, *r*	2	–
						(27)	(18)
Polymorphic Kiev (Ki) race (*g/m*, *h/i*, *k/o*)							
11	Skrygalov	52°03'20"N, 28°49'10"E	Ki	28	*g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*	1	–
			Ki	25	*g/m*, *h/i*, *k/o*, *n*, *p*, *q*, *r*	25	–
			H	26	*g*, *h*, *ik/ko*, *m*, *n*, *p*, *q*, *r*	1	–
						(27)	–
						199	80

chromosome identification was carried out after G-band staining procedure with trypsin (Seabright 1971) in accordance with the international common shrew chromosome nomenclature (Searle et al. 1991).

For statistical procedures, we proceeded from a single, two- and three- locus model with a codominant type of inheritance. The calculations were based on the matrix of individual karyotypes (Table 1). The frequency of karyotypes in the polymorphic populations of the Oktiabrskiy (*h/n*, *i/k*) and Svetlogorsk (*h/i*, *k/o*) races determine by combinations of four types of gametes (dihybrid segregation) and in the Białowieża ([*g/r* or *m/p*], *h/n*, *i/k*) and Kiev (*g/m*, *h/i*, *k/o*) races by combinations of eight types of gametes (trihybrid segregation).

According to the principles of Mendelian inheritance, nine different karyotypes are theoretically possible in polymorphic populations of the Oktiabrskiy or Svetlogorsk races (dihybrid segregation): (*hn*, *ik*), (*hn*, *i*, *k*), (*h*, *n*, *ik*), (*h*, *n*, *i*, *k*), (*hn*, *i/k*), (*h*, *n*, *i/k*), (*h/n*, *ik*), (*h/n*, *i*, *k*), and (*h/n*, *i/k*). In the case of a random combination of gametes and the absence of selection, the expected frequencies of genotypes (e. g. karyotypes) are constant, their values are given in genetic reference books. The expected frequency of the acrocentric (*h*, *n*, *i*, *k*) and homozygous (*hn*, *ik*) karyotypes should be 1/16 (0.0625), and heterozygous one (*h/n*, *i/k*) – ¼ (0.25). Similarly, in populations polymorphic by three metacentrics (Białowieża and Kiev races), 27 genotypes are theoretically possible (trihybrid segregation). The expected frequency of the acrocentric ([*g*, *r* or *m*, *p*] *h*, *n*, *i*, *k*) and homozygous ([*gr* or *mp*], *hn*, *ik*) karyotypes should be 1/64 (0.0156), and heterozygous one ([*g/r* or *m/p*], *h/n*, *i/k*) – 1/8 (0.125).

The expected frequency of homozygotes and heterozygotes was estimated by the Hardy–Weinberg equation: $p^2+2pq+q^2=1$, where p^2 is the proportion of homozygotes for one of the alleles (e. g. *hn*), p is the frequency of this allele, $2pq$ is the proportion of heterozygotes (*h/n*), q^2 is the proportion of homozygotes for the alternative allele (*h*, *n*), and q is the frequency of the corresponding allele. We used a variant of the Hardy–Weinberg equation for small samples (Li 1976).

Results

The distribution of the four chromosomal races in southern Belarus is shown in Fig. 1a. In southwestern Belarus, between the Pripyat and Neman Rivers, the complete set of race-specific metacentrics for the Białowieża race was known from populations eastward to Ganzevichi (26°25'E) (Fig. 1). Only two metacentrics, *hn* and *ik*, (Oktiabrskiy race) were found in populations 100 km eastward up to the Ptich and Pripyat Rivers (Fig. 1b, *4–8*), and up to the Berezina river (Fig. 1b, *3*). South of the Pripyat River, five metacentrics of the Białowieża race were found in Turov – Chvoyensk (27°56'E) (Fig. 1b, *9*, *10*).

We found rare hybrids of the Białowieża and Kiev races (recombinants *h/n*, *k/o* and complex heterozygotes *ik/ko*) in two localities of the Białowieża race distribution and in one locality inhabited by the Kiev race along the southern bank of the Pripyat river (Fig. 1b, *9–11*). The width of the hybrid zone between the Białowieża and Kiev races

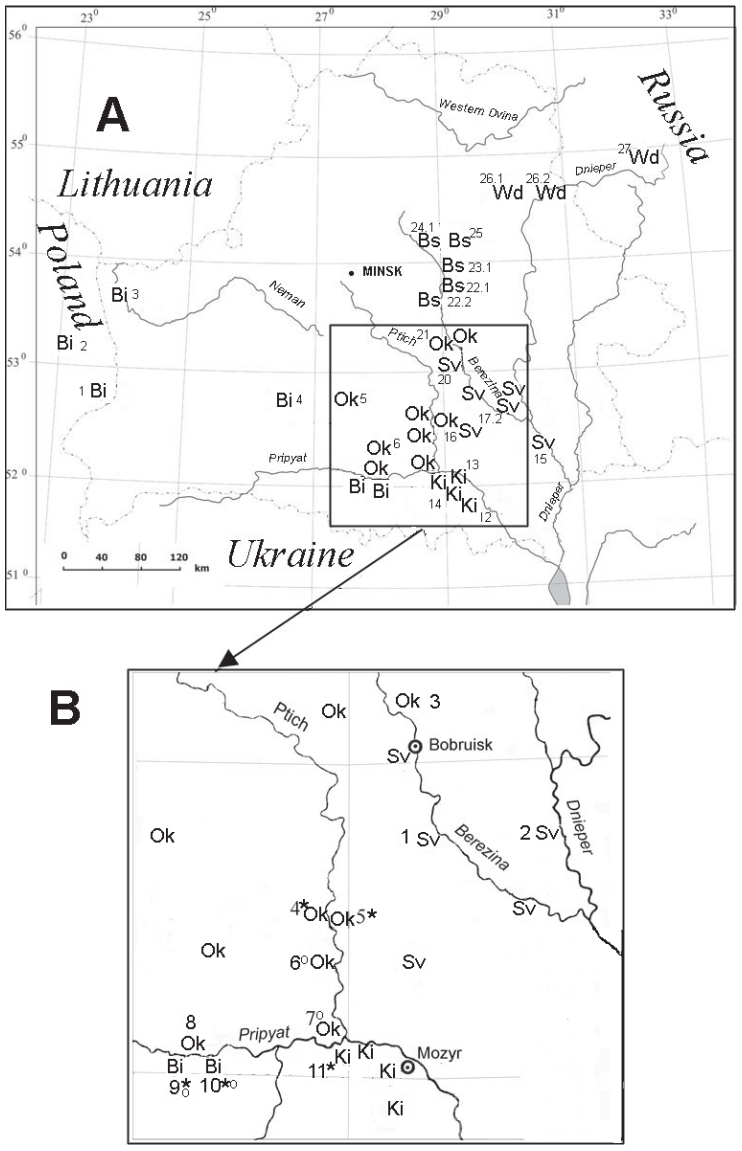

Figure 1. A Collection sites and distribution of chromosomal races *Sorex araneus* races in Belarus. Short abbreviations of races and their numbers indicate samples according to previously published (Borisov et al. 2016). Chromosomal races: Ki – Kiev; Sv – Svetlogorsk; Bi – Białowieża; Ok – Oktiabrskiy, Wd – West Dvina, Bs – Borisov **B** enlargement showing the sampling area. Site number as in Table 1, * – marked numbers of samples with single hybrids, o – marked numbers of samples with single karyotypes (*hk*) of the Borisov race.

can reach 70 kilometers. Three shrews with metacentric *hi* (Svetlogorsk race) and two hybrids of the Oktiabrskiy–Svetlogorsk races (*h/n*, *ko*) were found in localities of the Oktiabrskiy race distribution (Fig. 1b, *4*, *5*). The data allowed us to suppose that the Oktiabrskiy–Svetlogorsk hybrid zone stretches along the Ptich river.

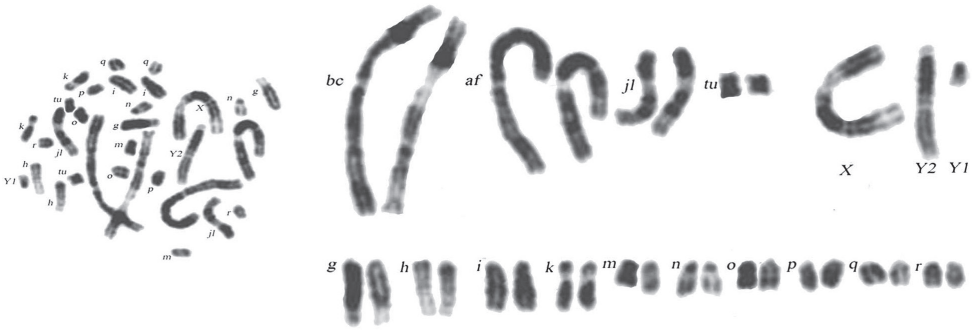

Figure 2. G-banded karyotypes of the acrocentric morph of the common shrew (male) from Konkovichi, Oktiabrskiy race.

Table 2. The frequency of karyotypes recorded in studied localities of the Oktiabrskiy and Svetlogorsk races; *O* – observed frequency, *E* – expected frequency (after Table 1).

Number of shrews	Karyotypes														
	Acrocentric			Heterozygous			Homozygous			Heterozygous			Homozygous		
	(Ok) *h*, *n*, *i*, *k* or (Sv) *h*, *i*, *k*, *o*			*h/n*, *i/k*			*hn*, *ik*			*h/i*, *k/o*			*hi*, *ko*		
	O	*E*	χ^2	*O*	*E*	χ^2	*O*	*E*	χ^2	*O*	*E*	χ^2	*O*	*E*	χ^2
Oktiabrskiy race (Ok)															
141	31	8.7	57.2	68	35.2	30.6	9	8.7	NS	–	–	–	–	–	–
	0.220	0.062	***	0.482	0.250	***	0.064	0.062							
Svetlogorsk race (Sv)															
21	4	1.3	5.6	–	–	–	–	–	–	17	5.2	26.8	–	1.3	–
	0.190	0.062	*							0.809	0.250	***		0.062	

Asterisks indicate the significance of differences: * $P < 0.05$, *** $P < 0.001$; NS – not reliable.

The studied samples allowed us to compare the observed and expected frequencies of three karyotypes (morphs) in each chromosomal race, including acrocentric karyotype, homozygous and heterozygous metacentric karyotypes in four races (Tables 2 and 3). Acrocentric karyotypes were found in all studied populations. The chromosomal formula of the acrocentric karyotype (morph): *XX / XY1Y2*, *af*, *bc*, *g*, *h*, *i*, *jl*, *k*, *m*, *n*, *o*, *p*, *q*, *r*, *tu* (Fig. 2). The area of distribution of this acrocentric karyotype in southern Belarus is undoubtedly much wider than the studied area. In all studied chromosomal races, deviations of the frequencies of karyotypes from those expected during dihybrid and trihybrid segregation were revealed. The observed frequencies of the acrocentric karyotype in two races with dihybrid segregation, Oktiabrskiy and Svetlogorsk race, are three times higher than expected and by an order of magnitude then expected in Białowieża race (trihybrid segregation). Only in the sample of the Kiev race, the difference in frequencies is not reliable, probably due to a small sample and a low expected frequency. The observed frequency of karyotype heterozygous for metacentrics in two races with dihybrid segregation are two to three times higher than expected and by an order of magnitude or more than expected in Białowieża and Kiev races (trihybrid segregation). The frequency of the karyotype homozygous for metacentrics *hn* and *ik* is

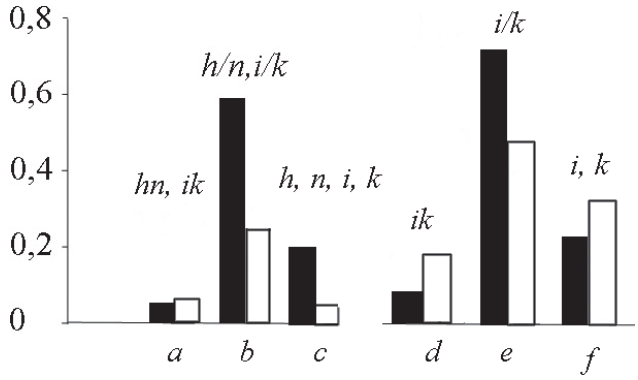

Figure 3. The frequencies of three karyotypes (the acrocentric karyotype and karyotypes homozygous and heterozygous for metacentrics) and homozygotes and heterozygotes for fusion *ik* recorded in five populations of the Oktiabrskiy race (after Table 2). Black bars – observed frequency, white – expected frequency: *a* – homozygous karyotype (*hn*, *ik*), *b* – heterozygous karyotype (*h/n*, *i/k*), *c* – acrocentric karyotype (*h*, *n*, *i*, *k*), *d* – homozygote *ik*, *e* – heterozygote *i/k*, *f* – homozygote *i*, *k*.

Table 3. The frequency of karyotypes recorded in studied localities of the Białowieża and Kiev races; *O* – observed frequency, *E* – expected frequency (after Table 1).

Number of Shrews	Karyotypes														
	Acrocentric (Bi) *g, r, m, p, h, n, i, k* or (Ki) *g, m, h, i, k, o*			**Heterozygous** [*g/r* or *m/p*] *h/n, i/k*			**Homozygous** [*gr* or *mp*], *hn, ik*			**Heterozygous** *g/m, h/i, k/o*			**Homozygous** *gm, hi, ko*		
	O	*E*	χ^2	*O*	*E*	χ^2	*O*	*E*	χ^2	*O*	*E*	χ^2	*O*	*E*	χ^2
Białowieża race (Bi)															
73	14	1.1	151.2	46	9.1	830	–	1.1	–	–	–	–	–	–	–
	0.192	0.016	***	0.630	0.125	***		0.016							
Kiev race (Ki)															
26	1	1.1	0.009	–	–	–	–	–	–	25	3.25	9.9	–	1.1	
	0.038	0.016	NS							0.961	0.125	***		0.016	

Asterisks indicate the significance of differences: *** $P < 0.001$; NS – not reliable.

close to that expected in the Oktiabrskiy race (Table 2), but such karyotypes were not found in other races due to small samples and low expected frequency. More clearly, the differences between the observed and expected frequencies in the Oktiabrskiy race are shown in Figure 3.

Not only the observed frequency of karyotypes with heterozygotes, but also the frequency of heterozygotes for fusion of acrocentric chromosomes significantly exceeded the expected by Hardy-Weinberg, while the observed frequency of homozygotes is less than expected (Table 4, Fig. 3d–f). This ratio of frequencies is maintained both for the two races as a whole and for individual samples. For example, in five samples of the Oktiabrskiy race, the ratios of the observed and expected number of heterozygotes *i/k*, calculated for small samples according to Li, 1976, are 17/13, 22/14, 15/8, 8/6, and 30/24, respectively. For homozygotes *i*, *k*, the ratios are 7/9, 8/12, 2/5, 6/7, 10/13 (Table 1, *3–7*).

Table 4. Tests for deviation from the Hardy–Weinberg equilibrium in the highly polymorphic samples of the Oktiabrskiy and Białowieża races; *O* – observed frequency, *E* – expected frequency.

Number of shrews	Homo-, heterozygotes	*O*	*E*	χ^2
214	*hn*	32	45	14.41 ***
		0.149	0.212	
	h/n	133	106	
		0.622	0.497	
	h, *n*	49	62	
		0.229	0.291	
	ik	15	38	63.30 ***
		0.070	0.177	
	i/k	150	104	
		0.701	0.487	
	i, *k*	49	72	
		0.229	0.335	

Asterisks indicate the significance of differences: *** $P < 0.001$.

Discussion

The studied polymorphic populations differ in two features from any other polymorphic populations of the common shrew:

1. The frequency of the acrocentric karyotype and karyotypes heterozygous for metacentrics both in dihybrid and trihybrid segregation turned out to be significantly higher than expected in case of random crossing.
2. The frequency of heterozygotes for fusion of acrocentric chromosomes turned out to be higher than expected according to Hardy-Weinberg in the case of random crossing, and the frequency of homozygotes, on the contrary, is less than expected.

