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Comparative cytogenetics among *Leporinus friderici* and *Leporellus vittatus* populations (Characiformes, Anostomidae): focus on repetitive DNA elements

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Abstract

Anostomidae are a neotropical fish family rich in number of species. Cytogenetically, they show a conserved karyotype with 2n = 54 chromosomes, although they present intraspecific/interspecific variations in the number and chromosomal location of repetitive DNA sequences. The aim of the present study was to perform a comparative description of the karyotypes of two populations of *Leporinus friderici* Bloch, 1794 and three populations of *Leporellus vittatus* Valenciennes, 1850. We used conventional cytogenetic techniques allied to fluorescence *in situ* hybridization, using 18S ribosomal DNA (rDNA) and 5S rDNA, a general telomere sequence for vertebrates (TTAGGG)n and retrotransposon (RTE) *Rex1* probes. The anostomids in all studied populations presented 2n = 54 chromosomes, with a chromosome formula of 32m + 22sm for *L. friderici* and 28m + 26sm for *L. vittatus*. Variations in the number and location of the 5S and 18S rDNA chromosomal sites were observed between *L. friderici* and *L. vittatus* populations and

species. Accumulation of *Rex1* was observed in the terminal region of most chromosomes in all populations, and telomere sequences were located just on all ends of the 54 chromosomes in all populations. The intraspecific and intergeneric chromosomal changes occurred in karyotype differentiation, indicating that minor chromosomal rearrangements had present in anostomid species diversification.

Keywords
Chromosomal differentiation, karyotype evolution, ribosomal DNA, retrotransposon

Introduction

Eukaryotic chromosomes can be classified into different DNA classes: single copy DNA, which are sequences found only once in a genome; and repetitive DNA, which are sequences repeated from a few tens to millions of times (Sumner 2003). Repetitive DNA can be classified into tandem repeats (multigene families and satellite, minisatellite, and microsatellite DNA) and transposable elements (TEs): transposons and retrotransposons with dispersed distribution in genomes (Sumner 2003).

Satellite DNA and TEs are responsible for a large part of the structural and functional organization of genomes (Sumner 2003, Feschotte 2008), and carry sequences containing DNA double-strand break hotspots, resulting in chromosome/genome reshuffle (Eichler and Sankoff 2003, Longo et al. 2009, Farré et al. 2011, Barros et al. 2017a, Glugoski et al. 2018). The movement of repetitive sequences within the genome promotes chromosomal differentiation, which has an important role on karyotype evolution (Wichman et al. 1991, Pucci et al. 2016, 2018a, 2018b, Lorscheider et al. 2018, do Nascimento et al. 2018).

Anostomids are neotropical fishes with a high number of species and diverse morphology (Garavello and Britski 2003, Graça and Pavanelli 2007, Britski et al. 2012, Ramirez et al. 2017a). Cytogenetically they present a conserved diploid number (2n) of 54 chromosomes, with mostly metacentric (m) and submetacentric (sm) chromosomes (Galetti Jr and Foresti 1986, Galetti Jr et al. 1991, 1995, Venere et al. 2004). Anostomidae species present differentiated karyotypes regarding the distribution of heterochromatin and repetitive sequences, presenting different localizations of heterochromatic bands and repetitive DNA sites (Martins and Galetti Jr 1999, Parise-Maltempi et al. 2007, Porto-Foresti et al. 2008, Hashimoto et al. 2009, Marreta et al. 2012, Borba et al. 2013).

Therefore, although they retain 2n = 54 chromosomes, anostomids present very high intra- and interspecific chromosomal/genetic variability, which is highly compatible with restricted gene flow (Parise-Maltempi et al. 2007, 2013, Ramirez et al. 2017a, 2017b, Silva-Santos et al. 2018). With the aim of better understanding the intra- and interspecific chromosomal differentiation due to accumulation of repetitive sequences, in the present study we performed a comparative evaluation of the karyotypes of two populations of *Leporinus friderici* (Bloch, 1794) and three populations of *Leporellus vittatus* (Valenciennes, 1850). Cytogenetic analysis was performed using Giemsa staining and C-banding, and chromosome mapping of repetitive DNAs using the ribosomal DNA (rDNA) 18S and 5S rDNA, the (TTAGGG)n sequence and the retrotransposon (RTE) *Rex1*.

Material and methods

Specimens of *Leporinus friderici* and *Leporellus vittatus* were collected from rivers belonging to different Brazilian hydrographic basins (Table 1). Fish capture was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio – license numbers 10538-1 and 15117-1) and the processing was performed in accordance with the Ethical Committee on Animal Use (CEUA 29/2016) of the Universidade Estadual de Ponta Grossa and current Brazilian legislation. The analyzed specimens were identified by taxonomists experts in the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia) museum, Universidade Estadual de Maringá (UEM).

Genomic DNA was extracted from the liver tissue, using the protocol of Doyle and Doyle (1990), from the *Megaleporinus obtusidens* (Ramirez et al. 2017a), described first time in the literature as *Leporinus obtusidens* (Valenciennes, 1837). The 18S rDNA amplification was performed using primers 18S Fw (5'-ccgctttggtgactcttgat-3') and 18S Rv (5'-ccgaggacctcactaaacca-3'), according to Gross et al. (2010). The 5S rDNA sequence was amplified using primers 5SA (5'-tcaaccaaccacaaagacattggcac-3') and 5S (5'-tagacttctgggtggccaaaggaatca-3'), according to Martins and Galetti (1999). The vertebrate telomere sequence (TTAGGG)n was obtained according to Ijdo et al. (1991). The non-long terminal repeats retrotransposon (non-LTR RTE) *Rex1* sequence was obtained by PCR using primers RTX1-F1 Fw (5'-ttctccagtgccttcaacacc-3') and RTX1-R1 Rv (5'-tccctcagcagaaagagtctgctc-3'), according to Volff et al. (1999, 2000). The sequences of the 5S rDNA, 18S rDNA and *Rex-1* were analyzed and their nucleotide identities were confirmed using BLASTn (National Center for Biotechnology Information) and the CENSOR tool for repeated sequences (Kohany et al. 2006). Finally, the sequences were deposited in GenBank (Sequences ID: MH697559, MH701851, MH684488, respectively).

Mitotic chromosomes were obtained according to Blanco et al. (2012) and stained with 5% Giemsa in phosphate buffer, pH 6.8. Heterochromatin detection was performed according to Sumner (1972), with modifications (Lui et al. 2009).

Table 1. Cytogenetic data of *Leporinus friderici* and *Leporellus vittatus* analyzed in the present study. SP = São Paulo State, PR = Paraná State, MG = Minas Gerais State, MT = Mato Grosso State, 2n = diploid number, FN = fundamental number, KF = karyotype formula, term = terminal sites.

Species	River/Basin/State/GPS	2n	FN	KF	5S sites	18S sites	*Rex1*
Leporinus friderici	Mogi-Guaçu River, Upper Paraná Basin – SP (21°58'52"S, 47°17'36"W)	54	108	32m+22sm	pairs 10 and 11	pair 1	term
	Jangada River, Iguaçu River Basin – PR (26°13'5.22"S, 51°16'17.40"W)	54	108	32m+22sm	pairs 3 and 11	pair 1	term
Leporellus vittatus	Mogi-Guaçu River, Upper Paraná Basin – SP (21°58'52"S, 47°17'36"W)	54	108	28m+26sm	pair 3	pair 5	term
	Aripuanã River, Aripuanã River Basin – MT (10°09'57.8"S, 59°26'54.9"W)	54	108	28m+26sm	pairs 6 and 8	pair 6	term
	São Francisco River, São Francisco Basin – MG (20°16'15"S, 45°55'39"W)	54	108	28m+26sm	pair 3	pair 6	term

The 18S rDNA was labeled with digoxigenin-11-dUTP, using the DIG-Nick Translation Mix (Roche Applied Science), according to the manufacturer's recommendations. The 5S rDNA sequence was labeled with biotin 16-dUTP by PCR, and *Rex1* and (TTAGGG)n sequences with digoxigenin-11-dUTP by PCR. PCR reactions were performed with 20 ng DNA template, 1× polymerase reaction buffer, 1.5 mM MgCl2, 40 μM dATP, dGTP and dCTP, 28 μM dTTP, 12 μM digoxigenin-11-dUTP or biotin 16 dUTP, 1 μM of each primer and 1 U of DNA Taq polymerase. The PCR program consisted of an initial step of denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 56 °C for 45 s, 72 °C for 2 min, and a final extension at 72 °C for 7 min.

The general protocol for FISH (Pinkel et al. 1986) followed under hybridization mixture (2.5 ng/μl probe, 50% formamide, 2×SSC, 10% dextran sulfate, at 37 °C for 16 h). Post-hybridization washes were performed in high stringency [50% formamide at 42 °C for 10 min (twice times), 0.1×SSC at 60 °C for 5 min (three times), and 4×SSC 0.05% Tween at room temperature for 5 min (two baths)]. Streptavidin Alexa Fluor 488 (Molecular Probes) and Anti-digoxigenin rhodamine fab fragments (Roche Applied Science) antibodies were used for probes detection. The chromosomes were stained with DAPI (0.2 μg/ml) in Vectashield mounting medium (Vector) and analyzed under epifluorescence microscopy.

Chromosome preparations were analyzed using the brightfield and epifluorescence microscope Zeiss Axio Lab 1, coupled to the Zeiss AxioCam ICM1 camera with the Zen Lite software and a resolution of 1.4 megapixels (Carl Zeiss). The karyotypes were organized and classified as metacentric (m) or submetacentric (sm) according to Levan et al. (1964).

Results

All anostomids evaluated in the present study presented 2n = 54 chromosomes and a fundamental number (FN) of 108 (Table 1). The two populations of *L. friderici* (Mogi–Guaçu and Jangada rivers) presented a karyotype formula (KF) of 32m + 22sm (Fig. 1a, b), and the three populations of *L. vittatus* (Mogi–Guaçu, Aripuanã and São Francisco rivers) a karyotype formula of 28m + 26sm (Fig. 1c, d, e). Sex chromosome heteromorphism was not detected in the populations/species analyzed.

C-banding showed discrete blocks of centromeric heterochromatin for *L. friderici*, with very evident blocks in the terminal regions of the long arms of just one homologue of chromosomes 1 and 5 for the population of the Mogi–Guaçu river (Fig. 2a); and, in the subterminal regions of pairs 1 and 17 for the population of the Jangada river (Fig. 2b). *Leporellus vittatus* showed blocks of heterochromatin in the pericentromeric or proximal regions of most chromosomes (Fig. 2c, d), which was very evident for the populations from the Mogi–Guaçu and Aripuanã rivers and less evident for the populations from the São Francisco river (Fig. 2e).