To date, such deviations of the observed frequencies from the expected ones have not been recorded in any polymorphic populations of the common shrew. This frequency of genotypes is not typical for hybrid zones of chromosomal races of the common shrew, in particular, with an "acrocentric peak" (Fedyk et al. 2019). The frequency of karyotypes in polymorphic populations of Belarus has not been previously analyzed, and the frequency of homozygotes and heterozygotes for fusions of acrocentric chromosomes in other polymorphic populations on the territory of Belarus did not differ from those expected according to Hardy-Weinberg (Borisov et al. 2010, 2014).

In the studied hybrid zones of the common shrew, the frequency of simple heterozygotes (CIII) does not differ from that expected in the case of random crossing, and the frequency of more complex heterozygotes is constantly lower than expected even taking into account the Wahlund effect (Orlov et al. 2020). In polymorphic populations and hybrid zones of the common shrew, the advantage of heterozygotes was never observed when their frequency was higher than expected.

Therefore, the polymorphism of the studied populations is not associated with the hybridization of metacentric races (Białowieża–Kiev or Oktiabrskiy–Svetlogorsk). We may suppose that this unusual polymorphism in populations of the common shrew and the origin of the Oktiabrskiy and Svetlogorsk races is caused by the hybridiza-

tion of metacentric races and the acrocentric population remained in the *ancient chromosomal form*. Two common shrew acrocentric populations without polymorphism were found at the southern border of the species range, in the Alps and the Balkans. These populations were described as chromosomal races Cordon (Hausser et al. 1991) and Pelister (Macholán et al. 1994). Probably, acrocentric populations were previously widespread in Europe and Asia. This is indicated not only by the surviving acrocentric chromosomal races (Cordon and Pelister), but also by the races with a single race-specific metacentric, Baikal, Carlit, Lemland, and Nogat (Bulatova et al. 2019). The possibility of the origin of new chromosomal races of the common shrew by means of the distribution of chromosomal rearrangements in populations with acrocentric chromosomes has been repeatedly noted by several authors (Wójcik 1993; Brünner et al. 2002; Schipanov and Pavlova 2017).

Therefore, the fitness of the acrocentric karyotype and heterozygous metacentric karyotype may be higher than homozygous metacentric karyotypes in the studied populations. It is the increased fitness that these karyotypes became the factor responsible for the disappearance of some metacentrics, their replacement by acrocentrics, and the origin of new chromosomal races. Previously, a decrease in the frequency of race-specific metacentrics of the West Dvina race from east to west, the Białowieża race from west to east, and race-specific metacentrics of the Kiev race – from south to north in Belarus was shown (Borisov et al. 2010, 2016). Such a change in the frequency of metacentrics may reflect the direction of dispersal of chromosomal races with metacentric chromosomes in Holocene and hybridization with local acrocentric populations.

In some populations of the peripheral parts of the Białowieża and Oktiabrskiy races, there are rare individuals that are heterozygous for the arm combination *hk* with karyotype *g*, *h/k*, *i*, *m*, *n*, *o*, *p*, *q*, *r* (Fig. 1b). This karyotype (morph) was previously described for one shrew as the Turov race from Turov town (Fig. 1b, *9*) (Mishta et al. 2000). As a specific arm combination, *hk* is distributed in ten chromosomal races that are spread from the Baltic Sea to the latitude south of Minsk (Bulatova et al. 2019). The arm combination *hk* is known in the Borisov race with a frequency of 0.833 (Borisov et al. 2010) in 150 km from populations of the Oktiabrskiy race with this morph (Fig. 1a, *22.2*). It is most likely that rare morphs *g*, *h/k*, *i*, *m*, *n*, *o*, *p*, *q*, *r*, enter the Oktiabrskiy race populations from the southern populations of the Borisov race. This is facilitated by the low frequency of race-specific metacentrics, 0.42–0.45, and the high frequency of acrocentric karyotypes in such populations. In our opinion, the Bobruysk race (*g*, *h/i*, *k*, *m*, *n*, *o*, *p*, *q*, *r*) described by two shrews from Bobruysk city (Mishta et al. 2000) is one of the morphs of the Svetlogorsk race In case of random dihybrid segregation, the morph *h/i*, *k*, *o* (Aab) should occur in populations of the Svetlogorsk race with a frequency of 0.125.

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RESEARCH ARTICLE

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Chromosomes of three gall wasps of the tribe Aylacini (Hymenoptera, Cynipidae)

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Abstract

Chromosomes of two species of the tribe Aylacini (Cynipidae), *Isocolus jaceae* (Schenck, 1863) and *I. scabiosae* (Giraud, 1859) (both have 2n = 18) were studied for the first time. In addition, 2n = 20 is confirmed in a member of the same tribe, *Aulacidea hieracii* (Bouché, 1834). All chromosomes of these gall wasps are biarmed; however, they gradually decrease in size in the case of *A. hieracii*, whereas a pair of large metacentrics is characteristic of karyotypes of both *Isocolus* Förster, 1869 species. Chromosomes of the two latter gall wasps are either metacentric or submetacentric, but elements with lower centromeric indices prevail in the karyotype of *A. hieracii*. Chromomycin A_3 (CMA_3)/DAPI staining revealed single CMA_3-positive bands on a particular pair of chromosomes of all species, and these bands apparently refer to the nucleolus organizing regions (NORs). However, localization of CMA_3-positive bands differs substantially between the studied members of *Isocolus* and *Aulacidea* Ashmead, 1897. Together with normal haploid and diploid mitotic divisions, several metaphase plates with 2n = 17 containing a peculiar dicentric chromosome were found in a single male specimen of *I. scabiosae*; this appears to be the first report of an obvious dicentric in the order Hymenoptera in general. Certain aspects of the chromosome diversity and karyotype evolution within the family Cynipidae and the tribe Aylacini in particular are briefly discussed.

Keywords

Aulacidea hieracii, chromosome, Cynipidae, dicentric, gall wasps, *Isocolus jaceae*, *Isocolus scabiosae*, karyotype

Introduction

Parasitoid Hymenoptera is an extremely species-rich, taxonomically complicated and economically important insect group (Forbes et al. 2018). The overwhelming majority of this group attack insects and some other arthropods; however, certain taxa of the 'parasitoid' Hymenoptera are in fact secondarily phytophagous (Quicke 1997). Among these herbivores, gall wasps of the family Cynipidae are the most diverse, with their world fauna exceeding 1400 species (Huber 2017). The tribe Aylacini s.l. (Ronquist et al. 2015) was previously considered a paraphyletic assemblage of the least advanced members of the monophyletic Cynipidae (Nieves-Aldrey 1994; Melika 2006). However, the recent analysis by Blaimer et al. (2020) shows that other cynipoid families render the latter group paraphyletic, recovering Aylacini as a basal monophyletic lineage of Cynipidae s.str.

Chromosomes of approximately 30 species of the family Cynipidae s.l. (sensu Blaimer et al. 2020) have been studied up to now (Dodds 1938; Sanderson 1988; Abe 1994, 1998, 2006; Gokhman et al. 2015). However, many aspects of karyotype evolution of gall wasps remain unknown due to lack of data on chromosome sets of many groups, especially basal ones. Specifically, karyotypes of only two members of the Aylacini, i.e., *Xestophanes potentillae* (Retzius, 1873) and *Aulacidea hieracii* (Bouché, 1834), are known at present (Dodds 1938). Moreover, chromosomes of these gall wasps were examined in the late 1930s, and therefore only chromosome numbers and other general features of karyotype structure were described. In addition, chromosomes of the large aylacine genus *Isocolus* Förster, 1869 which is most closely related to *Aulacidea* Ashmead, 1897 (Melika 2006), remained unknown up to now. We managed to study chromosomes of *A. hieracii* as well as of two *Isocolus* species, *I. jaceae* (Schenck, 1863) and *I. scabiosae* (Giraud, 1859) using chromosome morphometry and staining with base-specific fluorochromes. The results of this work are given below.

Materials and methods

Achene galls of *I. jaceae* as well as stem galls of *I. scabiosae* and *A. hieracii* were recovered from *Centaurea scabiosa* Linnaeus, *C. stoebe* Linnaeus and *Hieracium robustum* Fries (Asteraceae), respectively, in the wild in European Russia. Specifically, these galls were collected in Moscow (55°28'N, 36°52'E), the Dubovsky District of the Volgograd Province (49°01'N, 44°43'E) and in Saratov (51°33'N, 46°04'E) in 2019–2020 by V.E. Gokhman and M.I. Nikelshparg. After keeping the galls for about a month at 5 °C, immature stages of wasps were extracted from the dissected galls. Chromosomal preparations were obtained from cerebral ganglia and developing gonads of prepupae and early pupae, respectively, generally following the protocol developed by Imai et al. (1988) with a few modifications. Specifically, these organs were first dissected in 0.5% hypotonic sodium citrate solution containing 0.005% colchicine, and then transferred to fresh hypotonic solution and incubated for 30 min at room temperature. After that, 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 for approximately half an hour and stored at room temperature. For the routine chromosome staining, the preparations were stained with a freshly prepared 3% Giemsa solution in 0.05M Sorensen's phosphate buffer (Na_2HPO_4 + KH_2PO_4, pH 6.8) for a few hours.

Fluorochrome staining with chromomycin A_3 and 4', 6-diamidino-2-phenylindole (CMA_3/DAPI) was performed according to Schweizer (1976) with certain modifications. Specifically, the slide was flooded with CMA_3 staining solution (0.5 mg/ml in McIlvaine buffer containing 5 mM $MgCl_2$), covered with a coverslip, and incubated in the dark for about ten days. The coverslip was then removed, and the slide was briefly rinsed with distilled water and air-dried. The slide was then flooded with DAPI solution (2 μg/ml in McIlvaine buffer), covered with a coverslip, and stained in the dark for 30 min. The coverslip was then removed, and the slide was briefly rinsed with distilled water before being air-dried. The preparation was then mounted in a mixture of glycerin and McIlvaine buffer (1:1) containing 2.5 mM $MgCl_2$, and sealed with rubber cement. The slide was stored in the dark prior to examination for a minimum of three days.

Mitotic divisions were studied and photographed using an optic microscope Zeiss Axioskop 40 FL fitted with a digital camera Axiocam 208 color (Carl Zeiss, Germany). To produce illustrations, the resulting images were handled with the image processing programs ZEN version 3.0 (blue edition) and GIMP version 2.10. Mitotic chromosomes were measured on ten haploid metaphase plates of *A. hieracii* and *I. scabiosae* as well as on four diploid metaphase plates of *I. jaceae* using KaryoType software version 2.0 and then classified according to the guidelines provided by Levan et al. (1964).

Results

Males of *Isocolus scabiosae* generally have karyotypes with n = 9 (Fig. 1A), whereas females have 2n = 18 (Fig. 1B). The haploid chromosome set of this species harbors a large metacentric, with all other chromosomes (either metacentric or submetacentric) forming a more or less continuous gradation in length (Fig. 1A, B; Table 1). In addition, chromosome preparations obtained from testes of *I. scabiosae* contain a small proportion of apparently diploid cells. These cells can be found on analogous preparations from hymenopteran males of many species, but at least three metaphase plates from a particular male specimen of *I. scabiosae* had 2n = 17 and contained a characteristic dicentric chromosome (Fig. 1C). CMA_3 reveals a single subterminal band on the shorter arm of a larger (apparently third) chromosome (Fig. 2A). This band obviously represents a nucleolus organizing region (NOR). On the other hand, DAPI produces uniform staining of all chromosomes (Fig. 2B).

Apart from *I. scabiosae*, only female specimens with 2n = 18 of *Isocolus jaceae* were found during the present study. The karyotype structure and fluorochrome staining

Figure 1. Mitotic chromosomes of Aylacini. *Isocolus scabiosae* **A** haploid karyogram **B** diploid karyogram **C** diploid metaphase plate with dicentric chromosome (indicated by arrow); *I. jaceae* **D** diploid karyogram; *Aulacidea hieracii* **E** haploid karyogram **F** diploid karyogram. Scale bar: 10 μm.

Table 1. Relative lengths (RLs) and centromeric indices (CIs) of chromosomes of three species of the tribe Aylacini (mean ± SD).

Chromosome no.	*I. scabiosae*		*I. jaceae*		*A. hieracii*	
	RL	CI	RL	CI	RL	CI
1	19.54 ± 0.75	48.44 ± 1.35	18.87 ± 0.94	47.14 ± 1.67	12.52 ± 0.37	46.13 ± 2.00
2	11.52 ± 0.69	36.75 ± 2.10	11.73 ± 0.58	43.74 ± 2.50	11.95 ± 0.58	31.84 ± 3.41
3	11.36 ± 0.25	41.72 ± 3.87	11.16 ± 0.48	45.34 ± 3.61	11.02 ± 0.72	48.21 ± 1.79
4	10.60 ± 0.39	36.33 ± 3.33	10.80 ± 0.18	44.14 ± 3.44	10.61 ± 0.29	29.32 ± 2.06
5	10.27 ± 0.54	43.41 ± 3.60	10.25 ± 0.20	42.24 ± 5.18	10.01 ± 0.40	26.49 ± 2.92
6	9.69 ± 0.47	44.79 ± 3.48	9.93 ± 0.29	45.82 ± 2.83	9.37 ± 0.25	26.48 ± 3.88
7	9.48 ± 0.35	39.28 ± 3.07	9.77 ± 0.29	43.33 ± 4.29	9.16 ± 0.21	27.67 ± 2.71
8	8.96 ± 0.44	42.64 ± 3.72	9.29 ± 0.29	45.73 ± 1.69	8.73 ± 0.25	26.34 ± 3.46
9	8.58 ± 0.37	36.39 ± 3.80	8.20 ± 0.70	45.25 ± 5.42	8.45 ± 0.21	25.29 ± 3.79
10	–	–	–	–	8.18 ± 0.42	25.11 ± 3.66

of chromosomes of *I. jaceae* are similar to those of the previous species, probably except for chromosomes no. 2 and 4 which apparently have higher centromeric indices (Figs 1D, 2C, D; Table 1).

In *Aulacidea hieracii*, males and females have chromosome sets with n = 10 and 2n = 20, respectively (Fig. 1E, F). All chromosomes gradually decrease in size and are clearly biarmed (Table 1). Specifically, chromosomes no. 1 and 3 are metacentric, whereas chromosomes no. 2 and 4 are submetacentric. All other chromosomes are virtually intermediate between submetacentrics and subtelocentrics (Table 1). As in the first species studied in the present work, CMA_3 visualizes a characteristic positive band on a single chromosome of the haploid set of *A. hieracii*. However, this band (an apparent NOR) is situated on the longer arm near the centromere of the first chromosome (Fig. 2E). As in *I. scabiosae* and *I. jaceae*, DAPI also does not reveal any bands on chromosomes of this species (Fig. 2F).

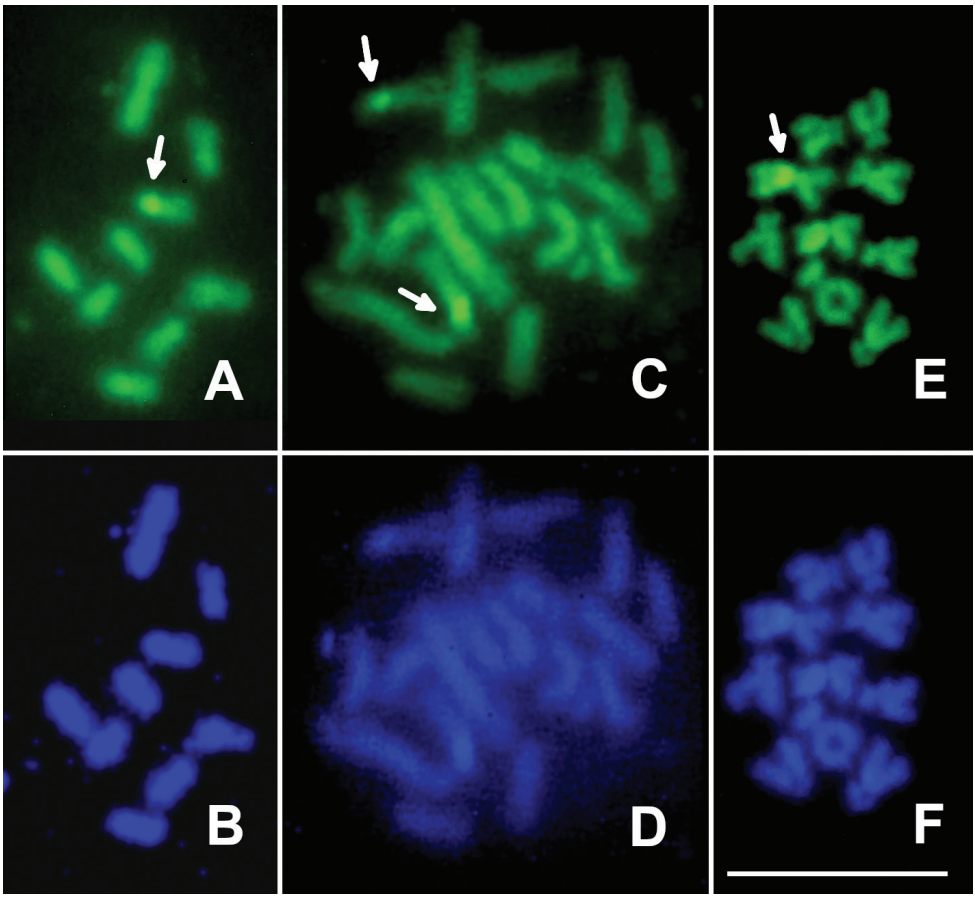

Figure 2. CMA_3/DAPI staining of chromosomes of Aylacini. *Isocolus scabiosae* (haploid metaphase plate) **A** CMA_3 staining **B** DAPI staining; *I. jaceae* (diploid metaphase plate) **C** CMA_3 staining **D** DAPI staining; *Aulacidea hieracii* (haploid metaphase plate) **E** CMA_3 staining **F** DAPI staining. Arrows indicate localization of CMA_3-positive bands. Scale bar: 10 μm.

Discussion

The above results show that *A. hieracii* and *I. scabiosae* are haplodiploid species, similar to bisexual generations of other Cynipidae studied in this respect (Sanderson 1988). As for *I. jaceae*, only female specimens were found during the present study, although males of this species are also known (Melika 2006).

The most frequent chromosome number in the family Cynipidae is n = 10 (Sanderson 1988), and this number is also confirmed for *A. hieracii*. Moreover, all chromosomes of the gall wasps studied in the present paper are biarmed. Despite karyotypes of many members of the Cynipidae s.str. sensu Blaimer et al. (2020) with n = 10 contain at least some biarmed chromosomes (Sanderson 1988), only acrocentrics were found in the chromosome sets of a few species, i.e. *Dryocosmus kuriphilus* Yasumatsu, 1951 (Abe 1994) and *Belonocnema kinseyi* Weld, 1921 (Gokhman et al. 2015, cited

as *B. treatae* Mayr, 1881; see Zhang et al. 2021). Interestingly, both *Dryocosmus* Giraud, 1859 and *Belonocnema* Mayr, 1881 represent relatively basal lineages within their clades, i.e., within the *Neuroterus*-group and *Cynips*-group respectively (sensu Liljeblad et al. 2008). Based on these data, we previously suggested that this karyotype structure is likely to be ancestral at least for members of their common clade (Gokhman et al. 2015), i.e., the tribe Cynipini (Ronquist et al. 2015). On the other hand, both *Aulacidea* and *Isocolus* belong to a separate clade, namely Aulacideini, which, in turn, is part of Aylacini s.l. (Ronquist et al. 2015; Blaimer et al. 2020). Unfortunately, further karyotypic data for the latter group, except for the chromosome number of n = 10 for *Xestophanes potentillae* (Dodds, 1938), are absent. Nevertheless, we can assume at this point that a haploid karyotype containing ten biarmed chromosomes could be ancestral for Aylacini s.l. and perhaps even for the family Cynipidae s.str. in general. In this case, chromosome sets of other members of the latter clade which contain at least some acrocentrics, might represent derived character states.

A large metacentric found in both *Isocolus* species apparently originated via chromosomal fusion, and this feature can be a synapomorphy either of the whole genus or just of *I. jaceae* and *I. scabiosae* which are very close to each other in terms of morphology (Melika 2006). Since it is difficult both to separate *Isocolus* from *Aulacidea* and to distinguish species of the former genus, chromosomal characters may help improving taxonomy of these taxa. Independent chromosomal fusions similar to those found in *Isocolus* were previously detected in other cynipoids that belong to the family Figitidae (Gokhman et al. 2011).

A characteristic dicentric chromosome found in a particular male specimen of *I. scabiosae* apparently deserves special attention. To my best knowledge, this is the first report of an obvious dicentric with two visible primary constrictions, i.e., centromeres, in the order Hymenoptera in general. This chromosomal mutation was obviously deleterious (Barra and Fachinetti 2018), since it was restricted to a small fraction of spermatogonial divisions in a single individual which otherwise produced karyotypically normal sperm cells. In *I. scabiosae*, the dicentric chromosome apparently originated via telomeric fusion of two smaller submetacentrics.

The present study also revealed single putative NORs in the haploid karyotypes of *I. scabiosae* and *A. hieracii*, in addition to the only paired NOR in the diploid set of *I. jaceae*. Among other Cynipidae, similar results were obtained for *Diplolepis rosae* (Linnaeus, 1758) using FISH with 18S rDNA probe (Gokhman et al. 2014) as well as for *B. kinseyi* (Gokhman et al. 2015). Taken together with the same number of NORs in other studied Cynipoidea, including members of the family Figitidae (Gokhman et al. 2016), presence of the single NOR is therefore characteristic of the Cynipoidea in general. However, localization of this region can substantially vary between different members of the superfamily (see also Gokhman et al. 2016).

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RESEARCH ARTICLE

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Differentiation of the frog sculpin *Myoxocephalus stelleri* Tilesius, 1811 (Actinopterygii, Cottidae) based on mtDNA and karyotype analyses

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Abstract

A molecular genetic and karyological study of the frog sculpin *Myoxocephalus stelleri* Tilesius, 1811 was carried out on an extensive sample from a large area of the species' range. A total of 42 specimens was sampled from the Sea of Japan, Sea of Okhotsk, and coastal waters off the southern Kuril Islands, which makes this sampling scheme the most comprehensive to date. The level of mtDNA polymorphism was found to be low. The haplotypes of the species formed three phylogenetic groups. The unique *M. stelleri* haplotype from the coast of Shikotan Island linked all the studied groups, indicating that it is likely ancestral. Robertsonian polymorphism was identified in the species. In all five cytotypes (I – 2n = 44, II – 2n = 43, III – 2n = 42, IV – 2n = 41, V – 2n = 40; NF = 44+2) were identified, all of which were present in the Sea of Japan. Only one (cytotype I) was found in the Sea of Okhotsk, which is probably the closest to the ancestral karyotype. The significant chromosomal polymorphism and the presence of common haplotypes in the studied samples indicate their recent origin from a common ancestor and/or relatively recent contacts within the range. The discrepancies between mtDNA and karyotypes in assigning the ancestral *M. stelleri* to the coastal waters off Shikotan Island (southern Kuril Islands) and the Sea of Okhotsk, respectively, can be explained by the different inheritance mechanisms and the rates of evolution of molecular genetic and karyological traits.

Keywords
16S rRNA, *COI*, cytochrome *b*, cytotype, haplotype, Myoxocephalinae, Robertsonian polymorphism, Sea of Japan, Sea of Okhotsk

Introduction

The genus *Myoxocephalus* Tilesius, 1811 is a large, taxonomically complex group of sculpins of the subfamily Myoxocephalinae (family Cottidae) (Neelov 1979). The modern world catalog (Fricke et al. 2020) lists 14 species and subspecies of this genus, which inhabit coastal and shelf zones of the Atlantic, Arctic, and Pacific Oceans (Neelov 1979; Parin et al. 2014; Mecklenburg et al. 2016). The northern Pacific Ocean is considered the center of diversification of this genus (Nazarkin 2000). The frog sculpin *Myoxocephalus stelleri* Tilesius, 1811 is one of the most common species of the genus, found across a wide geographic range from the Sea of Japan to the Gulf of Alaska (Mecklenburg et al. 2002). *M. stelleri* is characterized by significant morphological and ecological plasticity, as it tolerates substantial fluctuations in water temperature and salinity, being found even in river estuaries (Neelov 1979; Sokolovsky et al. 2011). Similar species, including *M. decastrensis* (Kner, 1865) and *M. raninus* Jordan et Starks, 1904 (Parin et al. 2014; Fricke et al. 2020), which are now considered to be junior synonyms of *M. stelleri*, have been described from various parts of the *M. stelleri* range.

Cytogenetic and genetic studies of *M. stelleri* have been described in two publications (Miller 2000; Podlesnykh and Moreva 2014). The only molecular genetic study thus far was based on a small number of specimens from Amur Bay, Sea of Japan, and Odyan Bay, Sea of Okhotsk (Podlesnykh and Moreva 2014). The study highlighted the limitations of using the short fragment of the *CO1* mitochondrial gene (525 bp) when constructing an adequate system of the *Myoxocephalus* species due to the lack of informative characters. The frog sculpin karyotype was first described from Amur Bay, Sea of Japan (Miller 2000). Unlike other *Myoxocephalus* species from the Sea of Japan and Sea of Okhotsk (Radchenko et al. 2020), *M. stelleri* exhibits chromosomal variation. Karyological analysis of morphologically indistinguishable individuals from Odyan Bay and Amur Bay revealed their differences not only in the diploid numbers (2n) (2n = 44 vs. 2n = 40; fundamental numbers (NF) 44 + 2), but also in the number and localization of active nucleolar organizers (NORs) (Podlesnykh and Moreva 2014). This led the authors to suggest revising the species affiliation of *M. stelleri* from the Sea of Japan (Podlesnykh and Moreva 2014). However, the same study showed that the *M. stelleri* clade is a combination of haplotypes of specimens not only from different geographic localities, but also with different karyotypes. Hence, the discrepancy between the genetic and cytogenetic results, along with the small geographic area of sampling and the limited resolution of the short *CO1* fragment, warrant further studies.

We conducted a study to determine the level of genetic and karyological differentiation within and between *M. stelleri* from the Sea of Japan, Sea of Okhotsk, and the

southern Kuril Islands. Using this karyological (N = 42) and molecular genetic data (N = 34), we also aimed to find the centers of species diversification. Our extensive sample included individuals of *M. stelleri* captured from waters near the site of the original species description – the estuary of the Bolshaya Zapadnaya Kamchatka River (Tilesius 1811) – and also from Chikhachev Bay (De Kastri), which is the type locality of *M. decastrensis* (Fricke et al. 2020). The results obtained allow assessment of the geographical variation of *M. stelleri* and identification of its probable causes.

Materials and methods

Sample collection

Fig. 1 and Table 1 show the sampling sites and number of specimens examined. Fish were collected from 2016 to 2019, at 20 localities in coastal waters of the Sea of Japan (7 localities), Sea of Okhotsk (10 localities), and off the southern Kuril Islands (3 localities). Species were identified by morphological traits (Neelov 1979). The Sea of Japan is divided into the northern and western parts according to Appendix to "Nekton of the Northwestern part of Japan (East) Sea" (Shuntov et al. 2004). The fish specimens are stored at the Ichthyological laboratory collection of the Institute of Biological Problems of the North, Far Eastern Branch, Russian Academy of Sciences, Russia (voucher numbers are listed in Table 1).

Ethics statements

This study utilizes samples collected with all applicable international, national and/or institutional guidelines for sampling, care and experimental use of organisms. The fishes studied here are not included in the IUCN Red List of Threatened Species, nor are they are listed as endangered, vulnerable, rare, or protected species in the Russian Federation. The sampling points are located beyond any protected areas.

DNA analysis

We obtained sequences for three mtDNA markers: *COI*, cytochrome *b*, and 16S rRNA. Total DNA was extracted from muscle tissues by standard phenol extraction (Maniatis et al. 1982) following tissue lysing with 1% SDS using Proteinase K (0.2 mg/mL). The following oligonucleotide primers were used to amplify and sequence the DNA markers: for *COI*, F-33 TCACAAAGACATTGGCACCCTA and R-1421 TTCACGTTTAGCAGCGAATGCTT (Moreva et al. 2017); for cytochrome *b*, L14795 CAATGGCAAGCCTACGAAAA and H15844 AGCTACTAGTGCATGACCATC (Radchenko 2005); for 16S rRNA, L2510 CGCCTGTTTATCAAAAACAT and H3080 CCGGTCTGAACTCAGATCACGT (Meyer 1993).

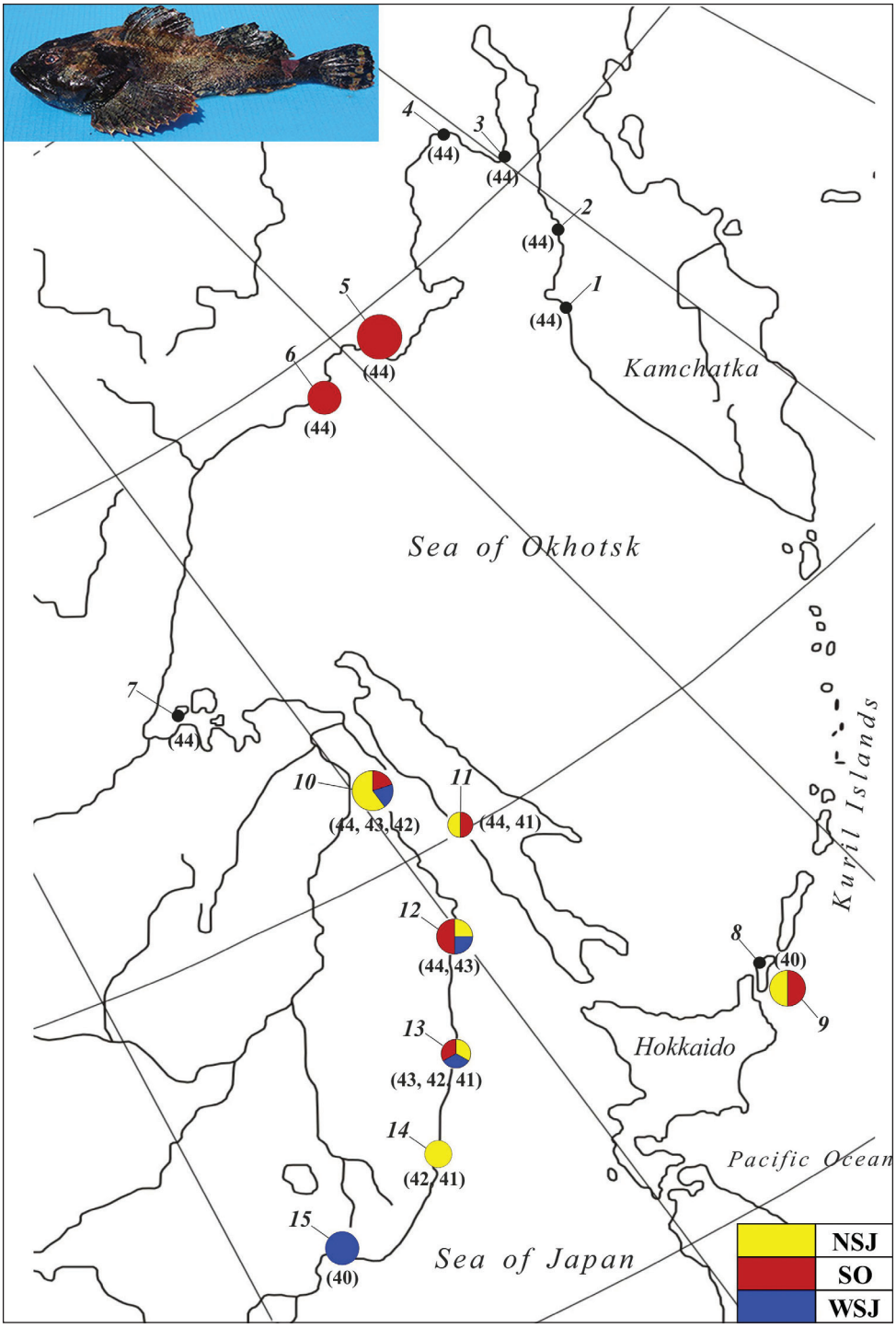

Figure 1. The sampling localities, distribution of haplotype groups (colored circles), and diploid numbers (2n in parentheses) in the cytotypes of *M. stelleri*. The locality numbers are listed in Table 1.