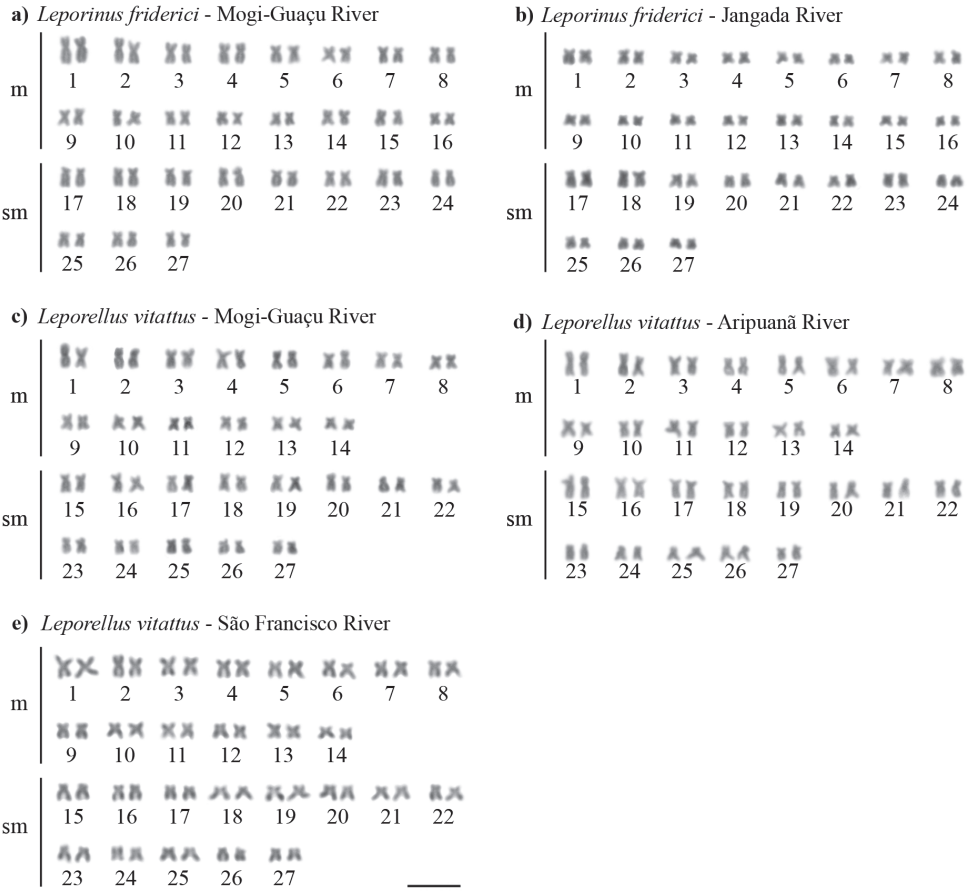

Figure 1. Karyotypes of *Leporinus friderici* (**a, b**) and *Leporellus vittatus* (**c, d, e**) after conventional Giemsa staining. Scale bar: 10 μm.

Double-FISH using 5S and 18S rDNA probes detected one 45S rDNA site in the short arm (p) of chromosome pair 1 for both populations of *L. friderici* (Fig. 3a, b). The 5S rDNA was located in the pericentromeric region of chromosome pair 10 and in the short arm (p) of pair 11 for *L. friderici* from the Mogi–Guaçu river (Fig. 3a), whereas it was located in the p arm of chromosome pairs 3 and 11 for *L. friderici* from the Jangada river (Fig. 3b). *Leporellus vittatus* from the Mogi–Guaçu river presented 45S rDNA in the terminal region of the long arm (q) of pair 5, and 5S rDNA was located in the proximal region of 3p pair (Fig. 3c). In *L. vittatus* from the Aripuanã river, the 45S rDNA was located in synteny with 5S rDNA in the chromosome pair 6, with terminal location 6q for 45S rDNA and proximal q arm site for 5S rDNA, and an additional 5S rDNA site in the proximal q arm of pair 8 (Fig. 3d). *Leporellus vittatus* from the São Francisco river presented the 45S rDNA in the terminal region of 6q, and the 5S

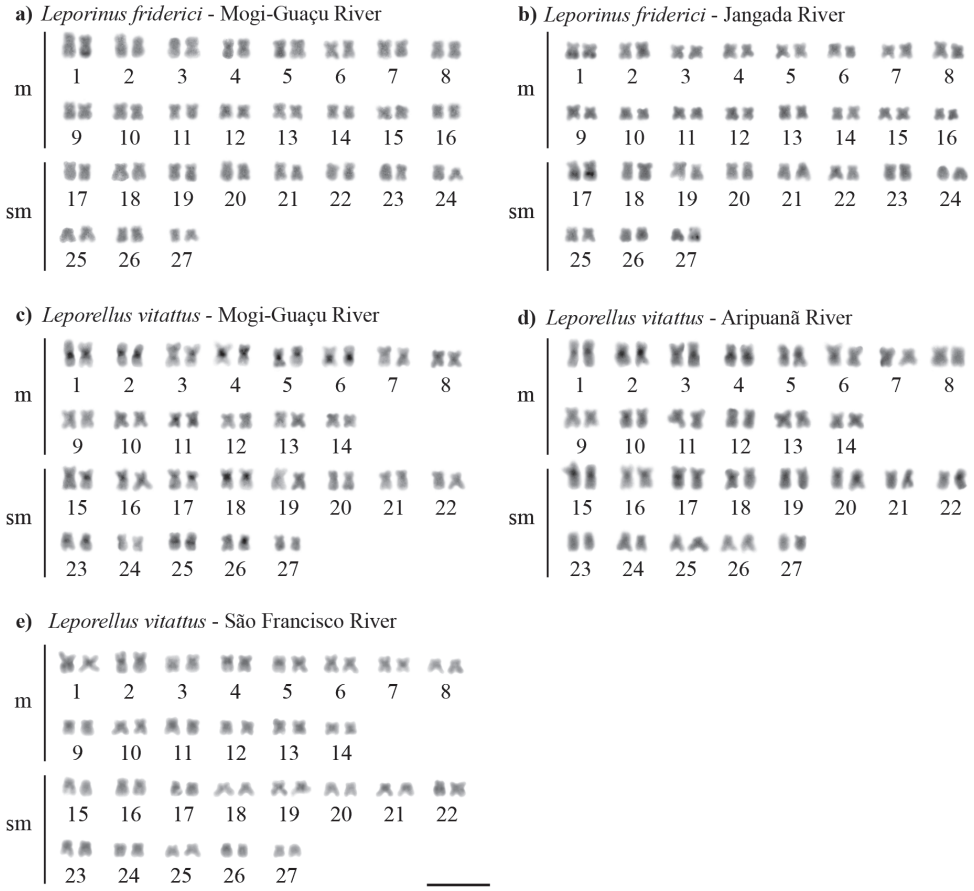

Figure 2. Karyotypes of *Leporinus friderici* (**a, b**) and *Leporellus vittatus* (**c, d, e**) after C-banding. Scale bar: 10 μm.

rDNA in the proximal region of 3p (Fig. 3e). *In situ* mapping of RTE *Rex1* (Fig. 4a–e) and (TTAGGG)n (Fig. 5a–e) showed signals in the terminal regions of all chromosomes for all populations of both *L. friderici* and *L. vittatus*. In *L. vittatus* from the Mogi-Guaçu river, the telomeres signals were tiny in all metaphases analyzed (Fig. 5c).

Discussion

The present cytogenetic analysis confirmed the conservation of the karyotype macrostructure of 2n = 54 chromosomes in *Leporinus friderici* and *Leporellus vittatus*, with metacentric and submetacentric chromosomes (FN=108). This karyotype structure is shared by most species belonging to Anostomidae (Galetti Jr et al. 1995, Venere et al. 2004). In addition, *L. friderici* and *L. vittatus* presented small differences in their karyo-

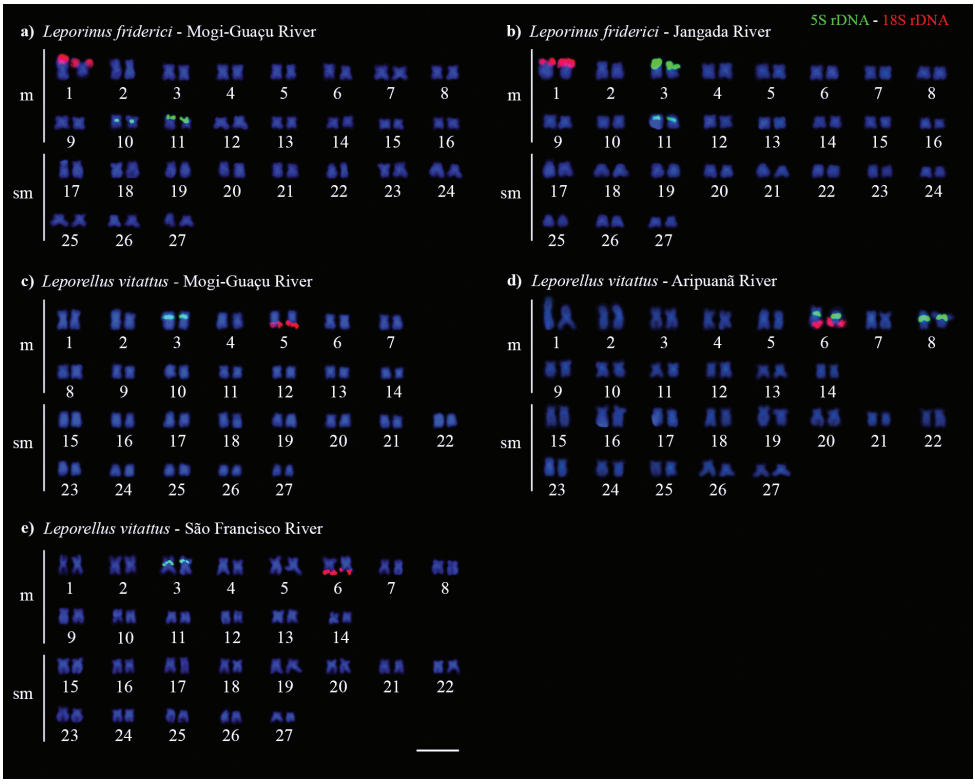

Figure 3. Karyotypes of *Leporinus friderici* (**a, b**) and *Leporellus vittatus* (**c, d, e**) submitted to fluorescence *in situ* hybridization with 18S rDNA and 5S rDNA probes. Scale bar: 10 μm.

type formulas resulted of the chromosome rearrangements such as pericentric inversions, translocations or centromere repositioning, which alters the chromosome morphology without any accompanying chromosomal rearrangements (Rocchi et al. 2012).

Some chromosomal markers presented some differentiation within and between species of anostomids. Intraspecific variations were observed in the chromosomal location and quantity of heterochromatin blocks, which were mainly located in pericentromeric regions in *L. vittatus* and terminal positions of chromosomes in *L. friderici*. These heterochromatin distribution in the chromosomes have also been observed for other anostomids (Pereira et al. 2002, Aguilar and Galetti Jr 2008, Barros et al. 2017b). Satellite DNA is one of the components of heterochromatin, which is also enriched in other dispersed repeated elements, including transposons (Mazzuchelli and Martins 2009, Vicari et al. 2010). It is usually accepted that the number of repetitive copies of a heterochromatin block may increase through mechanisms of homologous recombination, TEs invasion, or replication slippage for microsatellite expansion inside heterochromatin (Gray 2000, Kantek et al. 2009, Kelkar et al. 2011, Glugoski et al. 2018). These mechanisms may play a role in the microstructural differentiation

Figure 4. Karyotypes of *Leporinus friderici* (**a, b**) and *Leporellus vittatus* (**c, d, e**) submitted to fluorescence *in situ* hybridization with *Rex1* probe. Scale bar: 10 μm.

of heterochromatin chromosome blocks once no evident large heterochromatic blocks were observed in anostomids species analyzed.