Table 1. Specimens of *M. stelleri* and outgroup species examined (*specimens whose mtDNA was not studied).

Species (genetic voucher)	Locality	GenBank accession numbers		
		COI	Cyt *b*	16S rRNA
Sea of Japan				
M. stelleri (1754)	Peter the Great Bay, Vostok Bay (15)	KY062754	MH595735	KY062665
M. stelleri (1755)	Peter the Great Bay, Vostok Bay (15)	MN115304	MN115340	MN097160
M. stelleri (1756)	Peter the Great Bay, Vostok Bay (15)	MN115305	MN115341	MN097161
M. stelleri (2097)	Peter the Great Bay, Russky Island (15)	MN115306	MN115342	MN097162
M. stelleri (1991)	Olga Bay (14)	MN115311	MN115347	MN097167
M. stelleri (2081)	Olga Bay (14)	MN115312	MN115348	MN097168
M. stelleri (2044)	Zolotaya Bay (12)	MN115313	MN115349	MN097169
M. stelleri (2083)	Zolotaya Bay (12)	MT258533	MT253729	MT251919
M. stelleri (2149)	Zolotaya Bay (12)	MN115314	MN115350	MN097170
M. stelleri (2150)	Zolotaya Bay (12)	MN115315	MN115351	MN097171
M. stelleri (2047)	Dzhigit Bay (13)	MN115316	MN115352	MN097172
M. stelleri (2082)	Dzhigit Bay (13)	MN115317	MN115353	MN097173
M. stelleri (2148)	Dzhigit Bay (13)	MN115318	MN115354	MN097174
M. stelleri (1985)	Aleksandrovsky Bay (11)	MN115319	MN115355	MN097175
M. stelleri (2084)	Aleksandrovsky Bay (11)	MN115320	MN115356	MN097176
M. stelleri (2151)	Chikhachev Bay (10)	MN115321	MN115357	MN097177
M. stelleri (2152)	Chikhachev Bay (10)	MN115322	MN115358	MN097178
M. stelleri (2049)	Chikhachev Bay (10)	MN115323	MN115359	MN097179
M. stelleri (1973)	Chikhachev Bay (10)	MN115324	MN115360	MN097180
M. stelleri (2153)	Chikhachev Bay (10)	MN115325	MN115361	MN097181
Pacific Ocean, Shikotan Island				
M. stelleri (1736)	Gorobets Bay (9)	MN115307	MN115343	MN097163
M. stelleri (1737)	Krabovaya Bay (9)	MN115308	MN115344	MN097164
M. stelleri (1739)	Otradnaya Bay (9)	MN115309	MN115345	MN097165
M. stelleri (1740)	Otradnaya Bay (9)	MN115310	MN115346	MN097166
Sea of Okhotsk				
*M. stelleri**	Kunashir Island, Pervukhin Bay (8)	–	–	–
M. stelleri (1748)	Taui Bay, Uta River estuary (6)	MN115326	MN115362	MN097182
M. stelleri (1749)	Taui Bay, Shestakov Bay (6)	MN115327	MN115363	MN097183
M. stelleri (1778)	Taui Bay, Shestakov Bay (6)	MN115328	MN115364	MN097184
M. stelleri (1780)	Taui Bay, Shestakov Bay (6)	MN115329	MN115365	MN097185
M. stelleri (2077)	Taui Bay, Odyan Bay (5)	MN115330	MN115366	MN097186
M. stelleri (2078)	Taui Bay, Odyan Bay (5)	MN115331	MN115367	MN097187
M. stelleri (2133)	Taui Bay, Odyan Bay (5)	MN115332	MN115368	MN097188
M. stelleri (2134)	Taui Bay, Odyan Bay (5)	MN115333	MN115369	MN097189
M. stelleri (2131)	Taui Bay, Nedorazumeniya Island (5)	MN115334	MN115370	MN097190
M. stelleri (2132)	Taui Bay, Nedorazumeniya Island (5)	MN115335	MN115371	MN097191
*M. stelleri**	Feklistov Island (7)	–	–	–
	Feklistov Island (7)	–	–	–
	Shelikhov Bay, Gizhigin Bay (4)	–	–	–
	Shelikhov Bay, Gizhigin Bay (4)	–	–	–
	Shelikhov Bay, Penzhina Bay (3)	–	–	–
	Western Kamchatka, Kvachin Bay (2)	–	–	–
	Western Kamchatka, Kvachin Bay (2)	–	–	–
	Western Kamchatka, Pichgygyn Bay (1)	–	–	–
Outgroups				
M. jaok	Sea of Japan	MN115336	MN115372	MN097192
M. brandtii	Shikotan Island	MN115337	MN115373	MN097193
M. polyacanthocephalus	Shikotan Island	MN115338	MN115374	MN097194
M. ochotensis	Sea of Okhotsk	MN115339	MN115375	MN097195

DNA sequences were aligned in Clustal W (MEGA version X: Kumar et al. 2018) with default settings, and manually edited after visual inspection. To identify haplotypes and their relationships, a median network was built in SplitsTree4 v4.12.3 (Huson and Bryant 2006) using the median-joining method (Bandelt et al. 1999). Genetic distances (d) between haplotypes were calculated using *p*-distances in MEGA X. DNA sequences were first analyzed for each gene independently, then a concatenated matrix was created in which different sets of partition scenarios were investigated. For each gene, we used MEGA X under Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) to select the optimal models of nucleotide substitutions. Bayesian Inference trees were constructed using MrBayes v3.2.1 (Ronquist et al. 2012), with the prior set to fit the evolutionary models suggested by MEGA X but allowing the parameters to be recalculated during the run. The Markov Chain Monte Carlo process was set for four chains to be run simultaneously for 1,000,000 generations, with trees sampled every 100 generations. Out of the total 10,001 obtained trees, the first 1,001 with unstable parameters of the models of nucleotide substitutions were rejected. The Bayesian analysis dynamics were controlled in Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Nodes with posterior probabilities ≥0.95 were accepted as statistically significant (Leache and Reeder 2002). We used the following four congeneric species of the subfamily Myoxocephalinae as outgroups: *Myoxocephalus jaok* (Cuvier, 1829), *M. brandtii* (Steindachner, 1867), *M. polyacanthocephalus* (Pallas, 1814), and *M. ochotensis* Schmidt, 1929.

Karyological analysis

Chromosomes were prepared by the air-drying technique (Kligerman and Bloom 1977). Slides were stained with a 4% azure eosin solution (Giemsa, Merck, Germany) in 0,067 M Sorenson's buffer contains 4.53 g KH_2PO_4 and 4.72 g Na_2HPO_4 per liter of distilled water. Metaphase plates were examined under a Leica microscope. The best metaphase plates were photographed using an AxioCam HR CCD camera with the AXIOVISION software (Carl Zeiss MicroImaging GmbH, Germany). Microscopy and imaging were carried out at the Far Eastern Center of Electron and Light Microscopy, A.V. Zhirmunsky National Scientific Center of Marine Biology (NSCMB), FEB RAS, Vladivostok.

Chromosomes were classified according to the nomenclature of Levan et al. (1964). Metacentric chromosomes with equal arms and submetacentric chromosomes with unequal arms were referred to as bi-armed chromosomes; subtelocentric chromosomes with a very short second arm and acrocentric chromosomes with an invisible second arm were referred to as uni-armed chromosomes. Submeta-subtelocentrics were also distinguished in the cytotypes. The chromosomes of this pair are morphologically highly variable. In different cells, they may look like submetacentrics or subtelocentrics. For this reason, the number of chromosome arms (NF) is indicated with both possible variants: 44 + 2.

Results

DNA analysis

For *M. stelleri*, the length of the partial *COI* was 1,009 base pairs (bp), including 16 variable sites, 8 parsimony informative sites, and 5 non-synonymous substitutions. The length of the partial cytochrome *b* was 747 bp, including 16 variable sites, 10 parsimony informative sites, and 1 non-synonymous substitution. The length of the partial 16S rRNA was 600 bp, including 1 variable site and 1 parsimony informative site. All sequence data are deposited in GenBank/NCBI (www.ncbi.nlm.nih.gov) (for accession numbers, see Table 1). Based on the parsimony informative sites, 13 haplotypes were found (Table 2).

Haplotype polymorphism is determined by single-nucleotide mutations. The minimum difference (one substitution) was found between haplotypes 3b vs. 4b, 3b vs. 5b, 1c vs. 2c, and 2c vs. 3c; the maximum difference (14 substitutions) was found between haplotypes 2a vs. 4c and 3a vs. 4c (Fig. 2). Among the identified haplotypes, five were unique (1a, 1b, 5b, 6b, and 1c) with a total frequency of 14.7%, and three were common and widespread (2a, 3b, and 4b) with a total frequency of 53%.

The haplotype network for *M. stelleri* is a star-shaped structure with the central haplotype (1b) from Shikotan Island (Fig. 2). There were three haplogroups, each formed largely by the haplotypes from the same geographic area. Group NSJ (the Northern Sea of Japan group) includes three haplotypes, of which 2a is the most common. Haplotypes of this group are found in the northern Sea of Japan and in the coastal waters off the southern Kuril Islands. Group SO (the Sea of Okhotsk group) has six haplotypes, of which 3b is the most common. This group includes all specimens from the Sea of Okhotsk, as well as individuals from the northern Sea of Japan and the southern Kuril Islands. Group WSJ (the Western Sea of Japan group) has four haplotypes and includes all specimens from the western Sea of Japan and the northern part of the Sea of Japan.

Table 2. Haplotypes of *M. stelleri* (nucleotide substitutions indicating phylogenetic groups are highlighted in bold).

Haplotype	Parsimony informative nucleotide sites			Locality (see sampling site nos. in Table 1 and Fig. 1)
	COI	*Cyt b*	16S rRNA	
1a	C**T**A**A**TTC	T**C**TTGCTTC**T**	**A**	9
2a	C**T**A**A**CTTC	T**C**TTGCTTC**T**	**A**	9, 10, 12, 13, 14
3a	C**T**A**A**CTTC	A**C**TTGCTTC**T**	**A**	11, 14
1b	C**C**A**A**T**C**TC	T**C**TTACTTC**T**	**G**	9
2b	T**C**A**A**T**C**TC	T**C**T**C**ATTTC**T**	**G**	5, 6, 10
3b	C**C**A**A**T**C**TC	T**C**TTATCTC**T**	**G**	5, 9, 11, 12, 13
4b	C**C**A**A**T**C**TC	T**C**TTATCCC**T**	**G**	5, 6
5b	C**T**A**A**T**C**TC	T**C**TTATCTC**T**	**G**	12
6b	C**C**A**A**T**C**TC	A**C**TTATCTC**T**	**G**	5
1c	C**C**G**A**T**C**TC	T**T**C**T**G**C**TTC**C**	**G**	12
2c	C**C**G**A**T**C**CC	T**T**C**T**G**C**TTC**C**	**G**	13, 15
3c	C**C**G**A**T**C**CT	T**T**C**T**G**C**TTC**C**	**G**	10, 15
4c	C**C**G**G**T**C**CC	T**T**C**T**G**C**TTT**C**	**G**	15

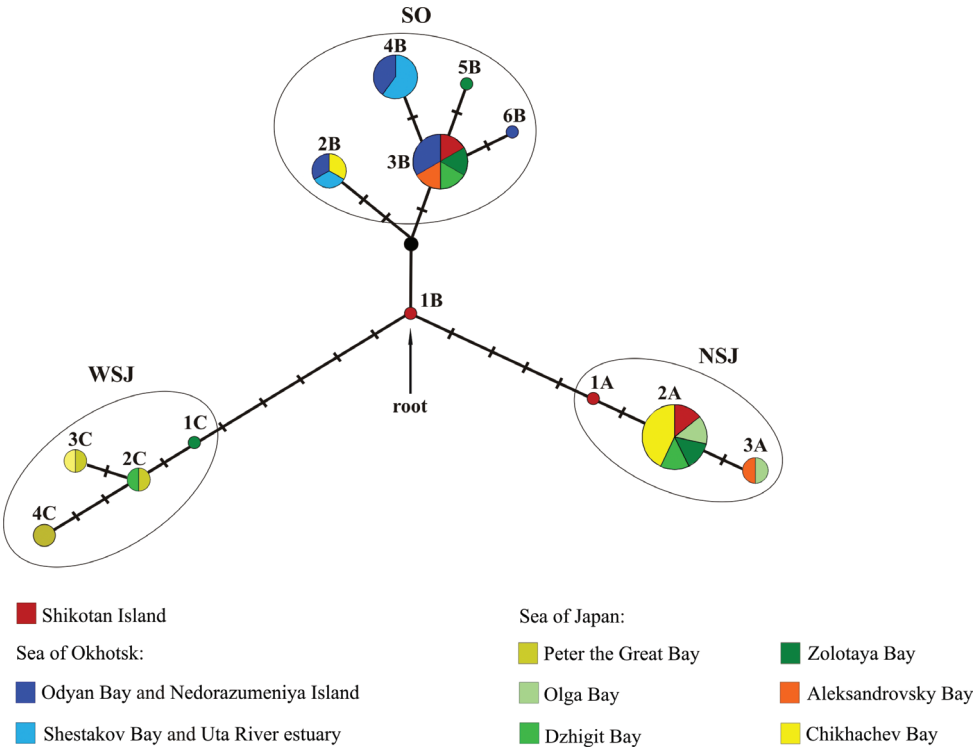

Figure 2. A median network of the *M. stelleri* haplotypes based on mtDNA sequences. Each haplotype is represented by a circle; its size corresponds to the number of individuals with this haplotype. Black circle indicates a hypothetical (unsampled) haplotype. Colors designate the geographic distribution of haplotypes. Markings on the branches are nucleotide substitutions. Ellipses are the haplotype groups: NSJ (Northern Sea of Japan group); SO (Sea of Okhotsk group); WSJ (Western Sea of Japan group).

We identified the nucleotide substitutions that distinguished the haplogroups. In Group NSJ, there is one substitution in the 16S rRNA gene (G → A at position 313) and one in *COI* (C → T at position 495). In Group SO, there is only one substitution in the cytochrome *b* gene (A → G at position 195). In Group WSJ, there are three substitutions: one in the *COI* gene (G → A at position 318) and two in cytochrome *b* (T → C at position 36, C → T at positions 75 and 639; nucleotide positions are according to our matrix).

The Bayesian tree (Fig. 3) shows three major clades that are congruent with the three haplogroups described above (NSJ, SO, and WSJ). Group WSJ is basal, while groups NSJ and SO are sister clades. The main nodes are supported by significant posterior probability values (≥0.95).

Karyological analysis

Five cytotypes were found in *M. stelleri*. Their major characteristics were described based on the analysis of 1,520 metaphase plates (Table 3 and Fig. 4).

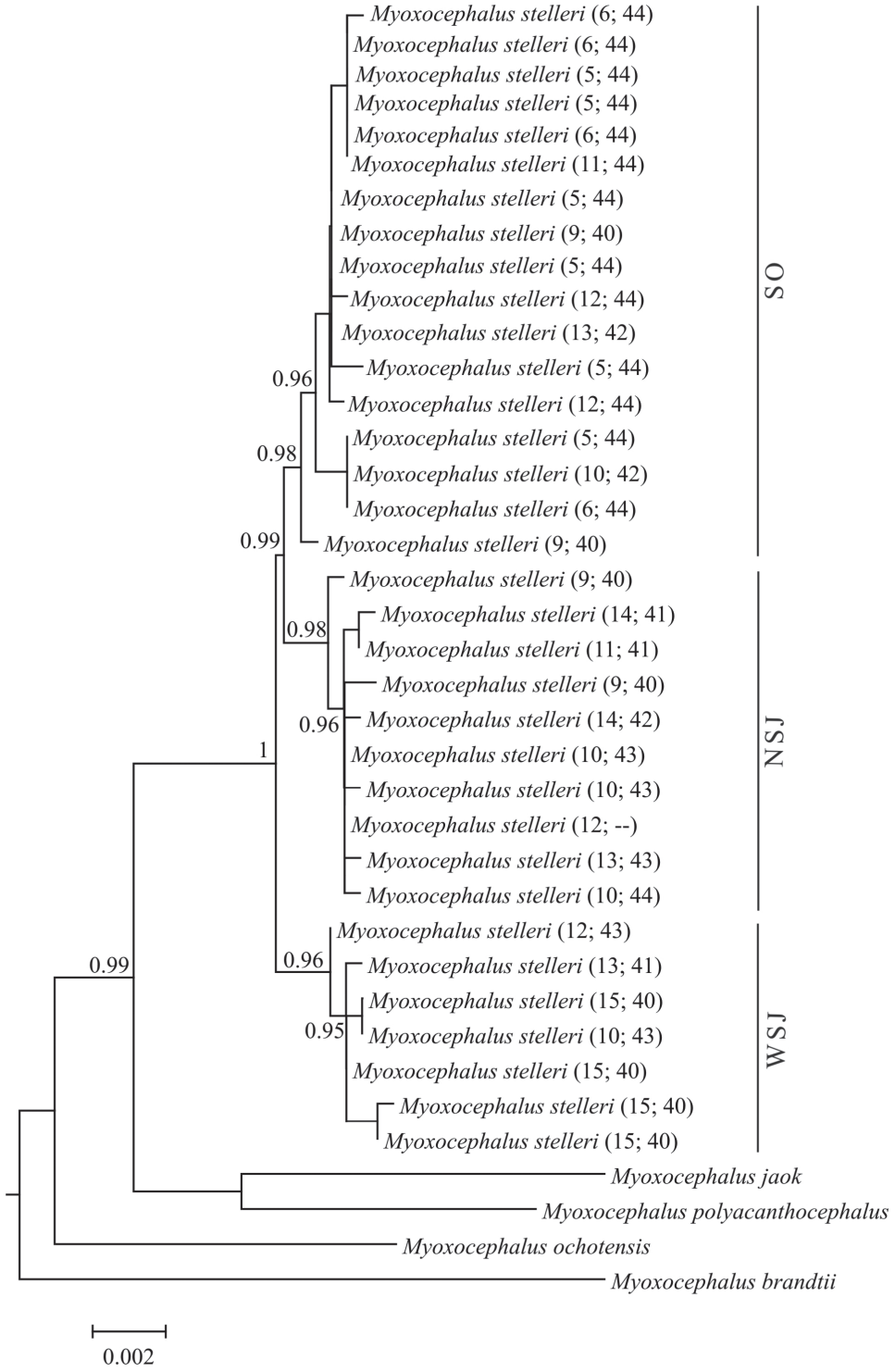

Figure 3. Bayesian Inference tree based on mtDNA marker sequences. The numerals at nodes are posterior probability values. The numerals in parentheses are the locality numbers and diploid numbers (2n) as in Fig. 1.

Table 3. The cytotypes of *M. stelleri* and their geographic distribution (juv = juvenile individuals).

No. of cytotype	Number / sex of individuals	Number of metaphase plates	Locality (see sampling sites nos. in Table 1 and Fig. 1)	Region
I	22 (10♀, 10♂, 2 juv)	551	1–7, 10–12	Sea of Okhotsk; northern Sea of Japan
II	5 (1 ♀, 2 ♂, 2 juv)	271	10, 12, 13	Northern Sea of Japan
III	3 (1 ♀, 1 ♂, 1 juv)	130	10, 13, 14	Northern Sea of Japan
IV	3 (2 ♂, 1 juv)	148	11, 13, 14	Northern Sea of Japan
V	9 (3 ♀, 5 ♂, 1 juv)	420	8, 9, 15	Western Sea of Japan; coastal waters off the southern Kuril Islands

Cytotype I: 2n = 44 chromosomes (Fig. 4A), with the first two chromosomes identified as submeta-subtelocentrics; the row of uni-armed chromosomes contains 28 subtelocentrics (pairs 2 to 15) and 14 acrocentrics (pairs 16 to 22).

Cytotype II: 2n = 43 (Fig. 4b), includes one odd submetacentric, two submeta-subtelocentrics (pair 1), 24 subtelocentrics (pairs 2 to 13), two non-homologous subtelocentrics (box), and 14 acrocentrics (pairs 14 to 20).

Cytotype III: 2n = 42 (Fig. 4c), includes two submetacentrics (pair 1), two submeta-subtelocentrics (pair 2), 24 subtelocentrics (pairs 3 to 14), and 14 acrocentrics (pairs 14 to 20).

Cytotype IV: 2n = 41 (Fig. 4d), includes one odd metacentric, two submetacentrics (pair 1), two submeta-subtelocentrics (pair 2), 20 subtelocentrics (pairs 3 to 12), two possibly non-homologous subtelocentrics (box), and 14 acrocentrics (pairs 13 to 19).

Cytotype V: 2n = 40 (Fig. 4e), includes two metacentrics (pair 1), two submetacentrics (pair 2), two submeta-subtelocentrics (pair 3), 20 subtelocentrics (pairs 4 to 13), and 14 acrocentrics (pairs 14 to 20).

All cytotypes have an equivalent number of chromosome arms: 44 + 2 (Fig. 4).

The markers of cytotypes III–V are two submetacentrics (Fig. 4c: pair 1; Fig. 4d: pair 1; Fig. 4e: pair 2), which are the largest of all chromosomes in these cytotypes; the markers of cytotype V are two metacentrics (Fig. 4e: pair 1), which are similar in size to four large subtelocentrics (Fig. 4e: pairs 4 and 5). The odd submetacentric of cytotype II (Fig. 4b) is similar in size to the large marker submetacentrics of cytotypes III–V (Fig. 4c, d: pair 1; Fig. 4e: pair 2). The size of the unpaired metacentric of cytotype IV (Fig. 4d) is close to those of the large marker metacentrics of cytotype V (Fig. 4e: pair 1). Two submeta-subtelocentrics (Fig. 4a, b: pair 1; Fig. 4c, d: pair 2; Fig. 4e: pair 3) and four large subtelocentrics (Fig. 4a, b: pairs 2 and 3; Fig. 4c, d: pairs 3 and 4; Fig. 4e: pairs 4 and 5) are clearly distinguishable in the metaphase plates and are the markers for all cytotypes. Cytotypes I and V have additional markers: two large subtelocentrics (Fig. 4A: pair 4; Fig. 4e: pair 6), which are smaller than the large marker subtelocentrics (Fig. 4A: pairs 2 and 3; Fig. 4e: pairs 4 and 5) but larger than all other uni-armed chromosomes of these cytotypes. The pairs of homologous subtelocentrics and acrocentrics in the presented karyograms are in a row from large to small (Fig. 4).

Figure 4. Karyograms of *M. stelleri*: (**a**) cytotype I, 2n = 44; (**b**) cytotype II, 2n = 43; (**c**) cytotype III, 2n = 42; (**d**) cytotype IV, 2n = 41; (**e**) cytotype V, 2n = 40 (according to Moreva and Borisenko 2017); NF = 44+2. The letter designations are as follows: m, metacentric; sm, submetacentric; sm-st, submeta-subtelocentric; st, subtelocentric; a, acrocentric chromosomes; the non-homologous subtelocentric chromosomes are boxed; the marker chromosomes are underlined once; the chromosomes similar in size to the marker metacentrics and submetacentrics are underlined twice. Scale bar: 10 μm.

Discussion

Our DNA data show that *M. stelleri* is genetically diverse at various levels: within the same locality, between geographically close localities, and between geographically distant areas. Each sample had 2 to 4 haplotypes, some of which were unique and others widespread; however, there were no haplotypes common to all studied samples. Several samples had specific haplotypes, e.g. haplotypes 4b (frequency 14.7%) and 6b were found only in the Sea of Okhotsk, while haplotype 4c (frequency 5.9%) was found only in Peter the Great Bay. Similarly, the unique haplotypes 1a and 1b were found in *M. stelleri* from the coastal waters off the southern Kuril Islands, and haplotypes 1c and 5b were found only in the Zolotaya Bay sample.

The most common haplotype (2a; frequency of 20.6%) was found in *M. stelleri* from the northern Sea of Japan (Chikhachev Bay, Zolotaya Bay, Dzhigit Bay, and Olga Bay) and from the coastal waters off the southern Kuril Islands. Haplotype 3b (17.7%) was distributed wider across the species range, from the southern Kuril Islands and the northern Sea of Japan to the Sea of Okhotsk. The less common haplotypes (3a, 2b, 2c, and 3c) were also found in more than one sample. The common haplotypes from the Sea of Okhotsk, Sea of Japan, and the southern Kuril Islands can be explained either by their origin from a common ancestor followed by dispersal from the same center, or by recent contact in various parts of the species range. A pattern of haplotype distribution with a few haplotypes being very common and others being rare or unique is frequently found in marine fish (Avise 2000).

In general, *M. stelleri* from the northern Sea of Japan exhibits higher genetic variation: eight haplotypes, many of which are shared with other geographic areas, and a low frequency of unique haplotypes. The variation between mtDNA sequences of *M. stelleri* from different localities is low, with the genetic distance between samples being approximately at the same level (Table 4). An exception is the southernmost sample from Peter the Great Bay, which is the most divergent (d = 0.34–0.52%).

The mtDNA haplotypes form three haplogroups, congruent with the three clades formed in the phylogenetic tree. These haplotypes belong to different phylogenetic groups: NSJ, SO, and WSJ. There were more differences within the Sea of Japan (groups NSJ and WSJ: d = 0.47%) than between the Sea of Okhotsk and the Sea of

Table 4. Genetic distances (d) between mtDNA of *M. stelleri* (%).

Localities (see sampling site nos. in Table 1 and Fig. 1)		1	2	3	4	5	6	7	8
1	Odyan Bay + Nedorazumeniya Island (5)								
2	Shestakov Bay + Uta River estuary (6)	0.10							
3	Peter the Great Bay (15)	0.45	0.47						
4	Olga Bay (14)	0.40	0.43	0.52					
5	Dzhigit Bay (13)	0.29	0.31	0.34	0.33				
6	Zolotaya Bay (12)	0.22	0.24	0.36	0.30	0.27			
7	Aleksandrovsky Bay (11)	0.21	0.24	0.46	0.22	0.28	0.23		
8	Chikhachev Bay (10)	0.36	0.35	0.40	0.24	0.30	0.28	0.26	
9	Shikotan Island (9)	0.24	0.26	0.44	0.24	0.28	0.24	0.21	0.26

Japan (SO and NSJ: d = 0.36%; SO and WSJ: d = 0.42%). The geographic distribution of haplotypes is not uniform (Fig. 1). *M. stelleri* from the Sea of Okhotsk have only SO haplotypes, while haplotypes from all phylogenetic groups were found in the Sea of Japan. For example, in the northern part of the sea, NSJ haplotypes were found at a frequency of 50%, SO haplotypes occurred at a frequency of 31%, and WSJ haplotypes at a frequency of 19%. However, in the western part, only WSJ haplotypes were found. In the Pacific coastal waters off the southern Kuril Islands, NSJ and SO haplotypes were found with equal frequencies.

Two unique haplotypes, 1a and 1b, were found in the coastal waters off Shikotan Island. In the Bayesian tree (Fig. 3), these haplotypes have a basal position in clades NSJ and SO; in the haplotype network (Fig. 2), haplotype 1b has a central position connecting all haplogroups, indicating that it is an ancestral haplotype. Such position of the Shikotan (Pacific) haplotypes probably shows the ancestral role that this population played in the formation of the *M. stelleri* species pattern, as evidenced by the star-shaped network with the central "founder" haplotype. This structure is typical when a population historically experienced an exponential increase in numbers after an earlier reduction in effective population size (bottleneck event) (Avise 2000).

The variation between haplotypes from different parts of the geographic range is most clear between the most distant localities: Shikotan Island vs. the western Sea of Japan, or the Sea of Okhotsk vs. the western Sea of Japan. Similar DNA differentiation has been reported for Cottidae species found in the Sea of Okhotsk and the Sea of Japan, documented both at the subspecies level, e.g. *Megalocottus platycephalus platycephalus* (Pallas, 1814) vs. *M. platycephalus taeniopterus* (Kner, 1868) (Radchenko and Petrovskaya 2019), and at the species level, e.g. species of the genera *Enophrys* Swainson, 1839 and *Porocottus* Gill, 1859 (Moreva et al. 2017, 2019). This trend has also been observed in other fish families: *Lycodes matsubarai* Toyoshima, 1985 (Zoarcidae; Sakuma et al. 2014), *Bothrocara hollandi* (Jordan et Hubbs, 1925) (Zoarcidae; Kodama et al. 2008), and the southern and northern forms of *Tribolodon hakonensis* (Günther, 1877) (Cyprinidae; Ryazanova and Polyakova 2012). In our case, the mtDNA differentiation of *M. stelleri* from the Sea of Okhotsk and the Sea of Japan is not higher than the intraspecific level (d = 0.21–0.47%).

The results of the karyological analysis are consistent with the conclusion drawn from the genetic data: *M. stelleri* is a heterogeneous species (Figs 1, 3). We have shown that *M. stelleri* has a higher chromosomal polymorphism than was previously assumed (Podlesnykh and Moreva 2014). The highest variation was found among individuals sampled from the northern Sea of Japan: 2n = 44 (cytotype I), 2n = 43 (cytotype II), 2n = 42 (cytotype III), and 2n = 41 (cytotype IV). In the western Sea of Japan, the coastal waters off the southern Kuril Islands (cytotype V; 2n = 40), and in the Sea of Okhotsk (cytotype I), individuals are stable in terms of diploid number (Table 3; Figs 1, 4).

All cytotypes of *M. stelleri* differed in the 2n (Fig. 4). Unlike other cytotypes, cytotype I lacks metacentrics and large submetacentrics. Cytotypes II–V contain different numbers of bi-armed chromosomes. All cytotypes differ in the number of subtelocentrics. Despite these differences, there are also traits common to all

chromosome sets (Fig. 4). These similarities, as well as NF (44 + 2), suggest that the metacentrics and submetacentrics in cytotypes II–V were formed through Robertsonian fusions. Karyological studies show that Robertsonian translocations are the main mechanism of structural changes in the chromosomes of marine sculpins of the family Cottidae (Vasilyev 1985; Terashima and Ida 1991; Moreva et al. 2017, 2019; Radchenko et al. 2020). Here we report the highest intraspecific variability in 2n (40 to 44) documented to date for this fish group (Arai 2011; Moreva et al. 2017, 2019; Moreva 2020).

The differences in the number of subtelocentrics between the chromosome sets (Fig. 4) indicate that these chromosomes have been involved in the formation of metacentrics and submetacentrics in cytotypes II–V. Cytotypes II–IV lack a pair of large subtelocentrics, which are the additional markers of cytotypes I and V. This fact, along with the size of the non-homologous chromosomes of cytotype II (Fig. 4b, boxed), suggests that the submetacentrics of cytotypes II–IV were formed through the Robertsonian fusion of large subtelocentrics (additional markers of cytotype I) with small subtelocentrics. Other subtelocentrics were involved in the formation of large submetacentrics of cytotype V; these were most likely the medium-sized subtelocentrics of cytotype I. Our data show that the large submetacentrics in cytotypes II–IV and cytotype V formed because of various Robertsonian fusions.

The low level of genetic differentiation in mtDNA between the studied *M. stelleri* (Fig. 3) confirms their close relationship. The karyological analysis suggests that the cytotype I (2n = 44) is close to the ancestral karyotype of the genus *Myoxocephalus* in general (Moreva and Borisenko 2017). Within Group SO (Fig. 3), the frequency of cytotype I was 76%. Among the individuals of this group that had 2n = 44, 77% were from the Sea of Okhotsk and 23% were from the northern Sea of Japan (Aleksandrovsky Bay, Zolotaya Bay). Cytotype I was not found among the individuals from the western Sea of Japan (Fig. 3, Group WSJ), which can be explained by their significant karyological divergence from the individuals from the Sea of Okhotsk and the northern Sea of Japan.