In situ location of ribosomal genes showed that these sites were also involved in the chromosomal changes, especially in the studied *L. vittatus* populations. The location of rDNA in different positions and number of chromosomal sites also supports the hypothesis of population differentiation. On the other hand, the location of rDNA sites was observed to be highly conserved in the karyotypes of some anostomids (Martins and Galetti Jr 1999, 2000, 2001). In the present study, consistent differences in the location of rDNA sites were observed between the *L. vittatus* populations evaluated. These differences are exclusive conditions due to population isolation and contribute to genomic diversification in this fish group.

Anostomids usually present only one pair of 45S rDNA (Martins and Galetti Jr 1999), being a common characteristic of this group. Previous studies observed polymorphisms in the number of 45S rDNA sites in *Leporinus taeniatus* Lütken, 1875, *Leporinus trifasciatus* Steindachner, 1876, *Rhytiodus microlepis* Kner, 1858 and *Schizodon fasciatus* Spix & Agassiz, 1829 (Barros et al. 2017b). In the present study, although

Figure 5. Karyotypes of *Leporinus friderici* (**a, b**) and *Leporellus vittatus* (**c, d, e**) submitted to fluorescence *in situ* hybridization with (TTAGGG)n probe. Scale bar: 10 μm.

this was also observed, differences in the chromosomal position of 45S rDNA were additionally observed between species, with signals in the terminal region of the p arm for *L. friderici* and in the q arm for *L. vittatus*. The rDNAs usually present high rates of karyotype rearrangements in evolutionary lineages (Symonová et al. 2013). These sequence movements within karyotypes have been proposed to occur by transposition and/or by transposon-mediated by TEs in a non-homologous recombination mechanism (Symonová et al. 2013, Barros et al. 2017a, Glugoski et al. 2018). The *L. vittatus* specimens from the Aripuanã river presented synteny of 45S rDNA and 5S rDNA, in contrast with the specimens from the Mogi–Guaçu and São Francisco rivers and the *L. friderici* populations corroborating to high evolutionary chromosomal change level to rDNA sites. The rDNA synteny was also observed in other anostomids, such as *L. trifasciatus*, *S. fasciatus* and *Laemolyta taeniata* (Kner, 1858), showing that it is a recurrent chromosomal characteristic of this group (Barros et al. 2017b).

Recently, some studies have proposed that the dispersal of ribosomal sites and changes in their chromosomal location may affect recombination rates in these specific sites, and that these changes can lead to rapid genome divergence (Symonová

et al. 2013). Therefore, these populational chromosome rearrangements due to rDNA transposition could promote differentiation (Symonová et al. 2013, Pucci et al. 2014, Barbosa et al. 2017), which may lead to speciation, as observed in the present study for Anostomidae.

The chromosomal mapping of the non-LTR retrotransposon family *Rex* (*Rex1*, *Rex3* and *Rex6*) has been conducted in the genomes of different teleost species (Volff et al. 1999, 2000, Cioffi et al. 2010, Valente et al. 2011, Borba et al. 2013, Sczepanski et al. 2013, among others). Although they may have a dispersed distribution (Ozouf-Costaz et al. 2004), in most cases, they show strong association with heterochromatic regions (Cioffi et al. 2010, Valente et al. 2011). Overall, the accumulation of RTE sequences in the terminal region of chromosomes has been well documented in *Drosophila melanogaster* (Meigen, 1830) and in *Sorubim lima* (Bloch & Schneider, 1801), a Neotropical catfish (Eickbush and Furano 2002, Sczepanski et al. 2013). The distribution of *Rex1* sequences in terminal regions of chromosomes in some species of Anostomidae was also detected by *in situ* mapping (Borba et al. 2013). Transpositions and DNA repair by non-homologous recombination involving repetitive sequences in the terminal regions of chromosomes are common during the Rabl configuration of cell division (Schweizer and Loid 1987, Sumner 2003). Furthermore, an efficient strategy to limit the damage caused by retrotransposition in the host genome is to direct the insertion in fairly safe regions, poor in genes, for example in heterochromatin or at telomeres (Okazaki et al. 1995, Zou et al. 1996, Takahashi et al. 1997).

Telomere shortening is usually prevented by telomerase, a reverse transcriptase which adds telomeric repeats to the chromosome ends, thus elongating telomeres (Makarov et al. 1997). The phylogeny involving telomerases and retrotransposons was confirmed after the discovery of a group of retrotransposons, called elements like Penelope, which encodes reverse transcriptase (RT) directly related to an enzyme telomerase (Arkhipova et al. 2003). In *Drosophila*, retrotransposons protect the ends of chromosomes, due to the absence of telomerase, which was possibly lost during evolution (Biessmann et al. 1990). TEs can play a role in the reorganization of the genome being co-opted or exapted to form new genomic functions (Feschotte 2008). This observation suggests the versatility of RT activity in counteracting the chromosome shortening associated with genome replication and that retrotransposons can provide this activity in case of a dysfunctional telomerase. In anostomids analyzed, the (TTAGGG) n sequence was detected in the chromosomal ends, indicating telomerase activity. The short telomere signals detected in *L. vittatus* from the Mogi-Guaçu population can be resulted of the somatic cells telomere shortens with each cell division or, due to inconsistent FISH detection in short telomere sequences. Finally, we observed absence of an interstitial telomeric sequence (ITS), together with the conserved karyotype of 2n = 54 chromosomes, indicating that just non-Robertsonian events may play a role in karyotype diversification in the studied species.

The present study showed intraspecific karyotype variation in populations with isolation of gene flow, and interspecific variation between populations of *L. friderici* and *L. vittatus*. This can be partly explained by genome reorganization due to move-

ment of heterochromatin blocks, ribosomal sites, satellite repetitive sequences, and transposable elements. Our results therefore confirm the conservation of the chromosome macrostructure and indicate karyotypic differentiation at the microstructural level during evolution in Anostomidae.

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Identification of sex chromosomes in *Eremias velox* (Lacertidae, Reptilia) using lampbrush chromosome analysis

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Abstract

Reptiles are good objects for studying the evolution of sex determination, since they have different sex determination systems in different lineages. Lacertid lizards have been long-known for possessing ZZ/ZW type sex chromosomes. However, due to morphological uniformity of lacertid chromosomes, the Z chromosome has been only putatively cytologically identified. We used lampbrush chromosome (LBC) analysis and FISH with a W-specific probe in *Eremias velox* (Pallas, 1771) to unequivocally identify the ZW bivalent and investigate its meiotic behavior. The heterochromatic W chromosome is decondensed at the lampbrush stage, indicating active transcription, contrast with the highly condensed condition of the lampbrush W chromosomes in birds. We identified the Z chromosome by its chiasmatic association with the W chromosome as chromosome XIII of the 19 chromosomes in the LBC karyotype. Our findings agree with previous genetic and genomic studies, which suggested that the lacertid Z chromosome should be one of the smaller macrochromosomes.

Keywords

meiosis, microdissection, sex chromosomes, lampbrush chromosomes, heterochromatin, lizard

Introduction

Reptiles represent a good model system for studying the evolution of sex determination, since different reptiles possess different sex determination systems. In some groups of reptiles (e.g., crocodiles, some turtles, some geckos), the sex of the offspring is determined by the temperature of egg incubation (TSD, temperature sex determination) (Viets et al. 1993). In other groups, various genetic sex determination (GSD) systems are found, ranging from GSD without heteromorphic sex chromosomes to prominently heteromorphic sex chromosome systems, some of which originated independently in different lineages from different ancestral autosomal pairs (Pokorná and Kratochvíl 2016). In some cases, different sex determination systems occur even in closely related species (Koubová et al. 2014, Gamble et al. 2015).

Several reptile lineages have sex chromosome systems common to the whole family or infraorder. These lineages include iguanas (Pleurodonta, or Iguanidae sensu lato) (Rovatsos et al. 2014), advanced snakes (Caenophidia) (Rovatsos et al. 2015), monitor lizards (Varanidae) and probably the whole anguimorph lizard group (Rovatsos et al. 2019), and lacertid lizards (Lacertidae) (Rovatsos et al. 2016a, b; see also Srikulnath et al. 2014). Comparative and evolutionary cytogenetics and genomics can determine the identities of different reptile sex chromosomes, and their homologs or syntenic chromosome regions in other animals' genomes (Deakin and Ezaz 2019).

Lacertids have a ZZ/ZW (female heterogametic) sex chromosome system. Their sex chromosomes were discovered in the early 1970s and have since been extensively studied (Ivanov and Fedorova 1973, Olmo et al. 1986, 1987, Odierna et al. 1993, Pokorná et al. 2011, Giovannotti et al. 2018). The W chromosome of lacertids is highly degenerate, and therefore can be easily identified in the karyotypes of most species by its size and/or differential staining and/or repetitive DNA content (Capriglione et al. 1994, Pokorná et al. 2011, Matsubara et al. 2015), although its exact size and DNA content vary strongly across species.

The lacertid Z chromosome is more difficult to identify. Lacertids typically have 18 pairs of extremely acrocentric or subtelocentric macrochromosomes, gradually decreasing in length, and a pair of microchromosomes (2n=38). The macrochromosomes can be roughly divided into two size groups: larger chromosomes 1–10 and smaller chromosomes 11–18 (Srikulnath et al. 2014). Differential staining techniques like G-banding, which gives chromosome-specific banding patterns in mammals, generally work poorly on reptiles.

Early studies yielded contradictory identifications of the lacertid "Z chromosome": it appeared as one of the largest chromosomes in some ideograms, and as one of the small chromosomes in others (Olmo et al. 1986, Odierna et al. 1993). Srikulnath et al. (2014) identified a putative Z chromosome of *Lacerta agilis* Linnaeus, 1758 as chromosome 5, based on Hoechst staining patterns. Recent works by Giovannotti et al. (2017) and Schmid et al. (2019) showed putative Z chromosomes of *Acanthodactylus erythrurus* (Schinz, 1933) and *Lacerta trilineata* Bedriaga, 1886, identified by FISH

with a telomeric probe and immunofluorescent localization of 5-methylcytosine, respectively, as small chromosomes.

Z-linked genes of many lacertid species were identified using transcriptome analysis and qPCR to detect genome regions with low coverage specific to one sex (Rovatsos et al. 2016a). Orthologues of all genes identified in various species are located in two microchromosomes in *Anolis carolinensis* Voigt, 1832 (Kichigin et al. 2016). Rovatsos et al. therefore suggested that the lacertids share the same Z chromosome, which is probably small. However, they did not visualize it directly. Therefore, the Z chromosomes of lacertids have not yet been unequivocally identified cytologically.