The significant chromosomal variability of individuals from the northern Sea of Japan may indicate their later divergence as compared to individuals from the western part of the sea. The assumption about different divergence times from the Okhotsk Sea is confirmed by the fact that the formation of submetacentrics of similar size in their cytotypes (II–IV and V) occurred because of different Robertsonian translocations. We assume that the polymorphism observed in individuals from the northern Sea of Japan could arise in the relatively recent past in the following way: the chromosomal rearrangement that took place in one or more individuals (Fig. 4b, cytotype II) then became fixed (Fig. 4c, cytotype III) and distributed among *M. stelleri* from the northern Sea of Japan. Cytotype IV (Fig. 4d) from the northern Sea of Japan could be formed because of subsequent hybridization of karyotypically different individuals from the northern and western Sea of Japan. Despite the lack of isolation and their geographic proximity to individuals from the northern Sea of Japan (cytotypes II–IV; Fig. 1), the modern *M. stelleri* from the western part of the sea have a uniform number of chromo-

somes (cytotype V) (Miller 2000; Podlesnykh and Moreva 2014). This may indicate the lack of secondary contact between *M. stelleri* from the western and northern Sea of Japan at present.

The frog sculpins from the coastal waters off the southern Kuril Islands deserve special attention. In the geological history of the basins of the Far Eastern seas, several long-lasting barriers existed during the regressions of the level of the World Ocean, separating the unified area of the species. One of them, extending along Sakhalin/Hokkaido/Honshu, kept the Japanese Sea and South Kuril populations separated for a long time. During this period of geographic isolation, the chromosome sets of individuals from the western Sea of Japan and the southern Kuril Islands could have formed independently of each other, despite their visual identity (cytotype V). One of the southern Kuril specimens has haplotype 1b, which connects all other haplotypes found in *M. stelleri*, and may thus be the closest to the ancestral haplotype. Contrary to the results of mtDNA analysis, the karyological data point to a significant divergence of *M. stelleri* (2n = 40) from the coastal waters off the southern Kuril Island. This discrepancy may be caused by the high rate of change in karyological traits compared to that of DNA markers (Lukhtanov and Kuznetsova 2009).

Our results do not support the assumption that the individuals of *M. stelleri* from the Sea of Japan and the Sea of Okhotsk belong to different species (Podlesnykh and Moreva 2014). Judging by the values of genetic and chromosomal polymorphism, the low level of genetic differentiation, and the frequency and spatial distribution of haplotypes, this species is relatively young. It can be suggested that the fragmentation of the single range of *M. stelleri*, which took place during the last Quaternary glaciation 20–25 thousand years ago, resulted in the geographic isolation of populations from the Sea of Japan and Sea of Okhotsk. The accumulation of Robertsonian translocations in *M. stelleri* from the western Sea of Japan presumably occurred during this period. We suggest that, as the glaciation ended, the ocean transgression and restoration of connections between the seas of the Northwest Pacific (Nishimura 1964; Lindberg 1972; Yokoyama et al. 2007; Matsuzaki et al. 2018) allowed the secondary colonization of *M. stelleri*, which likely resulted in a significant chromosomal polymorphism in the northern Sea of Japan. The paleoclimate events also influenced the distribution of genetic variation in *M. stelleri*. The formation of the three phylogenetic groups is likely associated with the geographic isolation of ancestral forms in different parts of the species range, and with the limited gene exchange and secondary contact because of migrations after the last ice age (Briggs 2003; Altukhov 2005).

Conclusions

Our data have revealed a similarity in chromosome sets, as well as low levels of differentiation in mtDNA between *M. stelleri* from the Sea of Okhotsk, the Sea of Japan, and the coast of Shikotan Island (southern Kuril Islands), thus, confirming that these

represent a single, yet variable, species across its geographic range. The significant chromosomal polymorphism and the presence of common haplotypes in the studied samples indicate their recent origin from a common ancestor and/or relatively recent contacts within the range. The contribution of different Robertsonian translocations to the formation of cytotypes (II–IV and V) of individuals from the northern and western Sea of Japan allows us to conclude that they diverged from the Sea of Okhotsk *M. stelleri* independently of each other. The star-shaped structure of the haplotype network with a central ancestral haplotype indicates a connection between all constituent haplotypes and the ancestral position of the southern Kuril Islands haplotype (1b). The discrepancy in assessments of the divergence of individuals from the Sea of Okhotsk and waters off the southern Kuril Islands can be attributed to the different mechanisms of inheritance and rates of evolution of karyological traits and mtDNA markers.

The results of our study demonstrate the necessity of further detailed analysis of the widely sampled *M. stelleri* populations from the Pacific part of the species range and from the southern Sea of Okhotsk. Such studies should include differential chromosome staining and SNP markers, as well as comparative morphological and osteological analyses using up-to-date methods.

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Chromosomal and DNA barcode analysis of the *Melitaea ala* Staudinger, 1881 species complex (Lepidoptera, Nymphalidae)

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Abstract

The species of the *Melitaea ala* Staudinger, 1881 complex are distributed in Central Asia. Here we show that this complex is a monophyletic group including the species, *M. ala*, *M. kotshubeji* Sheljuzhko, 1929 and *M. enarea* Fruhstorfer, 1917. The haploid chromosome number n=29 is found in *M. ala* and *M. kotshubeji* and is, most likely, a symplesiomorphy of the *M. ala* complex. We show that *M. ala* consists of four subspecies: *M. ala zaisana* Lukhtanov, 1999 (=*M. ala irtyshica* Lukhtanov, 1999, **syn. nov.**) (South Altai, Zaisan Lake valley), *M. ala ala* (Dzhungarian Alatau), *M. ala bicolor* Seitz, 1908 (North, East, Central and West Tian-Shan) and *M. ala determinata* Bryk, 1940 (described from "Fu-Shu-Shi", China). We demonstrate that *M. kotshubeji kotshubeji* (Peter the Great Mts in Tajikistan) and *M. kotshubeji bundeli* Kolesnichenko, 1999 (Alai Mts in Tajikistan and Kyrgyzstan) are distinct taxa despite their geographic proximity in East Tajikistan. *Melitaea enarea* is widely distributed in the southern part of Central Asia and is sympatric with *M. kotshubeji*.

Keywords

chromosome, *COI*, DNA barcode, karyosystematics, *Melitaea*, taxonomy

Introduction

This work is a continuation of a series of publications (Lukhtanov and Kuznetsova 1989; Pazhenkova et al. 2015; Pazhenkova and Lukhtanov 2016; Lukhtanov 2017) devoted to the analysis of chromosomal and mitochondrial haplotype diversity and taxonomy of butterflies of the species-rich butterfly genus *Melitaea* Fabricius, 1807. The combination of molecular and cytogenetic methods is a useful tool for taxonomic studies (Lukhtanov et al. 2015; Pazhenkova and Lukhtanov 2019) and can be a good addition to morphological analysis of taxonomically complicated groups of species (Lukhtanov et al. 2016). In our previous papers, we applied analysis of the DNA barcodes and karyotypes to study the genetic and taxonomic structure of the *M. didyma* (Esper, 1779) (Pazhenkova et al. 2015; Pazhenkova and Lukhtanov 2016) and *M. persea* Kollar, 1849 (Lukhtanov 2017) species complexes. The aim of this work is to study a complex of species close to *M. ala* Staudinger, 1881.

The species of this complex are distributed in Central Asia (Kolesnichenko 1999). According to Kolesnichenko (1999), this complex consists of the following species: *Melitaea ala* Staudinger, 1881, *M. kotshubeji* Sheljuzhko, 1929, *M. ninae* Sheljuzhko, 1935, *M. chitralensis* Moore, 1901, and *M. enarea* Fruhstorfer, 1917. According to van Oorschot and Coutsis (2014), this complex consists of the following species: *M. acraeina* Staudinger, 1881, *M. ninae* Sheljuzhko, 1935, *Melitaea ala* Staudinger, 1881, *M. didymina* Staudinger, 1895, *M. chitralensis* Moore, 1901, *M. enarea* Fruhstorfer, 1917, *M. bundeli* Kolesnichenko, 1999, *M. kotshubeji* Sheljuzhko, 1929, *M. sutschana* Staudinger, 1881 and *M. yagakuana* Matsumura, 1927 (the latter taxon is usually considered a subspecies of *M. sutschana*, e.g. see Higgins, 1941).

Molecular phylogenetic analysis (Leneveu et al. 2009) demonstrated that *M. ala* and *M. enarea* (cited in the article as *M. permuta* Higgins, 1941) are sister species, and *M. acraeina* is a phylogenetically distant species which is a sister to the lineage (*M. ala* + *M. enarea*). *Melitaea sutschana* was found as a member of the *M. didyma* species complex which is a sister to the lineage ((*M. acraeina* + (*M. ala* + *M. enarea*)) (Leneveu et al. 2009). In our study, we focused on the analysis of the *M. ala* lineage. We did not include *M. ninae*, *M. didymina* and *M. chitralensis* in the analysis, since for these species there has been no material suitable for chromosomal and molecular studies.

Materials and methods

Chromosomal analysis

Karyotypes of four samples of *M. kotshubeji kotshubeji* were studied as previously described (Lukhtanov et al. 2014; Vishnevskaya et al. 2016). Briefly, gonads were removed from the adult male abdomen and placed into freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing the butterfly in the field. Testes were stored in the fixative for 3–36 months at +4 °C. Then the gonads were

stained in 2% acetic orcein for 5–10 days at +18–20 °C. Different stages of male meiosis, including metaphase I (MI) and metaphase II (MII) were examined using an original two-phase method of chromosome analysis (Lukhtanov et al. 2006, 2008). Leica DM2500 light microscope equipped with HC PL APO 100×/1.44 Oil CORR CS lens and S1/1.4 oil condenser head was used for bright-field microscopy analysis. A Leica DM2500 light microscope equipped with HC PL APO 100×/1.40 OIL PH3 lens was used for phase-contrast microscopy analysis.

Molecular methods and DNA barcode analysis

Standard *COI* barcodes (658-bp 5' fragment of *mitochondrial cytochrome oxidase subunit I*) were studied as previously described (Lukhtanov et al. 2014; Vishnevskaya et al. 2016). *COI* sequences were obtained from 34 specimens representing the *M. ala* species group and outgroups (*M. telona* Fruhstorfer, 1908 and *M. alatauica* Staudinger, 1881). Legs were used as a source for DNA isolation

Legs from 6 specimens (*M. kotshubeji bundeli* Kolesnichenko, 1999) were processed in the Department of Karyosystematics of Zoological Institute of the Russian Academy of Sciences using primers and protocols described by Shapoval et al. (2017). Sequencing was carried out at the Research Resource Center for Molecular and Cell Technologies of St. Petersburg State University.

Legs from 28 specimens of *Melitaea* spp. were processed in the the Canadian Centre for DNA Barcoding (**CCDB**, Biodiversity Institute of Ontario, University of Guelph) using their standard high-throughput protocol described by Hajibabaei et al. (2005), Ivanova et al. (2006) and deWaard et al. (2008). The set of voucher specimens of butterflies is kept in the Zoological Institute of the Russian Academy of Science (St. Petersburg) and in the McGuire Center for Lepidoptera and Biodiversity (**MGCL**), Florida Museum of Natural History, University of Florida, Gainesville, Florida, USA. Photographs of these specimens, as well as collecting data are available in the of Life Data System (BOLD), projects Butterflies of Palearctic (BPAL) and Butterflies of Palearctic Part B (BPALB) at http://www.boldsystems.org/.

We also used 30 published *COI* sequences for DNA barcode analysis (Leneveu et al. 2009; Lukhtanov et al. 2009; Ashfaq et al. 2013; Pazhenkova et al., 2015; Pazhenkova and Lukhtanov 2016; Lukhtanov 2017) (Table 1).

Sequences were aligned using the BioEdit software (Hall 1999) and edited manually. Phylogenetic hypotheses were inferred using Bayesian inference as described previously (Vershinina and Lukhtanov 2010; Przybyłowicz et al. 2014; Lukhtanov et al. 2016). Briefly, the Bayesian analysis was performed using the program MrBayes 3.2 (Ronquist et al. 2012) with default settings. Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. We checked runs for convergence and proper sampling of parameters [effective sample size (ESS) > 200] using the program Tracer v1.7.1 (Rambaut et al. 2018). The first 25% of each run was discarded as burn-in. The consensus of the obtained trees was visualized using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 1. Specimens of *Melitaea* spp. used in the DNA barcode analysis.

Species and subspecies	Species name as found in GenBank	Field code or BOLD number	GenBank number	Country	Locality	Reference
M. acentria	*M. acentria*	BOLD:BPAL2191-13	**KY777529**	Israel	Hermon	Lukhtanov 2017
M. acraeina	*M. acraeina*	BOLD:GBLN1879-09	**FJ462229**	Uzbekistan	Komsomolobad	Leneveu et al. 2009
M. ala ala	*M. ala*	BPALB179-16; CCDB-25458_G12	**MW672072**	Kazakhstan	Dzhungarian Mts, Kopal, 45.08°N, 79.07°E	This study
M. ala ala	*M. ala*	BOLD:BPAL039-10	**MW672074**	Kazakhstan	Taldy-Kurgan region, Kysylagash	This study
M. ala ala	*M. ala*	BOLD:BPAL3407-16	**MW672077**	Kazakhstan	Taldy-Kurgan region, Kyzylagash	This study
M. ala bicolor	*M. ala*	BOLD:GBLN1877-09	**FJ462231**	China	Tian-Shan	Leneveu et al. 2009
M. ala bicolor	*M. ala bicolor*	BOLD:LOWA355-06	**FJ663775**	Kyrgyzstan	Moldatoo Mts, 41.5°N, 74.62°E	Lukhtanov et al. 2009
M. ala bicolor	*M. ala bicolor*	BOLD:LOWA356-06	**FJ663774**	Kyrgyzstan	Moldatoo Mts, 41.5°N, 74.62°E	Lukhtanov et al. 2009
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL2288-14	**MW672075**	China	Xinjiang, Kunges Valley	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL2289-14	**MW672076**	China	Xinjiang, Kunges Valley	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL012-10	**MW672079**	Kazakhstan	Kirgizsky Mts, Kaindy	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL013-10	**MW672080**	Kazakhstan	Kirgizsky Mts, Kaindy	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL026-10	**MW672081**	Kyrgyzstan	Talassky Mts, Kara-Bura Pass	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL027-10; RPVL-00027	**MW672082**	Kyrgyzstan	Talassky Mts, Kara-Bura Pass	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL3499-16	**MW672089**	Kyrgyzstan	Talassky Mts, Kara-Bura Pass	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL3500-16	**MW672090**	Kyrgyzstan	Talassky Mts, Kara-Bura Pass	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL3501-16	**MW672091**	Kyrgyzstan	Talassky Mts, Kara-Bura Pass	This study
M. ala bicolor	*M. ala bicolor*	BOLD:BPAL009-10; CCDB-03024-RPVL-00009	**MW672078**	Kazakhstan	Kirgizsky Mts, Merke River	This study
M. ala irtyshica	*M. ala*	BOLD:BPALB181-16	**MW672073**	Kazakhstan	Zyryanovsk region, 49.62°N, 83.62°E	This study
M. ala irtyshica	*M. ala*	BOLD:BPAL3481-16	**MW672083**	Kazakhstan	Zyryanovsk region, 49.62°N, 83.62°E	This study
M. ala irtyshica	*M. ala*	BOLD:BPAL3483-16	**MW672085**	Kazakhstan	Zyryanovsk region, 49.62°N, 83.62°E	This study
M. ala irtyshica	*M. ala*	BOLD:BPAL3484-16; CCDB-25456_F04	**MW672086**	Kazakhstan	Zyryanovsk region, 49.62°N 83.62°E	This study
M. ala irtyshica	*M. ala*	BOLD:BPAL3485-16	**MW672087**	Kazakhstan	Zyryanovsk region, 49.62°N, 83.62°E	This study
M. ala irtyshica	*M. ala*	BOLD:BPAL3486-16	**MW672088**	Kazakhstan	Zyryanovsk region, 49.62°N 83.62°E	This study
M. ala zaisana	*M. ala zaisana*	BOLD:LOWA174-06	**FJ663777**	Kazakhstan	Kurchumski Khrebet 48.47°N, 84.12°E	Lukhtanov et al. 2009
M. ala zaisana	*M. ala zaisana*	BOLD:LOWA175-06	**FJ663776**	Kazakhstan	Kalgutynski Pass, 48.47°N 84.12°E	Lukhtanov et al. 2009
M. alatauica	*Mellicta alatauica*	BOLD:PALB177-16	**MW672064**	Kazakhstan	Dzhungarian Mts, Kopal, 45.08°N, 79.07°E	This study
M. alatauica	*Mellicta alatauica*	BOLD:LOWA273-06	**FJ663811**	Kazakhstan	Dshungarski Alatau, Koksu, 44.72°N, 79.0°E	Lukhtanov et al. 2009
M. alatauica	*Mellicta alatauica*	BOLD:LOWA274-06	**FJ663810**	Kazakhstan	Dshungarski Alatau, Koksu, 44.72°N, 79.0°E	Lukhtanov et al. 2009
M. casta	*M. casta*	BOLD:BPAL2306-14	**KY777552**	Iran	Lorestan	Lukhtanov 2017
M. deserticola	*M. deserticola*	BOLD:BPAL3124-15	**KY086157**	Israel	Jerusalem	Pazhenkova and Lukhtanov 2016
M. didyma	*M. didyma*	BOLD:BPAL2495-14	**KT874733**	Austria	Tirol	Pazhenkova et al. 2015
M. didymoides	*M. didymoides*	BOLD:BPAL3493-16	**KY086178**	Russia	Buryatia	Pazhenkova and Lukhtanov 2016

Species and subspecies	Species name as found in GenBank	Field code or BOLD number	GenBank number	Country	Locality	Reference
M. enarea	*M. enarea*	BOLD:BPAL2656-14	**MW672065**	Tajikistan	Tabakchi, 37.85° N, 68.98°E, 1200 m	This study
M. enarea	*M. enarea*	BOLD:BPAL2657-14	**MW672066**	Tajikistan	Chaltau, 37.9550°N, 69.1403°E; 1041m	This study
M. enarea	*M. enarea*	BOLD:BPAL2658-14	**MW672067**	Tajikistan	Chaltau, 37.9550°N, 69.1403°E; 1041m	This study
M. enarea	*M. enarea*	BOLD:BPAL2659-14; CCDB-17967_H10	**MW672068**	Tajikistan	Chaltau, 37.9550°N, 69.1403°E; 1041m	This study
M. enarea permuta	*M. enarea permuta*	BOLD:GBLN1837-09	**FJ462272**	Uzbekistan	Ghissar Mts	Leneveu et al. 2009
M. gina	*M. gina*	BOLD:BPAL3083-15	**KY086152**	Iran	35.32°N, 47.15°E	Pazhenkova and Lukhtanov 2016
M. higginsi	*M. higginsi*	BOLD:BPAL2469-14	**KY777548**	Afghanistan		Lukhtanov 2017
M. interrupta	*M. interrupta*	BOLD:BPAL3019-15	**KY086139**	Georgia	Bakuriani	Pazhenkova and Lukhtanov 2016
M. kotshubeji bundeli	*Melitaea ala bundeli*	GA161	**MW672092**	Tajikistan	Alai Mts, 39.42°N, 71.62°E	This study
M. kotshubeji bundeli	*Melitaea ala bundeli*	GA162	**MW672093**	Tajikistan	Alai Mts, 39.42°N, 71.62°E	This study
M. kotshubeji bundeli	*Melitaea ala bundeli*	GA163	**MW672094**	Tajikistan	Alai Mts, 39.42°N, 71.62°E	This study
M. kotshubeji bundeli	*Melitaea ala bundeli*	GA164	**MW672095**	Tajikistan	Alai Mts, 39.42°N, 71.62°E	This study
M. kotshubeji bundeli	*Melitaea ala bundeli*	GA165	**MW672096**	Tajikistan	Alai Mts, 39.42°N, 71.62°E	This study
M. kotshubeji bundeli	*Melitaea ala bundeli*	GA166	**MW672097**	Tajikistan	Alai Mts, 39.42°N, 71.62°E	This study
M. kotshubeji kotshubeji	*M. ala kotshubeji*	BOLD:BPAL2308-14	**MW672069**	Tajikistan	Peter I Range, Garm	This study
M. kotshubeji kotshubeji	*M. ala kotshubeji*	BOLD:BPAL2484-14; CCDB-17966 B02	**MW672070**	Tajikistan	Peter I Range, 7 km S Tajikobad	This study
M. kotshubeji kotshubeji	*M. ala kotshubeji*	BOLD:BPAL2485-14	**MW672071**	Tajikistan	Peter I Range, Garm	This study
M. latonigena	*M. latonigena*	BOLD:BPAL3476-16	**KY086170**	Russia	Altai	Pazhenkova and Lukhtanov 2016
M. mauretanica	*M. didyma*	NW107-5; BOLD:GBLN1855-09	**FJ462253**	Morocco		Leneveu et al. 2009
M. mixta	*M. chitralensis*	BOLD:MABUT253-11	**KC158427**	Pakistan	35.8333°N, 71.7667°E	Ashfaq et al. 2013
M. mixta	*M. chitralensis*	BOLD:MABUT254-11	**KC158426**	Pakistan	35.8333°N, 71.7667 °E	Ashfaq et al. 2013
M. mixta	*M. didyma*	BOLD:BPAL2509-14	**KT874722**	Tajikistan	Farob	Pazhenkova et al. 2015
M. neera	*M. neera*	BOLD:BPAL3482-16	**MW672084**	Kazakhstan	Zyryanovsk region, 49.62°N, 83.62°E	This study
M. neera liliputana	*M. didyma*	CCDB-17968 E10; BOLD:BPAL2718-14	**KT874744**	Israel	Hermon	Pazhenkova at al. 2015
M. occidentalis	*M. didyma*	RVcoll.08-L832	**GU676247**	Spain	Comunidad_de_Madrid	GenBank
M. persea	*M. persea*	BOLD:BPAL2349-14	**KY777522**	Iran	Tehran	Lukhtanov 2017
M. persea paphlagonia	*M. persea*	BOLD:BPAL2959-15	**KY777526**	Iran	Shahrud	Lukhtanov 2017
M. saxatilis	*M. saxatilis*	NW120-8; BOLD:GBLN1828-09	**FJ462281**	Iran	Tehran	Leneveu et al. 2009
M. sutschana	*M. sutschana*	BOLD:BPAL2543-14	**KT874696**	Russia	Chita	Pazhenkova et al. 2015
M. telona	*M. ornata telona*	BOLD:BPAL3126-15	**MW672062**	Israel		This study
M. turkestanica	*M. didyma*	BOLD:BPAL2770-15	**KY086115**	Kazakhstan	Saikan	Pazhenkova and Lukhtanov 2016

Results

Karyotype

The haploid chromosome number n=29 was found in prometaphase I, MI and MII cells of four studied individuals of *M. kotshubeji kotshubeji* (Table 2, Fig. 1). All chromosome elements formed a gradient size row. The karyotype contained no exceptionally large or small chromosomes.

DNA barcode analysis

DNA barcode analysis revealed *M. ala*, *M. kotshubeji* and *M. enarea* as highly supported monophyletic entities. Together, these three species formed a monophyletic lineage (the *M. ala* species complex) (1 in Fig. 2). In relation to the *M. ala* species complex, *M. acraeina* was found as a phylogenetically distant sister group (2 in Fig. 2). Taxa close to *M. didyma* (the *M. didyma* species complex) also formed a clade, but

Table 2. Chromosome number in studied samples of *Melitaea kotshubeji kotshubeji.*

Code number of the specimen	Chromosome number	Locality, date and collector	Number of cells checked
VLcoll.17-AB028	n=29	Tajikistan, Peter the Great Mts, Ganishou, 2200 m, 30.VI.2017, E. Pazhenkova leg.	5
VLcoll.17-AB080	n=29	Tajikistan, Peter the Great Mts, Muk, 2800 m, 25.VII.017, V. Lukhtanov leg.	7
VLcoll.17-AB086	n=29	Tajikistan, Peter the Great Mts, Muk, 2800 m, 26.VII.2017, V. Lukhtanov leg.	11
VLcoll.17-AB087	n=29	Tajikistan, Peter the Great Mts, Muk, 2800 m, 26.VII.2017, V. Lukhtanov leg.	14

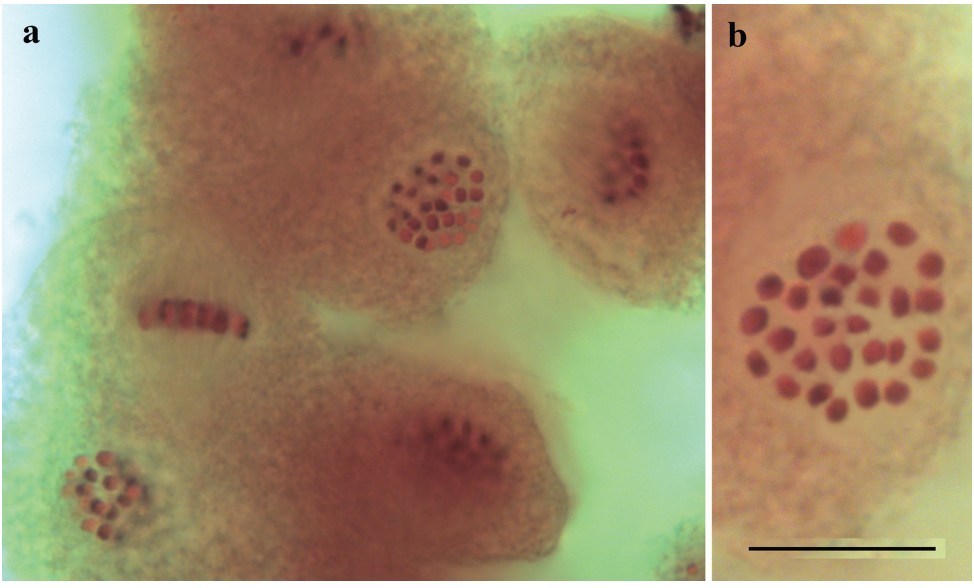

Figure 1. Karyotype of *M. kotshubeji* **a** general view of six MI cells in a spermatocyst **b** *M. kotshubeji*, AB080, MI, n=29. Scale bar: 10 μm.

Figure 2. The Bayesian 50% majority rule consensus tree of the analyzed samples of *Melitaea* inferred from *COI* sequences. *Melitaea alatauica* and *M. telona* sequences are used to root the tree. Museum ID numbers, GenBank accession numbers, species and subspecies names, and localities are shown to the right of the branches. Bayesian posterior probabilities higher than 0.75 are shown next to the recovered branches. ***b1*** is *M. ala bicolor*, clade 1. ***b2*** is *M. ala bicolor*, clade 2. ***i*** *is M. ala irtyshica.* ***k*** is *M. kotshubeji kotshubeji*. ***z*** is *M. ala zaisana* **l** is the *M. ala* species complex **2** is *M. acraeina* **3** is the *M. didyma* species complex. **4** is *M. deserticola*. 5 is the *M. persea* species complex. I is *M. didyma* species group. II is *M. persea* species group.

Table 3. Intragroup uncorrected *COI p*-distances revealed within *M. ala*.

Group	Minimum p-distance	Maximum p-distance
irtyshica	0%	0.2%
zaisana	0%	0%
(irtyshica+zaisana)	0%	0.5%
ala	0%	0%
bicolor1	0%	0.6%
bicolor2	0%	0.2%
(bicolor1+bicolor2)	0%	0.8%

Table 4. Uncorrected *COI p*-distances between the groups revealed within *M. ala*.

Group 1	Group 2	Minimum p-distance	Maximum p-distance
irtyshica	zaisana	0.3%	0.5%
(irtyshica+zaisana)	ala	0.9%	1.5%
(irtyshica+zaisana)	bicolor1	0.9%	1.5%
(irtyshica+zaisana)	bicolor2	0.9%	1.5%
ala	bicolor1	0.9%	1.3%
ala	bicolor2	0.9%	1.5%
bicolor1	bicolor2	0.3%	0.8%
(irtyshica+zaisana)	(bicolor1+bicolor2)	0.9%	1.5%
ala	(bicolor1+bicolor2)	0.9%	1.5%

its support was relatively low (3 in Fig. 2). The species *M. deserticola* formed an independent lineage within the *M. didyma* species group (4 in Fig. 2). Together, these four lineages (*M. ala* complex + *M. acraeina* + *M. didyma* complex+ *M. deserticola*) formed the well-supported *M. didyma* species group (I in Fig. 2). The species of the *M. persea* group also formed a supported clade, sister to the *M. didyma* group (5 and II in Fig. 2).

Within the *M. ala* clade, five supported (Bayesian posterior probabilities ranged from 0.9 to 1.0), relatively weakly differentiated subclades were found. These are (1) *M. ala ala*, (2) *M. ala irtyshica*, (3) *M. ala zaisana*, (4) *M. ala bicolor* (clade *b1*) and (5) *M. ala bicolor* (clade *b2*). We also calculated the uncorrected *COI p*-distances within (Table 3) and between (Table 4) the revealed clades and groups.

Melitaea kotshubeji kotshubeji and *M. kotshubeji bundeli* were found to differ by four fixed nucleotide substitutions in the *COI* barcode region.

Discussion

Chromosome number variation

The genus *Melitaea* (Fabricius, 1807) has relatively low interspecific chromosome number variation. The representatives of basal clades (see phylogeny in Leneveu et al. 2009), the taxa of *M. cinxia* (Linnaeus, 1758), *M. diamina* (Lang, 1789), *M. athalia* (Rottemburg, 1775), *M. trivia* ([Denis et Schiffermüller], 1775) and *M. phoebe* ([Denis et Schiffermüller], 1775) species groups demonstrate n=30–31 (Federley 1938; de Lesse 1960; Robinson 1971; Larsen 1975; Hesselbarth et al. 1995). These haploid

Table 5. Chromosome nmbers of taxa close to *M. didyma*.

Species complex	Taxon	Chromosome number	Country	Locality	Reference
Melitaea didyma species complex	*M. didyma*	n=28	Italy	Abruzzi	de Lesse 1960
	M. didyma neera	n=28	Kazakhstan	Altai	Lukhtanov and Kuznetsova 1989
	M. didyma neera	n=27	Russia	N Caucasus, Pyatigorsk	Lukhtanov and Kuznetsova 1989
	M. interrupta	n=29	Turkey		de Lesse 1960
	M. interrupta	n=29	Azerbaijan, Nakhichevan	Zangezur Mts	Lukhtanov and Kuznetsova 1989
	M. latonigena	n=29–30	Kazakhstan	Altai	Lukhtanov and Kuznetsova 1989
	M. gina	n=28	Iran	W Azerbaijan	Pazhenkova and Lukhtanov 2016
Melitaea deserticola species complex	*M. deserticola*	n=29	Lebanon		Larsen 1975
Melitaea ala species complex	*M. ala*	n=29	Kazakhstan		Lukhtanov and Kuznetsova 1989
	M. kotshubeji	n=29	Tajikistan		This study
Melitaea persea species complex	*M. persea*	n=27	Iran		de Lesse 1960
	M. acentria	n=27	Israel		Lukhtanov 2017

numbers are modal ones not only for *Melitaea*, but also for the family Nymphalidae and for the order Lepidoptera in whole (Robinson 1971; Lukhtanov 2000, 2014). Most likely, one of them (probably, n=31, see Lukhtanov 2014) represents an ancestral lepidopteran state preserved in the basal lineages of *Melitaea*.

The *Melitaea didyma* species group is one of the younger lineages of *Melitaea* (Leneveu et al. 2009). This group is found to have lower chromosome numbers varying from n=27 to n=29–30 (Table 5). *Melitaea didyma* species complex is characterized by chromosome numbers from n n=27 to n=30, with n=28 and n=29 as modal numbers. In the *Melitaea deserticola* species complex, only one species (*M. deserticola*) is karyotyped (n=29). In the *Melitaea persea* species complex, n=27 is found in two species. In the *Melitaea ala* species complex, n=29 is found in two species studied.

Based on the distribution of the known chromosome numbers (Table 3) relative to the phylogeny (Fig. 2) and on the frequency of their occurrence, we can assume that n=29 is an ancestral state for the species of the *M. didyma* group. Thus, for the species of the *M. ala* complex n=29 is a symplesiomorphy.

Intraspecific taxonomy of the *M. ala* species group

The five identified clades within the species *M. ala* have relatively high support (Fig. 2) and can be considered as taxa, at least from the standpoint of the phylogenetic species concept (Cracraft 1989; Coyne and Orr 2004), in which diagnosable entities can be classified as species regardless of whether there is reproductive isolation between them or not. To assess the possibility of interpreting these clades as species or subspecies, we compared the level of *COI* divergence between the clades with the level of variability within the clades (Tables 3, 4). We found that in all cases, the distances between these clades were lower than 'standard' DNA-barcode species threshold (3%) (Hebert et al. 2003).