In our study, we rely on the existence of a chiasmatic association between the Z chromosome and the easily detectable W chromosome in meiotic prophase I. To visualize the sex bivalent, we obtained lampbrush chromosome (LBC) preparations from the rapid racerunner (*Eremias velox* (Pallas, 1771)). LBCs represent a specific condition of meiotic chromosomes which is found in maturing oocytes of birds, reptiles, fishes, and amphibians (Callan 1986). They are widely used in amphibian and bird cytogenetics. LBC spreads from lacertids have been reported before (Lukina 1994), but the sex chromosomes were not identified. The W chromosome of *E. velox* was previously studied by Pokorná et al. (2011). It is relatively large, but totally heterochromatic and harbours many satellite repeat sequences. To confirm the identification of the sex bivalent, we performed FISH with a microdissected probe of the W chromosome, obtained from the mitotic metaphase plate.

Material and methods

Samples and DNA barcoding

Two adult and two juvenile *E. velox* were obtained from private keepers. The adults were used for LBC preparation, and the juveniles were used for fibroblast cultures. All manipulations with live animals and euthanasia were approved by the Saint Petersburg State University Ethics Committee (statement #131-03-2) and the Institute of Molecular and Cellular Biology Ethics Committee (statement #01/18 from 05.03.2018). To confirm the species identity, we carried out DNA barcoding. DNA was extracted from ethanol-preserved blood of one of the adult specimens by the conventional phenol-chloroform technique (Sambrook et al. 1989). Primers and PCR conditions for the amplification of the fragment of the mitochondrial COI gene were as described earlier (Nagy et al. 2012). After PCR, the products were purified by electrophoresis in 1% agarose gel, cut from the gel and extracted by a commercial DNA gel extraction kit (BioSilica, Novosibirsk, Russia). The amplicons were Sanger sequenced using the BigDye3.1 reagent (ThermoFisher Scientific, USA), and the sequence was processed using MEGA7 (https://megasoftware.net). Then the sequence was analyzed using the distance-based and tree-based identification tools of the BOLD v.4 database (Ratnasingham and Hebert 2007; http://boldsystems.org/).

Lampbrush chromosome preparation

LBCs of *E. velox* were manually dissected from previtellogenic and early vitellogenic oocytes (each ovary contained 15–16 such oocytes) using the standard avian lampbrush technique described by Saifitdinova et al. (2017) with slight modifications: namely, $MgCl_2$ was excluded from the buffer solutions and EDTA was added to a final concentration of 0.01% to better disrupt the oocyte nucleus content. After centrifugation, preparations were fixed in 2% paraformaldehyde, then in 50% and in 70% ethanol. After dehydration in 96% ethanol, preparations were air-dried and mounted in antifade medium (1–1.2% DABCO, 2× SSC, 50% glycerol) with DAPI (50 ng/mL). After acquiring the DAPI and phase contrast images, the preparations were washed in 2× SSC, dehydrated in ethanol series (70%, 80%, 96%), air-dried and subjected to FISH.

Cell cultures and metaphase chromosome preparation

Primary fibroblast cell lines were established in the Laboratory of Animal Cytogenetics, the Institute of Molecular and Cellular Biology, Russia, using enzymatic treatment of tissues as described previously (Stanyon and Galleni 1991, Romanenko et al. 2015). All cell lines were deposited in the IMCB SB RAS cell bank ("The general collection of cell cultures", 0310-2016-0002). Metaphase chromosome spreads were prepared from chromosome suspensions obtained from early passages of primary fibroblast cultures as described previously (Yang et al. 1999, Graphodatsky et al. 2000, 2001).

Microdissection and FISH

Candidate chromosomes were manually microdissected from the Giemsa-stained metaphase plates using an Olympus IX-51 microscope equipped with an Eppendorf Transferman NK2 micromanipulator. Since the W chromosome does not have specific morphological features, we dissected 26 chromosomes of appropriate size from 3 metaphase plates. The dissected chromosomes were amplified and labelled with biotin- and digoxigenin-dUTP (Roche) using the commercial GenomePlex Whole Genome Amplification (WGA-1) kit (Sigma). The probes obtained were checked and characterized by FISH on metaphase chromosome preparations as described in Liehr et al. (2017). The recognized W chromosome-specific probe was used for FISH on LBCs, which was carried out as described above, omitting the RNAse and pepsin treatment stages.

Microscopy and image processing

DAPI and phase contrast images were acquired with a Leica DM4000B microscope installed at the "Chromas" Resource Centre, Saint Petersburg. The FISH

preparations were analyzed with an Axioplan 2 Imaging microscope (Carl Zeiss) equipped with a CCD camera (CV M300, JAI), CHROMA filter sets, and the ISIS4 image processing package (MetaSystems GmbH). The brightness and contrast of all images were enhanced using Corel PaintShop Photo Pro X6 (Corel Corp). The lengths of the LBCs were measured using MicroMeasure 3.3 software (Reeves 2001).

Results

The DNA sequence (GenBank accession number MK558359) showed that the specimens analyzed belong to the "eastern" clade of *E. velox* (the nominative subspecies *E. velox velox* (Pallas, 1771)). The mitotic karyotype of the lizards studied was typical of Lacertidae and was in agreement with previous studies (Kupriyanova and Arronet 1969; Pokorná et al. 2011). It consisted of 38 uniarmed chromosomes gradually decreasing in length. The W chromosome was DAPI-positive, and one of the microdissected probes showed a very strong hybridization signal on it (Fig. 1). It also gave several additional hybridization signals in the telomeres and centromeres of some autosomes, but no other chromosome showed a hybridization signal across its whole length. This probe was concluded to be W-specific.

The contents of the oocyte nuclei after the removal of the nuclear envelope formed a dense ball, and its full dispersal was more difficult to achieve than with birds and amphibians. Thus, most LBC sets showed insufficient spreading, and only one finely spread and complete chromosome set was obtained. The lampbrush karyotype of *E. velox* consisted of 19 bivalents, with the bivalent XIX (the only microchromosome) significantly smaller than the others (Suppl. material 1: Fig. S1). This agrees with the mitotic karyotype. The bivalents typically had one or two terminal or subterminal chiasmata. The total number of chiasmata per spread was estimated as 35 to 38. Interestingly, the microchromosomal bivalent (XIX) had two chiasmata.

Although LBCs were isolated from previtellogenic oocytes, prominent lateral loops were absent on most bivalents, which is in accordance with a previous observation made in lacertids by Lukina (1994). This fact probably reflects that the oocytes which are large enough for LBC preparations are at relatively late diplotene stages in small lizards (Lukina 1994). In one of the bivalents, the homologues were different in length and chromatin state. One of the homologues consisted of dense chromomeres, resembling other chromosomes. The other homologue was decondensed and showed long chromatin loops. The W-specific probe labelled the decondensed homologue, thus confirming that this is the sex bivalent (Fig. 2). The sex bivalent had only a single chiasma, which was located terminally, suggesting a physically very short pseudoautosomal region. The measurements of the relative lengths of the LBCs showed that the Z chromosome is chromosome XIII in the lampbrush karyotype, thus belonging to the fraction of small chromosomes (Fig. 3).

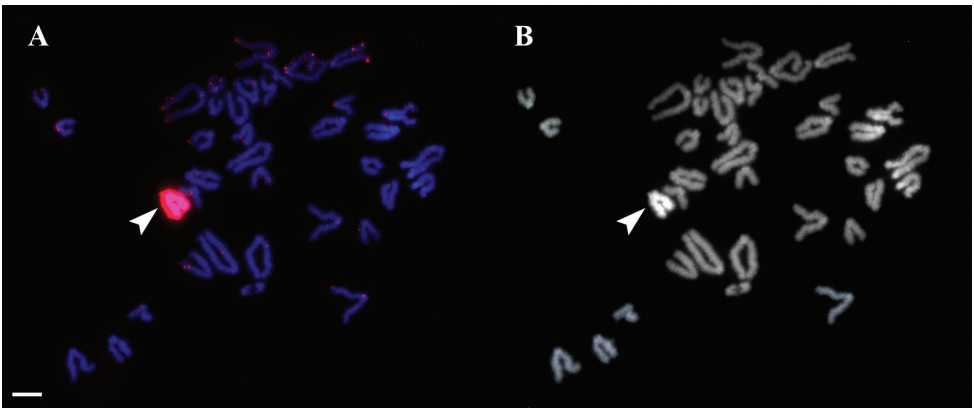

Figure 1. FISH with the microdissected W-specific probe on mitotic chromosomes of *Eremias velox* **A** DAPI (blue), W-specific probe (red) **B** DAPI channel separately. Arrowhead indicates W chromosome. Scale bar: 10 μm.

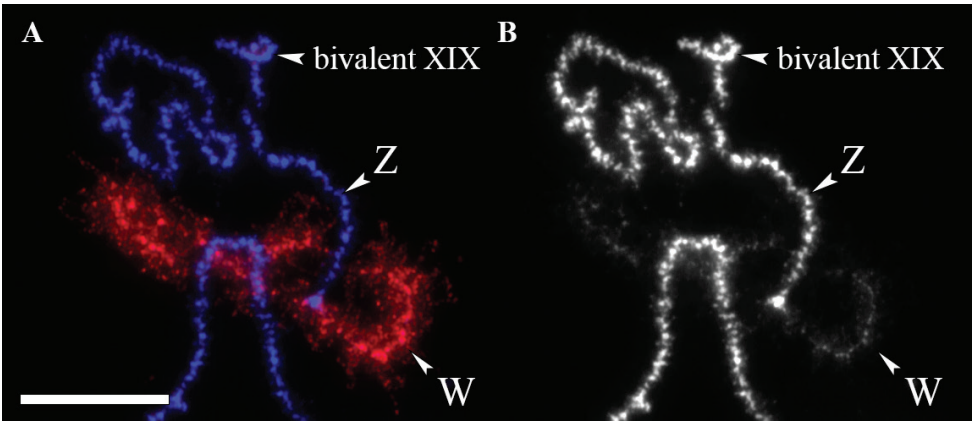

Figure 2. FISH with the microdissected W-specific probe on lampbrush sex bivalent of *Eremias velox*. **A** DAPI (blue), W-specific probe (red) **B** DAPI channel separately. Scale bar: 15 μm.

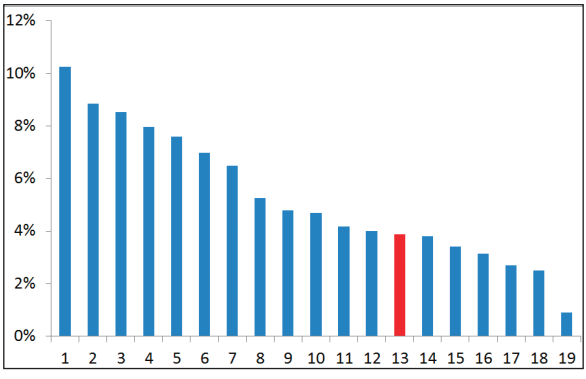

Figure 3. Ideogram of lampbrush karyotype of *Eremias velox*. Red indicates Z chromosome. X axis indicates size ranks. Y axis indicates relative length.