An especially low level of differentiation (0.3–0.5%) was found between the clades *M. ala zaisanica* and *M. ala irtyshica*. Therefore, we are inclined, especially taking into

account the geographical proximity of these lineages, to consider them as a single taxonomic unit, M. *ala zaisanica* (= *M. ala irtyshica*).

A slightly higher average level of differentiation (0.3–0.8%) was found between the *b1* and *b2* clades (Fig. 2, Table 4). However, in this case, a rather high level of intragroup variability was observed (Table 3), and the maximum values of intragroup variability exceeded the minimum intergroup differences. Therefore, taking into account the geographical proximity of these lineages, we decided to consider them as a single taxonomic unit, *M. ala bicolor*.

Thus, within the studied populations, three subspecies can be distinguished. These are *M. ala ala*, *M. ala bicolor* and *M. ala zaisana*.

Melitaea ala ala is distributed in the Dzhungarian Alatau in East Kazakhstan (Fig. 3). This subspecies is characterized by darkening of the veins on the underside of the hind wing. These darkened veins form clear cells in the region of the median band (Fig. 4a).

Melitaea ala bicolor Seitz, 1908 is distributed in the North, East, Central and West Tian-Shan in SE Kazakhstan, NW China and Kyrgyzstan (Fig. 3). In this subspecies the veins on the underside of the hind wing are not strongly darkened. The cells of the median band are not highlighted. They are only marked with dark brackets on the outside of the median band (Fig. 4b). The specimens from the Tyshkantau Mts (SE part of the Dzhungarian Alatau in Kazakhstan) (Tuzov and Churkin 2000) and the eastern most part of the Tian-Shan (Kolesnichenko 1999) are intermediate between *M. ala ala* and *M. ala bicolor*.

With regards to DNA barcodes, *M. ala zaisana* Lukhtanov, 1999 (Fig. 4c) is distinct from the geographically closest *M. ala ala*. With regards to the wing pattern, *M. ala zaisana* is more similar to *M. ala bicolor* than to *M. ala ala*. Interestingly, the northernmost population of *M. ala* from Oktyabrsk (Kazakhstan) (Fig. 3d) is intermediate in its appearance between *M. ala ala* and *M. ala zaisana*. This population was described as *M. ala irtyshica* Lukhtanov, 1999 (Lukhtanov 1999) and was later erroneously synonymized with *M. latonigena* Eversmann, 1847 (Lukhtanov et al. 2007). DNA barcode analysis demonstrates that this population is similar to *M. ala zaisana*.

Currently, there is a tendency to consider as a species any group of populations with a minimum set of fixed differences. We are almost certain that, given this trend, the subspecies discussed above will be interpreted by some authors as species in the future. Nevertheless, in our opinion, in accordance with the subspecies criteria (Lukhtanov et al. 2016; De Queiroz, 2020), they should be treated as subspecies of the same species.

Melitaea kotshubeji bundeli (Fig. 4h, i) was described as subspecies of *Melitaea kotshubeji* (Fig. 4j) (Kolesnichenko 1999), but later was treated as a distinct species (van Oorschot and Coutsis 2014) or alternatively as a synonym (Tshikolovets 2003, 2005). Our study demonstrates that these two taxa are not only distinct in the wing pattern, but also differ by four fixed nucleotide substitutions in the DNA barcode region, indicating the relative long independent evolution of these two sublineages. Interestingly, the distribution areas of these two allopatric taxa are in close proximity to each other and are separated by a narrow valley of the Surkhob River (in Kyrgyzstan, this river is called the Kyzylsu).

In our work we do not consider the intraspecific structure of *M. enarea* (Fig. 4k, l) due to the lack of molecular data for the northern populations of this species.

Figure 3. Locations of the analyzed samples of *M. ala*, *M. kotshubeji* and *M. enarea* **1** type-locality of *M. ala irtyshica* (Kazakhstan, Zyryanovsk district, Oktyabrsk, 49.62°N, 83.62°E) **2** type-locality of *M. ala zaisanica* (Kazakhstan, Kurtchumski Mts, 48.47°N, 84.12°E) **3** *M. ala ala* (Kazakhstan, Dzhungarian Alatau, Kyzylagash and Kopal) **4** *M. ala bicolor* (clade *b1*) (China, Kyrgyzstan) **5** *M. ala bicolor* (clade *b2*) (Kyrgyzstan, Kara-Bura Pass; Kazakhstan, Kirgizski Mts) **6** *M. kotshubeji kotshubeji* (Tajikistan, Peter the Great Mts) **7** *M. kotshubeji bundeli* (Tajikistan, border with Kyrgyzstan, Alai Mts, 39.42°N, 71.62°E) **8** *M. enarea* (Tajikistan).

The taxa described by Bryk (1940)

Bryk (1940) described four taxa (all as subspecies of *M. didyma*) that should be assigned to *M. ala*. The types of these taxa were studied by the first author of this article in 2007 during a visit to Swedish Museum of Natural History.

The taxon described by Bryk (1940) as *M. didyma allah* Bryk, 1940 has the wing pattern with clear characters of *M. ala ala* (Fig. 5a, b), but not of the subspecies *M. ala zaisana* (Fig. 4c) as supposed by Tuzov and Churkin (2000). Thus, *M. didyma allah* should be synonymized with *M. ala ala* as suggested by Kolesnichenko (1999). We agree with Kolesnichenko (1999) that the label data of the syntype of *M. didyma allah* (Fig. 5c) are probably wrong.

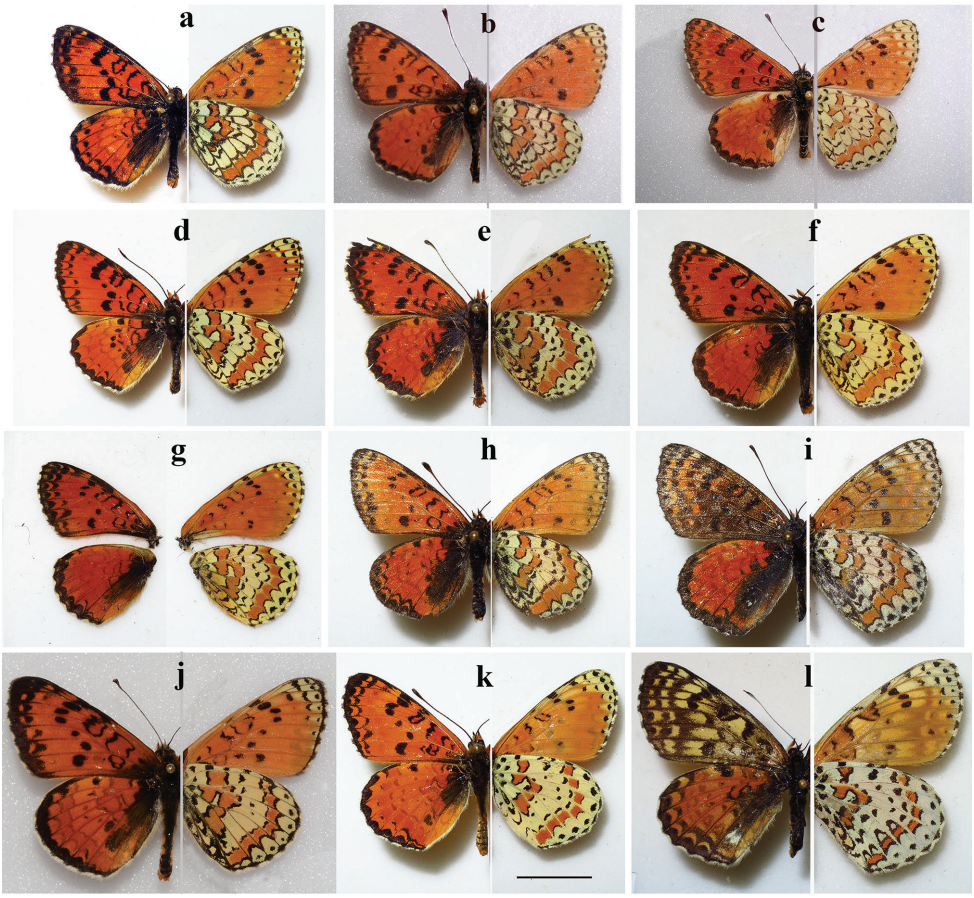

Figure 4. Butterflies of the *Melitaea ala* species complex **a** *M. ala ala*, male, BPALB179-16 (CCDB-25458_G12), Kazakhstan, Dzhungarian Alatau, Kopal, 45.04°N, 79.06°E, 1800–1900 m, 13.VI.2016, V. Lukhtanov leg. **b** *M. ala bicolor*, clade *b1*, male, Kyrgyzstan, Moldatoo Mts, 41.5°N, 74.62°E, 2100 m, 29.VI.1996, V. Lukhtanov leg. **c** *M. ala zaisana*, male, LOWA174-06, Kazakhstan, Kurchumski Khrebet, Kalgutinski Pass, 600 m, 48.47°N, 84.12°E, 9.VI.1998, V. Lukhtanov leg. **d** *M. ala irtyshica*, male, BPAL3484-16 (CCDB-25456_F04), Kazakhstan, Zyryanovsk distr., Oktyabrsk, 49.6178°N, 83.6219°E, 420 m, 08.VI.1999, V. Lukhtanov leg. **e** *M. ala bicolor*, clade *b2*, male, CCDB-03024-RPVL-00009, Kazakhstan, Kirgizski Mts, Merke, 42.69°N, 73.25°E, 1500m, 13.VI.2000, V. Lukhtanov leg. **f** *M. ala bicolor*, clade *b2,* male, BPAL027-10 (RPVL-00027), Kyrgyzstan, Talassky Mts, Kara-Bura pass, 42.27°N, 71.57°E, 2000m, 30.VI.2000, V. Lukhtanov leg. **g** *M. ala bicolor*, clade *b2*, male, BPAL026-10 (RPVL-00026), Kyrgyzstan, Talassky Mts, Kara-Bura pass, 42.27°N, 71.57°E, 2000m, 30.VI.2000, V. Lukhtanov leg. **h** *M. kotshubeji bundeli*, male, GA161, Tajikistan, Alai Mts, Kichi-Karamuk, 39.4258°N, 71.6125°E; 3150 m, 03.VIII.2019, V. Lukhtanov leg. **i** *M. kotshubeji bundeli*, female, GA166, Tajikistan, Alai Mts, Kichi-Karamuk, 39.4258°N, 71.6125°E; 3150 m, 03.VIII.2019, V. Lukhtanov leg. **j** *M. kotshubeji kotshubeji*, male, BPAL2484-14 (CCDB-17966 B02), Tajikistan, Peter I Range, 7 km S Tajikobad, 14.VIII.2003 **k** *M. enarea*, male, BPAL2656-14 (CCDB-17967_H07), Tajikistan, Tabakchi Mts, 37.85°N, 68.98°E, 1150 m, 01.V.2014, V. Lukhtanov leg. **l** *M. enarea*, female, BPAL2659-14 (CCDB-17967_H10), Tajikistan, Chaltau Mts, 37.9550°N, 69.1403°E, 1041m, 02.V.2014, V. Lukhtanov leg. Scale bar: 10 mm

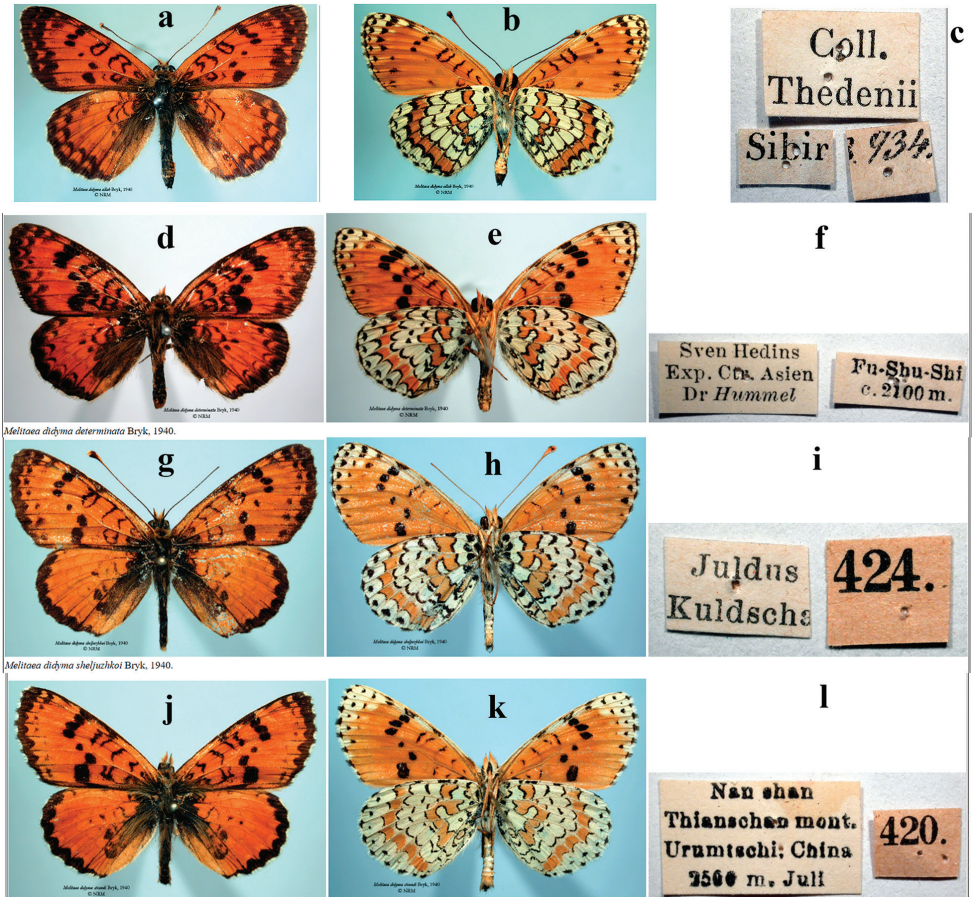

Figure 5. Syntypes of the taxa of the *Melitaea ala* species complex, originally described by Felix Bryk (1940) as subspecies of *M. didyma*. All specimens are deposited in Swedish Museum of Natural History (Naturhistoriska riksmuseet, NRM) **a** *M. didyma allah*, upperside **b** *M. didyma allah*, underside **c** *M. didyma allah*, labels **d** *M. didyma determinata*, upperside **e** *M. didyma determinata*, underside **f** *M. didyma determinata*, labels **g** *M. didyma sheljuzhkoi*, upperside **h** *M. didyma sheljuzhkoi*, underside **i** *M. didyma sheljuzhkoi*, labels **j** *M. didyma strandi*, upperside **k** *M. didyma strandi*, underside **l** *M. didyma strandi*, labels.

The taxa described by Bryk (1940) as *M. didyma sheljuzhkoi* Bryk, 1940 (Fig. 5g–i) and *M. didyma strandi* (Fig. 5j–l) have the wing pattern with characters of *M. ala bicolor*. Most likely, they represent synonyms of *M. ala bicolor*.

The taxon from "Fu-Shu-Shi" (China) described by Bryk (1940) as *M. didyma determinata* Bryk, 1940 is characterized by the well-developed black wing pattern on both wing upper- and underside (Fig. 5d–f). Most likely, it represents a distinct subspecies. Unfortunately, we do not have material for molecular study to test this hypothesis.

Probably erroneous species identifications in the *M. ala* complex

The specimens identified as *Melitaea ninae* (sample NW113-10, FJ462269, Kyrgyzstan), *M. enarea* (sample NW113-15, FJ462256, Tajikistan) (Leneveu et al. 2009; Long et al. 2014) and *M. chitralensis* (samples KC158426 and KC158427) (Ashfaq et al. 2013) were reported in the cited molecular phylogenetic analyses of the genus *Melitaea*. According to the DNA barcodes of these samples, they most likely belong to *M. turkestanica* Sheljuzhko, 1929 (NW113-10) and *M. mixta* Evans, 1912 (NW113-15, KC158426 and KC158427).

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