Discussion

For many years, amphibian and avian LBCs have been serving as a spectacular model for studying chromosome organization and genome functioning. In squamate reptiles, which also have a hypertranscriptional type of oogenesis, LBCs have scarcely been studied before. The initial descriptions of LBCs of *Lacerta agilis*, *Zootoca vivipara* (Lichtenstein, 1823), *Darevskia armeniaca* (Méhely, 1909) and *Podarcis tauricus* (Pallas, 1814) were made by Lukina (1994). However, no full karyotypes were described and the sex chromosomes were not identified. We are the first to describe the complete lacertid karyotype in the lampbrush form, and identify the sex bivalent by a molecular cytogenetic approach.

We noted above that most lampbrush bivalents had one or two chiasmata. The observed number exceeds the mean numbers of recombination nodules in male meiosis in *Darevskia* Arribas, 1999, identified by immunolocalization of SYCP3 and MLH1 proteins at pachytene, which equaled 24–29 in different species (Spangenberg et al. 2017, 2019). In particular, the occurrence of two chiasmata, like those observed in bivalent XIX (Fig. 2), is extremely rare in the microchromosomes of male lizards (Lisachov et al. 2017, 2019). This suggests the occurrence of more crossovers in female than male meiosis in lacertids (heterochiasmy). Different types of heterochiasmy, including more crossovers in one sex than in another, and/or different crossover localizations, are known in many species (Mank 2009). However, since our sample size is limited to one spread, more data are required to draw firm conclusions about crossover numbers. The terminal and sub-terminal localization of most chiasmata is also consistent with the previously obtained data on lacertid lizards and many other animal species (Mézard et al. 2015).

The decondensed state of the heterochromatic W chromosome in *E. velox* contrasts with the lampbrush sex bivalents of birds, in which the heterochromatic W chromosome is much more condensed than the Z and autosomes (Solovei et al. 1993). Numerous lateral loops indicate that the W chromosome of *E. velox* is transcriptionally active at the lampbrush stage. Due to the transcriptional activity of LBCs, an enormous amount of RNA is synthesized in the oocyte nucleus, mainly of sequences that do not encode proteins, e.g. transposable and some satellite repeated sequences (Gaginskaya et al. 2009). These transcripts could have functions in regulatory mechanisms involved in embryonic development, epigenetic processes, maintaining chromatin structure, or other functions (Gaginskaya et al. 2009). More detailed analysis of sex chromosome behavior in meiosis in *E. velox* and other lacertids is required to determine whether the high transcriptional activity of the W chromosome is common to all lacertids, what these transcripts represent and their biological roles, what is the extent of "degeneration" and heterochromatinization of the W chromosome, and its possible "junk" repetitive sequences accumulated.

This study is the first unequivocal cytological identification of a lacertid lizard Z chromosome. The size ranks of LBCs do not always correlate precisely with the sizes of the mitotic chromosomes, or their relative genomic lengths (Daks et al. 2010). Given

the similar sizes of the small macrochromosomes in the lacertid karyotypes (Srikulnath et al. 2014), the Z chromosome of the rapid racerunner may not be its 13th largest chromosome, but it is evident that it belongs to the group of small macrochromosomes.

Our identification is in good agreement with the previous recent putative cytological identifications of Z chromosomes in *A. erythrurus* and *L. trilineata* (Giovannotti et al. 2017, Schmid et al. 2019) using FISH and immunostaining, and with the genetic identifications: in several lacertid species using the qPCR approach mentioned above (Rovatsos et al. 2016a), and in *Podarcis muralis* (Laurenti, 1768) using coverage differences between genome sequences from male and female samples (Andrade et al. 2019). Chromosome 5, which belongs to the group of large macrochromosomes and was suggested to be the sex chromosome in *L. agilis* (Srikulnath et al. 2014), is apparently not a sex chromosome in *E. velox*.

Identification of the *E. velox* sex chromosomes should lead to further studies of sex chromosome evolution and function in Lacertidae, including estimates of the extent of W chromosome genetic degeneration and its time course. Reliable identification of the *E. velox* Z chromosome will facilitate obtaining Z-derived chromosome-specific and region-specific probes for cytogenetic and genomic studies, including via LBC microdissection.

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Supplementary material 1

Figure S1

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RESEARCH ARTICLE

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Molecular and cytogenetic differentiation within the *Lariophagus distinguendus* (Förster, 1841) species complex (Hymenoptera, Pteromalidae)

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Abstract

Several strains of the apparently well-known cosmopolitan synanthropic parasitoid of coleopteran stored-product pests, *Lariophagus distinguendus* (Förster, 1841) from Western Europe, were studied using DNA sequencing and chromosomal analysis. The presence of at least two cryptic species with different COI sequences and chromosome numbers (n = 5 and 6) was supported. The species with n = 6 is associated with the drugstore beetle *Stegobium paniceum* (Linnaeus, 1758), whereas the other one with n = 5 mostly develops on the granary weevil *Sitophilus granarius* (Linnaeus, 1758). A phylogenetic study revealed that the karyotype with n = 6 represents an ancestral character state in this complex. Consequently, the chromosome set with n = 5 which is characteristic of a particular internal clade, apparently originated via chromosomal fusion which was probably preceded by a pericentric inversion. If this is true, inverted chromosome segments could accumulate a number of genetic loci responsible for certain interspecific differences.

Keywords

Pteromalidae, *Lariophagus distinguendus*, cryptic species, phylogeny, COI sequencing, karyotype

Introduction

Parasitoid Hymenoptera are among the most diverse, taxonomically complicated and economically important insect groups (Heraty 2017, Forbes et al. 2018). Over 80 thousand species of parasitoid wasps have already been described (Huber 2017). Furthermore, at least one million parasitoid species might still be unknown (Bebber et al. 2014, also see Quicke 1997). In addition to the poor knowledge of tropical fauna of parasitoid wasps, this high number of undescribed species apparently results from the phenomenon of the so-called cryptic lineages (Quicke 2002, Heraty 2017), which are very similar or virtually identical in morphology but differ considerably in genetic, ecological, behavioral, and other characteristics. Due to certain features of the parasitoid lifestyle, the latter phenomenon appears to be widespread among these insects (see Gokhman 2018 for review). Moreover, successful resolution of cryptic species complexes has important implications both for parasitoid wasp taxonomy and biological pest control (Heraty 2017).

The vast superfamily Chalcidoidea, which contains nearly 23 thousand described species (Huber 2017), is one of the largest groups among parasitoid Hymenoptera. Pteromalidae is one of the most species-rich chalcid families, comprising over 3,500 described species (Huber 2017). Although Pteromalidae *sensu lato* never recovers as a monophyletic group in all modern studies (see, e.g., Munro et al. 2011 and Heraty et al. 2013) and is going to be subdivided into a number of separate families, monophyly of the so-called pteromaloid complex, including Pteromalinae and few related subfamilies, has constantly been supported by recent cladistic analyses (e.g., Peters et al. 2018). Moreover, Pteromalinae include several known complexes of cryptic species. For example, the taxonomic revision of the genus *Anisopteromalus* Ruschka, 1912 has led to the description of a new cosmopolitan synanthropic species, *Anisopteromalus quinarius* Gokhman & Baur, 2014 which, together with the well-known *A. calandrae* (Howard, 1881) usually attacks various beetles that feed on stored products (Baur et al. 2014). Recently, cryptic lineages have also been detected in another cosmopolitan parasitoid from the subfamily Pteromalinae, *Lariophagus distinguendus* (Förster, 1841) (König et al. 2015) with an analogous biology. Specifically, a particular lineage is apparently specialized on the drugstore beetle *Stegobium paniceum* (Linnaeus, 1758) (Coleoptera, Ptinidae) occurring in households, while strains of the other lineage were collected on weevils of the genus *Sitophilus* Schönherr, 1838 (Coleoptera, Dryophthoridae) in grain stores. To define the taxonomic status of these lineages, we have undertaken an extensive study of synanthropic populations of *L. distinguendus* from Western Europe using research of partial mitochondrial cytochrome oxidase I (COI) DNA sequences and chromosomal analysis. The results of this study are given below.

Materials and methods

Origin of parasitoid wasps

In total, fourteen strains of *L. distinguendus* were studied including nine strains described in König et al. (2015). Four new strains (CAN-D I, CAN-D III, OST-D I, and

STU-D II) were collected by volunteers as part of a citizen science project in 2017 and 2018. In this project, bait boxes equipped with pellets of koi fish food (Hikari Friend, Kamihata Fish Industry Group, Kyorin Corporation, Japan) infested by *St. paniceum* were used. An additional strain (FRI-D I), also attacking *St. paniceum*, was sent to us by a private person. All strains were reared either on *St. paniceum* or *Sitophilus granarius* (Linnaeus, 1758) depending on their host preferences, as described in König et al. (2015) (see Table 1 for the list of studied strains and specimens).

DNA extraction and sequencing

DNA from *L. distinguendus* strains CAN-D I, CAN-D III, OST-D I, FRI-D I, BIR-D I and STU-D II was extracted and purified following the manufacturer's instructions using Nexttec 1-Step DNA Isolation Kit – Tissue & Cell (Biozym, Hessisch Oldendorf, Germany) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). PCR amplification, bidirectional sequencing, processing and editing of the partial COI fragment was performed as described in König et al. (2015). We used the primer pair C1-J-2183 5′-CAACATTTATTTTGATTTTTTGG-3′ and TL2-N-3014 5′-TCCAATGCACTAATCTGCCATATTA-3′ from Simon et al. (1994). The thermocycler program started with a denaturation temperature 95 °C / 2 min, followed by 40 cycles at 94 °C / 1 min, 58 °C / 1 min and 72 °C / 1.5 min. The final extension was 10 min at 72 °C. Positive PCR products were bidirectionally sequenced by Seqlab (Göttingen, Germany). Each chromatogram was checked for ambiguous positions and possible double peaks to avoid potential nuclear copies of mitochondrial sequences (NUMTs) (see Bensasson et al. 2001). All sequences were assembled using the program GENtle version 1.9.4 (by Magnus Manske, University of Cologne, Germany, released under GPL 2003). The obtained DNA sequences were translated into amino acid ones using the program "Virtual Ribosome" (Wernersson 2006) based on the code for invertebrate mitochondria to check for unexpected stop codons or gaps. The resulting consensus DNA sequences lacked ambiguity at all base pairs, and were finally aligned in MAFFT version 7 (Katoh and Standley 2013) with the G-INS-i algorithm (Katoh et al. 2005). Newly obtained sequences were submitted to GenBank (Table 1).

Phylogenetic analysis

Phylogenetic analyses were conducted in MEGA X (Kumar et al. 2018) by first checking for the best-fit substitution model and subsequently constructing a maximum likelihood (ML) tree including 1000 bootstrap replications (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The model for nucleotide substitutions [Hasegawa-Kishino-Yano (Hasegawa et al. 1985) allowing some sites to be evolutionarily invariable] was

Table 1. Strains and specimens of *L. distinguendus* used in the molecular and chromosome study.

Strain	Host	Locality	Country/region	COI GenBank accession numbers	No. of specimens for chromosome study (male/female)	Haploid/diploid chromosome number
BIR-D I 1 BIR-D I 2	*St. paniceum*	Stuttgart Birkach	Germany/Baden-Württemberg	MK572719 MK572720	1(2) / 10(44)	6/12
BYG-DK I1 BYG-DK I2	*S. granarius*	Bygholm	Denmark	KJ867379 KJ867380	3(47) / 1(4)	5/10
CAN-D I1 CAN-D I2	*St. paniceum*	Stuttgart Bad Cannstatt	Germany/Baden-Württemberg	MK572723 MK572724	2(19) / 6(38)	6/12
CAN-D III 1	*St. paniceum*	Stuttgart Bad Cannstatt	Germany/Baden-Württemberg	MK572726	1(4) / 1(3)	6/12
FRI-D I1 FRI-D I2	*St. paniceum*	Fritzlar	Germany/Hessen	MK572717 MK572718	4(19+1‡) / 2(9)	6, 7‡/12
OST-D I2 OST-D I3	*St. paniceum*	Ostfildern	Germany/Baden-Württemberg	MK572721 MK572722	2(7) / 6(24+2†)	6/12, 13†
PFO-D I1 PFO-D I2	*S. granarius*	Pforzheim	Germany/Baden-Württemberg	KJ867383 KJ867384	4(32) / 2(10)	5/10
RAV-D I1 RAV-D I2	*St. paniceum*	Ravensburg	Germany/Baden-Württemberg	KJ867387 KJ867388	1(3) / 2(8)	6/12
SAC-D I1 SAC-D I2	*S. granarius*	Sachsen	Germany/Saxony	KJ867381 KJ867382	1(25) / 2(10)	5/10
SAT-D I1 SAT-D I2	*S. granarius*	Satrup	Germany/Schleswig-Holstein	KJ867375 KJ867376	1(10) / –	5/–
SLO-GB I1 SLO-GB I2	*S. granarius*	Slough	UK/Berkshire	KJ867377 KJ867378	4(28) / 1(13)	5/10
STU-D I1 STU-D I2	*St. paniceum*	Stuttgart Bad Cannstatt	Germany/Baden-Württemberg	KJ867385 KJ867386	2(18) / 1(1)	6/12
STU-D II1	*St. paniceum*	Stuttgart Mitte	Germany/Baden-Württemberg	MK572725	– / –	– / –
WAG-D I1 WAG-D I2	*St. paniceum*	Wageningen	The Netherlands	KJ867389 KJ867390	1(1) / 2(4)	6/12
–	–	F_1 hybrids (RAV × PFO)	–		– / 6(29)	–/11
–	–	Male progeny of F_1 hybrids	–		3+4(23+23) / –	5, 6/–

†An aberrant female karyotype with a smaller acrocentric fragment.
‡An aberrant male karyotype with an apparently fragmented acrocentric chromosome.

selected by applying the Bayesian Information Criterion (BIC) in MEGA X. The present analysis involved 27 nucleotide sequences and included 679 positions in the final dataset. Uncorrected *p*-distances were calculated using MEGA X.

Chromosomal analysis

Chromosome preparations were obtained from cerebral ganglia of male and female prepupae of *L. distinguendus* following the protocol developed by Imai et al. (1988) with a few modifications (see e.g. Gokhman et al. 2017). Specifically, ganglia were extracted from insects dissected in 0.5% hypotonic sodium citrate solution containing 0.005% colchicine. The extracted ganglia were then transferred to a fresh portion of hypotonic solution and incubated for 30 min at room temperature. The material was transferred onto a pre-cleaned microscope slide using a Pasteur pipette and then gently flushed with

Fixative I (glacial acetic acid: absolute ethanol: distilled water 3:3:4). The tissues were disrupted using dissecting needles in an additional drop of Fixative I. Another 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 chromosome staining, the preparations were usually left overnight in a freshly prepared 3% Giemsa solution in 0.05M Sorensen's phosphate buffer (Na_2HPO_4 + KH_2PO_4, pH 6.8). Mitotic divisions were studied and photographed using an optic microscope Zeiss Axioskop 40 FL fitted with a digital camera AxioCam MRc (Carl Zeiss, Oberkochen, Germany). To obtain karyograms, the resulting images were prepared with image processing software: Zeiss AxioVision version 3.1 and Adobe Photoshop version 8.0. Mitotic chromosomes were measured on 20 haploid metaphases of each species using KaryoType software version 2.0. We report relative lengths (RL: 100 × length of each chromosome divided by total length of the set) and centromeric indices (CI: 100 × length of shorter arm divided by total length of a chromosome) for both species. On the karyograms, chromosomes were initially subdivided according to their measurements into elements characteristic of a particular chromosome set (columns 1–3) and those shared by the two main karyotypes (columns 4–7; see below). Within both groups, chromosomes were arranged in decreasing order of size. In addition, chromosomes were further classified into metacentric, subtelocentric or acrocentric according to the guidelines provided by Levan et al. (1964).

Results

Molecular data

Three main clades (*Stegobium* Clade 1, *Sitophilus* Clade 1, *Stegobium* Clade 2) were recovered within the *L. distinguendus* species complex (Fig. 1), including a particular one (*Stegobium* Clade 2) which can be considered as an outgroup to all previously studied strains (König et al. 2015). All strains from *Stegobium* Clades 1 and 2 were collected on *St. paniceum* in pantries or were trapped with *St. paniceum* samples as baits. In turn, all strains from the *Sitophilus* Clade 1 originate from samples from grain stores, which were infested with *S. granarius*. The average numbers of base differences per site for all sequence pairs of different clades were 0.137 between *Stegobium* Clade 1 and *Sitophilus* Clade 1, 0.155 between *Stegobium* Clades 1 and 2, and 0.147 between *Sitophilus* Clade 1 and *Stegobium* Clade 2. Sequence differences within the clades were low (*Stegobium* Clade 1 = 3.0%, *Sitophilus* Clade 1 = 1.6%, *Stegobium* Clade 2 = 0.1%).

Cytogenetic data

Chromosome study of all studied strains revealed two main karyotypes with different chromosome numbers, n = 5 (2n = 10) and 6 (2n = 12) (Fig. 2a–d). The karyotype

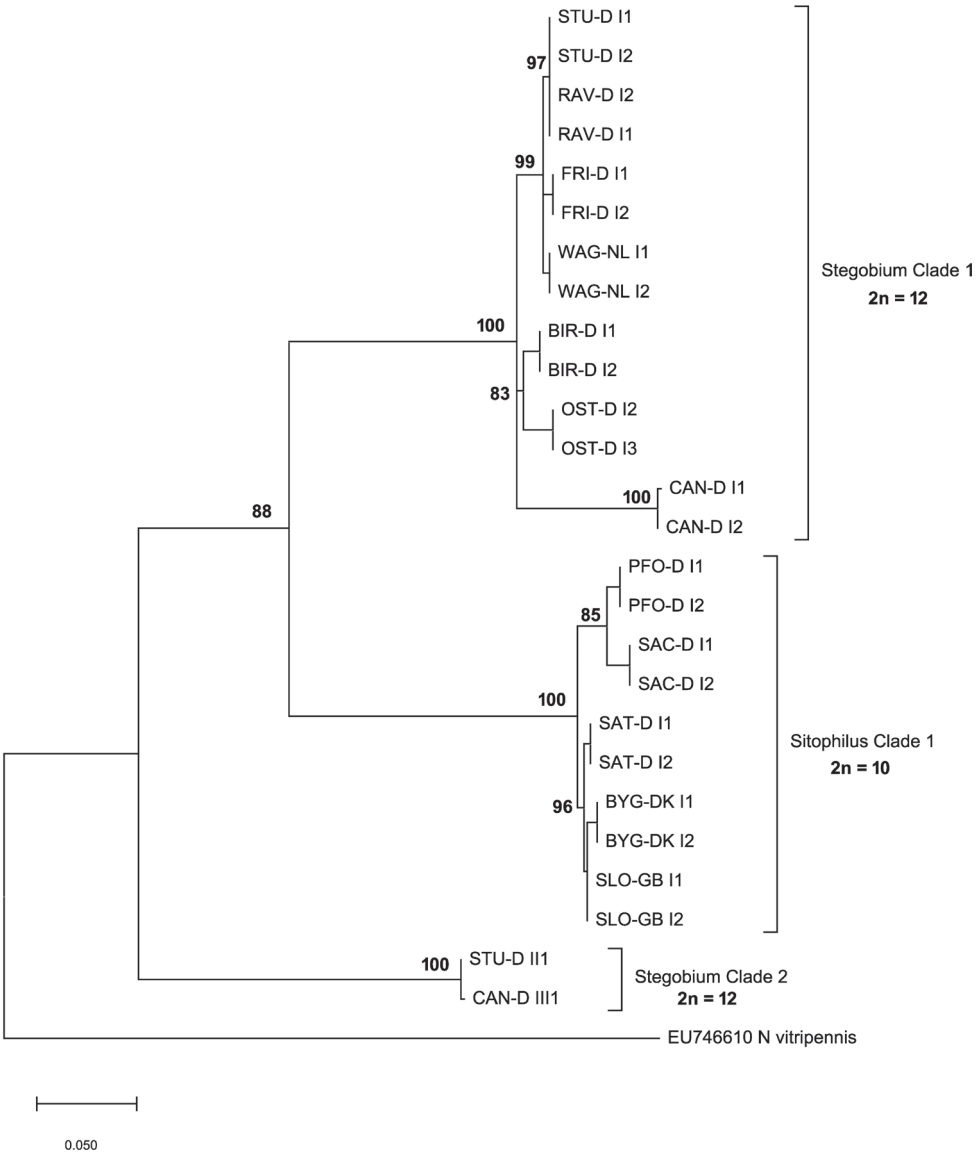

Figure 1. Evolutionary relationships of different strains of *L. distinguendus* based on a partial COI fragment. The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). The tree with the highest log likelihood (-2312.56) is shown. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 65.23% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

of hybrid females contained 11 chromosomes (2n = 11), whereas their male progeny had either n = 5 or 6 (Fig. 2e–g). Preliminary measurements indicated that four metacentric chromosomes within both haploid karyotypes were similar. In addition, the

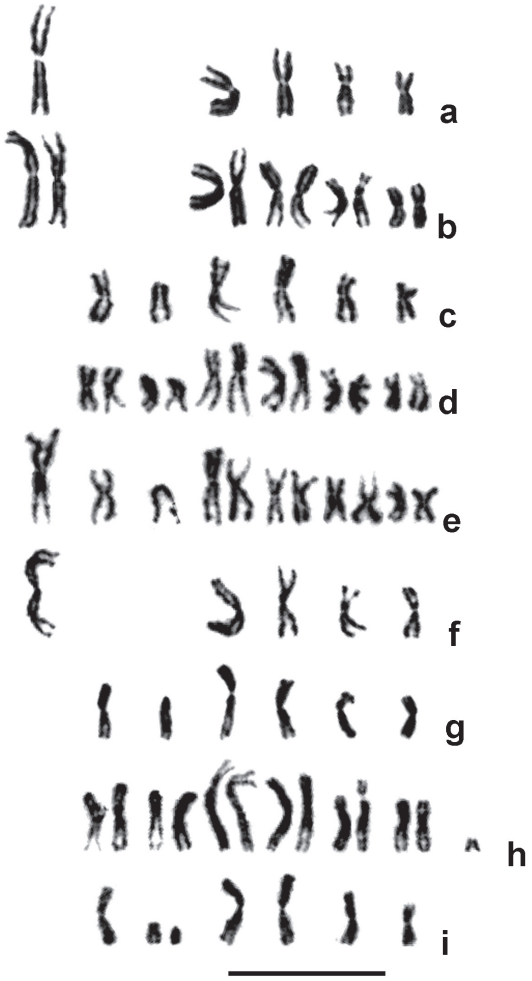

Figure 2. Karyotypes of different strains of the *Lariophagus distinguendus* species complex (see Table 1 for details). **a** PFO-D I, male (n = 5) **b** SLO-GB I, female (2n = 10) **c** OST-D I, male (n = 6) **d** OST-D I, female (2n = 12) **e** F_1 hybrid RAV-D I × PFO-DI, female (2n = 11) **f** progeny of F_1 hybrid RAV-D I × PFO-D I, male (n = 5) **g** ditto (n = 6) **h** OST-D I, female, aberrant karyotype (the same individual as in **d** 2n = 13) **i** FRI-D I, male, aberrant karyotype (n = 7). Scale Bar: 10 μm.

karyotype with n = 5 contained the largest metacentric in the chromosome set, while the karyotype with n = 6 contained a smaller metacentric and the only acrocentric chromosome. These results were also confirmed by the detailed morphometric study (see Table 2 and Fig. 3). Moreover, four similar metacentrics were clearly paired within female karyotypes of F_1 hybrids, whereas the other three elements were represented by single copies (Fig. 2e). This suggests that certain unpaired chromosomes from different karyotypes correspond to each other. This assumption is further corroborated by the fact that combined RLs of the two smaller chromosomes (no. 2 and 3) in the karyotype

Table 2. Measurements of mitotic chromosomes on haploid metaphase plates of the *L. distinguendus* complex with n = 5 and 6 (N = 20; mean ± SD).

Karyotype / chromosome no.		1	2	3	4	5	6	7
n = 5	RL	29.48 ± 1.77	–	–	23.03 ± 1.15	19.19 ± 0.75	14.98 ± 0.91	13.32 ± 0.86
	CI	47.06 ± 3.16	–	–	46.13 ± 2.25	47.25 ± 1.79	43.74 ± 3.83	44.49 ± 4.58
n = 6	RL	–	16.68 ± 0.89	12.86 ± 0.94	22.55 ± 1.28	19.86 ± 1.05	15.35 ± 0.83	12.70 ± 0.83
	CI	–	45.45 ± 4.08	0	43.27 ± 3.30	46.96 ± 2.69	45.83 ± 2.64	46.10 ± 3.23

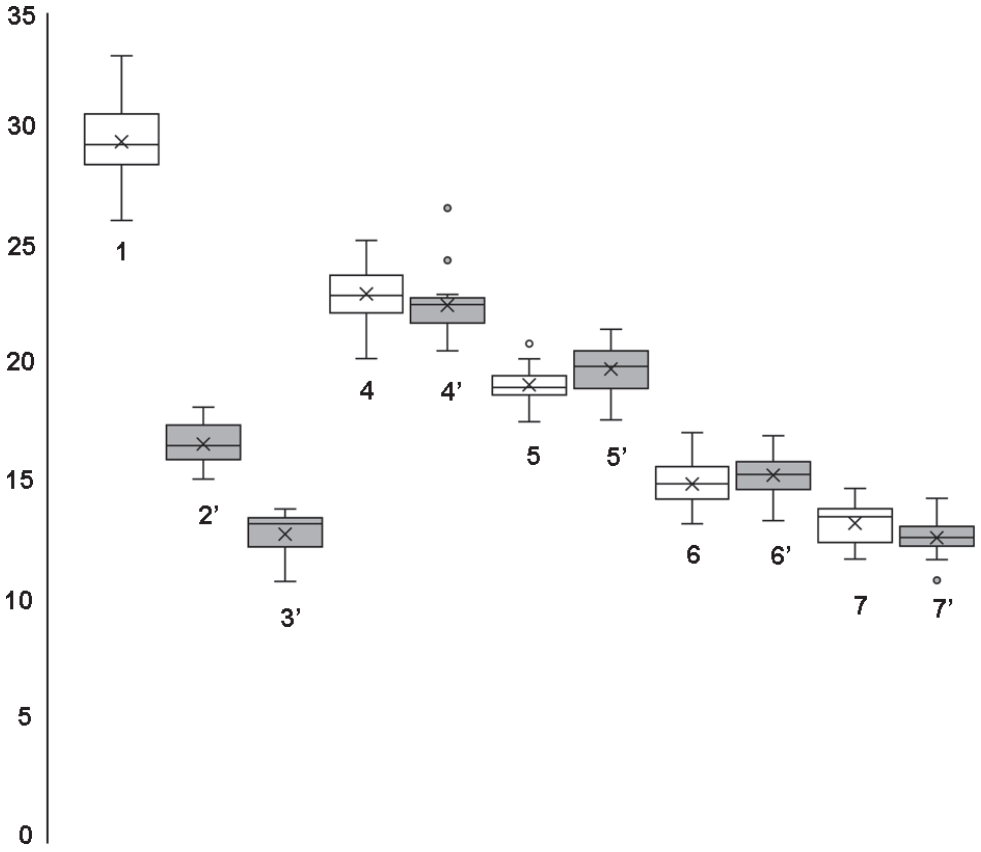

Figure 3. Box-and-whisker plot of relative lengths of chromosomes of different species of the *L. distinguendus* complex (based on data of the chromosome measurements also used in Table 2). The means, medians, second and third quartiles as well as variation ranges of RLs are represented by X signs, horizontal lines within boxes, boxes and whiskers respectively. 1, 4 etc. – numbers of chromosomes of the species with n = 5; 2', 3' etc. – numbers of chromosomes of the species with n = 6.

with n = 6 were almost exactly equal to the RL of the largest metacentric (chromosome 1) in the karyotype with n = 5 (see Table 2). In addition, these chromosomes were again segregated in the male progeny of F_1 hybrid females that contained both karyotypes with n = 5 and 6 in similar proportions (Table 1, Fig. 2f–g).

A few aberrant mitotic divisions were also detected. Specifically, most metaphase plates from a particular female individual of OST-D I strain had the normal karyotype with 2n = 12 (Fig. 2d), whereas a few cells carried a small additional, apparently acrocentric element (Fig. 2h). On the other hand, almost all metaphase plates of another male specimen of FRI-D I strain also showed a normal chromosome set with n = 6, although a single mitotic division with n = 7 was found (Fig. 2i). A detailed study of the latter karyotype suggests that it carries two smaller elements, a subtelocentric and an acrocentric. In this case, chromosome morphometrics demonstrates that the two chromosomes probably result from fragmentation of the medium-sized acrocentric of the normal karyotype.

Discussion

Molecular phylogeny

Phylogenetic analysis of COI sequences revealed a clear separation of the strains into three main clades, supported by high bootstrap values (Fig. 1). The molecular divergence between the clades was remarkably high (13.7% – 15.5%) in contrast to the low genetic differences within the clades. Interestingly, the position of the strains in the cladogram was correlated with their host preference, and was independent of their geographic origin (Table 1). All strains from *Stegobium* Clades 1 and 2 were associated with drugstore beetles (*St. paniceum*), whereas all strains from *Sitophilus* Clade 1 were collected on weevils of the genus *Sitophilus* in grain stores (König et al. 2015). The fact that *Stegobium* Clade 2 is basal to all other main clades suggests that *St. paniceum* or a closely related species can be the ancestral host, and that *Sitophilus* Clade 1 evolved by a host shift to *Sitophilus*. This agrees with the hypothesis by König et al. (2015) on the evolution of the two cryptic lineages of *L. distinguendus*. Remarkably, this host shift was probably related to the ability to learn host-related cues (König et al. 2015).

Chromosome study

Apart from a few aberrant metaphase plates, two main karyotypes with n = 5 (2n = 10) and n = 6 (2n = 12) were detected. Specifically, the latter chromosome set is characteristic of the strains of *Stegobium* Clades 1 and 2 which originated from samples developing on *St. paniceum*, while karyotype with n = 5 was found in all members of *Sitophilus* Clade 1 from grain stores which were associated with weevils of the genus *Sitophilus* (König et al. 2015; Fig. 1, also see above). These data indicate that n = 6 is the ancestral character state for the *L. distinguendus* species complex, and the chromosome set with n = 5 is derived, although this is in contrast to the idea that n = 5 is apparently ancestral for members of Pteromalidae (Gokhman 2009). Chromosome measurements (Table 2) indicate that the karyotype with n = 5 in *L. distinguendus* most

likely originated from fusion of chromosomes 2 and 3 of the karyotype with n = 6, yielding chromosome 1, the largest metacentric in the karyotype with n = 5. Together with some other recent studies (see e.g. Gokhman et al. 2017), the present work thus demonstrates substantial importance of molecular research for the phylogenetic reconstruction of karyotype evolution of parasitoid wasps.

Our recent hypothesis that the decrease in the chromosome number in the *L. distinguendus* species complex occurred through chromosomal fusion is further corroborated by the results of the karyotypic study of F_1 hybrids between these forms (Fig. 2e). As far as possible rearrangements underlying the above-mentioned decrease in the chromosome number are concerned, either central or tandem chromosomal fusion can be proposed (White 1973, Gokhman 2009). In the case of central fusion, it must be preceded by a pericentric inversion in the smaller metacentric of the chromosome set with n = 6. If this is true, the two species of the *L. distinguendus* complex also differ by this inversion, in addition to the chromosomal fusion. Interestingly, accumulation of genetic loci responsible for certain differences between closely related forms within inverted chromosomal segments now became a key feature of the so-called "supergene concept", a popular approach in modern evolutionary genetics (see e.g. Thompson and Jiggins 2014). This concept is based on the fact that chromosome inversions interfere with the process of crossing-over, thus preventing recombination within the inverted segments (White 1973). Nevertheless, one-step rearrangement, i.e., a tandem fusion between the acrocentric and the metacentric chromosome accompanied by centromere inactivation in the longer arm of the resulting larger metacentric, is also possible (White 1973, Gokhman 2009).

Taxonomic implications of the molecular and cytogenetic studies

All obtained information, together with data on reproductive relationships and host specificity of the studied strains (König et al. 2015), suggests that the *L. distinguendus* complex harbors at least two cryptic species. However, no reliable morphological difference between these species was found to date (Wendt et al. 2014). This information, as well as their karyotypic similarity and the possibility of interspecific hybridization indicates that these cryptic species are closer to each other than e.g. those of the genus *Anisopteromalus* (Baur et al. 2014). Nevertheless, genetic differences between members of the *L. distinguendus* complex together with our preliminary data on the decreased production of hybrid offspring from crossings between forms with different karyotypes confirm that this complex harbors separate species. Our results thus describe the first case of hybridization between two cryptic parasitoid species with different chromosome numbers. On the other hand, relatively strong differences in the structure of COI sequences between certain strains with the same karyotype do not necessarily indicate their species status (see e.g. Hernández-López et al. 2012, Korenko et al. 2018). Further molecular studies, which should also include nuclear markers for those strains that were not previously examined in this respect, are therefore needed (see König et al. 2015).

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DATA PAPER

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Rapid chromosomal evolution in enigmatic mammal with XX in both sexes, the Alay mole vole *Ellobius alaicus* Vorontsov et al., 1969 (Mammalia, Rodentia)

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Abstract

Evolutionary history and taxonomic position for cryptic species may be clarified by using molecular and cytogenetic methods. The subterranean rodent, the Alay mole vole *Ellobius alaicus* Vorontsov et al., 1969 is one of three sibling species constituting the subgenus *Ellobius* Fischer, 1814, all of which lost the Y chromosome and obtained isomorphic XX sex chromosomes in both males and females. *E. alaicus* is evaluated by IUCN as a data deficient species because their distribution, biology, and genetics are almost unknown. We revealed specific karyotypic variability (2n = 52–48) in *E. alaicus* due to different Robertsonian translocations (Rbs). Two variants of hybrids (2n = 53, different Rbs) with *E. tancrei* Blasius, 1884 were found at the Northern slopes of the Alay Ridge and in the Naryn district, Kyrgyzstan. We described the sudden change in chromosome numbers from 2n = 50 to 48 and specific karyotype structure for mole voles, which inhabit the entrance to the Alay Valley (Tajikistan), and revealed their affiliation as *E. alaicus* by cytochrome *b* and fragments of nuclear *XIST* and *Rspo1* genes sequencing. To date, it is possible to expand the range of *E. alaicus* from the

Alay Valley (South Kyrgyzstan) up to the Ferghana Ridge and the Naryn Basin, Tien Shan at the north-east and to the Pamir-Alay Mountains (Tajikistan) at the west. The closeness of *E. tancrei* and *E. alaicus* is supported, whereas specific chromosome and molecular changes, as well as geographic distribution, verified the species status for *E. alaicus*. The case of *Ellobius* species accented an unevenness in rates of chromosome and nucleotide changes along with morphological similarity, which is emblematic for cryptic species.

Keywords

speciation, hybridization, chromosome painting, cytochrome *b* gene, nuclear *XIST* and *Rspo1* genes, Robertsonian translocations, synaptonemal complex, *Ellobius*

Introduction

An origin of species due to chromosome changes is still debatable (King 1993, Castiglia 2014, Dobigny et al. 2017). The problem of chromosomal speciation is closely connected with the phenomenon of sibling species. Mole voles of the genus *Ellobius* Fischer, 1814, and some other rodents, such as *Mus* Linnaeus, 1758, *Nannomys* Peters, 1876 (Gropp et al. 1972, Capanna et al. 1976, Capanna and Castiglia 2004, Veyrunes et al. 2010, Garagna et al. 2014), and subterranean *Spalax* Guldenstaedt, 1770, *Fukomys* Kock et al., 2006, *Ctenomys* Blainville, 1826 etc. (Wahrman et al. 1969, Nevo et al. 2000, Van Daele et al. 2007, Deuve et al. 2008, Kryštufek et al. 2012, Buschiazzo et al. 2018), demonstrate a broad chromosome variability at the species and intraspecies levels without morphological differences (Lyapunova et al. 1980). The lack of clear morphological characters, by which specimens can be easily distinguished in museum collections, as well as in nature, makes such species problematic for study and protection. New molecular methods, especially DNA sequencing and cross-species chromosome painting, can be a precise approach for studying the most intriguing groups (Graphodatsky et al. 2011).

The genus *Ellobius* divides into two subgenera: *Bramus* Pomel, 1892 and *Ellobius* Fischer, 1814 (Musser, Carleton 2005). The subgenus *Bramus* includes two species: *E. fuscocapillus* Blyth, 1843 (2n = 36, XX♀–XY♂), and *E. lutescens* Thomas, 1897 (2n = 17, X0♀-X0♂) (Matthey 1953, Vorontsov et al. 1969, Lyapunova, Vorontsov 1978). Species of the subgenus *Ellobius* (*E. talpinus* Pallas, 1770, *E. tancrei* Blasius, 1884, and *E. alaicus* Vorontsov et al. 1969) are cryptic ones, indistinguishable by morphological features (Yakimenko and Vorontsov 1982), the main diagnostic features are distant karyotypes. *E. talpinus*, *E. tancrei*, and *E. alaicus* are unique in mammals. Along with autosomal changes, the species lost the Y chromosome, the *Sry* gene, and obtained isomorphic XX chromosomes in both males and females (Lyapunova and Vorontsov 1978, Vorontsov et al. 1980, Kolomiets et al. 1991, Just et al. 1995, Romanenko et al. 2007, Bakloushinskaya et al. 2012, Bakloushinskaya and Matveevsky 2018). The study of *E. lutescens* and *E. talpinus* whole genomes was not able to reveal any sex determining factors (Mulugeta et al. 2016). The first signs of sex chromosomes heteromorphism in *E. talpinus* and *E. tancrei* were observed in the meiotic behaviour of XX chromosomes in males (Kolomiets et al. 1991 2010, Matveevsky et al. 2016 2017).

The northern mole vole, *E. talpinus*, with 2n = NF = 54 (Ivanov 1967, Romanenko et al. 2007), has no described chromosomal variability, but significant mtDNA vari-

ability was revealed recently along its wide range (Bogdanov et al. 2015). The eastern mole vole, *E. tancrei* has stable 2n = 54, NF = 56 in most of its range, and demonstrates enormous karyotype variability (2n = 54-30) in the Pamir-Alay region (Vorontsov and Radzhabli 1967, Lyapunova et al. 1984 2010, Bakloushinskaya et al. 2013). The third species was described first as a chromosomal form of *E. talpinus* sensu lato (a chromosomal form of *E. tancrei* from the modern point of view) with one pair of large metacentric chromosomes and small submetacentrics, specific 2n = 52, NF = 56 (Vorontsov and Radzhabli 1967), and later it was designated as the Alay mole vole *E. alaicus* (Vorontsov et al. 1969, Lyapunova and Vorontsov 1978). The Alay Valley, the *terra typica* of the Alay mole vole, extending appr. 180 km from Tajikistan in the west to China in the east between two mountain systems: the Tien Shan and the Pamir. Range of the species was limited to the Alay Valley and the Northern slopes of the Alay Ridge, Tien-Shan (Kyrgyzstan). *E. alaicus* was listed by IUCN as data deficient species; cytogenetic data are scarce, no molecular study has been made ever (Gerrie and Kennerley 2016).

We studied the G-band structure of the *E. alaicus* karyotype previously and described a morphological homology for one pair of large metacentrics of the species to the Robertsonian metacentrics of *E. tancrei* from the Pamir-Alay (Bakloushinskaya 2003). We also discovered different forms of *E. alaicus* and their hybrids with *E. tancrei* with 2n = 50-53 from other parts of the Inner Tien-Shan (Lyapunova et al. 1985, Bakloushinskaya, Lyapunova 2003). But the study was incomplete, and application of modern cytogenetical and molecular techniques is required to confirm the karyotype structure, validity of *E. alaicus* as a species and its distribution.

The main objectives of this study were to reveal the chromosomal variability in *E. alaicus* and prove species affiliations for mole voles from adjacent to the Alay Valley territories of the Inner Tien-Shan and the Pamir-Alay Mountains. To bring a phylogenetic framework to the delimiting species, we examined the phylogeny of the subgenus *Ellobius* using the mitochondrial DNA marker, complete cytochrome *b* gene, *cytb*, and two nuclear DNA markers, fragments of the *XIST* (X-inactive specific transcript) and *Rspo1* (R-spondin 1) genes.

Material and methods

We analyzed karyotypes or *cytb* structure, or both, of 116 specimens of *E. alaicus* and *E. tancrei* mole voles from 27 localities across the Alay Valley and adjacent territories, as well as 7 *E. talpinus* specimens from 6 localities of Russia (Fig. 1, Table 1). Fragments of the *XIST* and *Rspo1* genes were studied for nine specimens of three species.

Samples

We used samples from the Joint collection of wildlife tissues for fundamental, applied and environmental researches of the Koltzov Institute of Developmental Biology RAS,

the state registration number AAAA-A16-116120810085-1, which is a part of the Core Centrum of the Koltzov Institute of Developmental Biology RAS, the state registration number 6868145. Tissues and chromosome suspensions were collected during our field trips in 1981–1983, 2008, 2010, 2013, and 2015–2018. For *cytb* sequencing we also used dried skins of specimens S132130*, S132131*, S132133*, S132135* deposited to the Zoological Museum of Lomonosov Moscow State University (Table 1) and originated from the *terra typica* of the Alay mole vole.

Animals were treated according to established international protocols, as in the Guidelines for Humane Endpoints for Animals Used in Biomedical Research. All the experimental protocols were approved by the Ethics Committees for Animal Research of the Koltzov Institute of Developmental Biology RAS in accordance with the Regulations for Laboratory Practice in the Russian Federation. All efforts were made to minimize animal suffering.

Mitotic and meiotic chromosomes

Chromosomes from bone marrow were prepared according to Ford and Hamerton (1956) for all animals listed with chromosome numbers in Table 1. G-banding was achieved using trypsin digestion (Seabright 1971). Samples from 3 animals (25610, 25611, 25612, Table 1) were used for tissue culture (Stanyon and Galleni 1991, Romanenko et al. 2015). All cell lines were retrieved from the IMCB SB RAS cell bank ("The general collection of cell cultures", № 0310-2016-0002). Full sets of paints derived from flow-sorted chromosomes of the field vole *Microtus agrestis* Linnaeus, 1761 were used (Sitnikova et al. 2007). FISH was performed according to previously published protocols (Yang et al. 1999, Graphodatsky et al. 2000). G-banding was carried out for metaphase chromosomes prior to FISH. The same procedures were used previously for specimens from localities 11, 12, 16, 17, and 18 (Bakloushinskaya et al. 2010, 2012, 2013, Matveevsky et al. 2015). It was not possible to use Zoo-FISH on material gathered in the 1980s, but the pictures of G-banded karyotypes were suitable for comparative analyses. Karyological data, obtained from 1981 to 2008, were re-examined in accordance with a new nomenclature for the Rb translocations in *E. tancrei* (Bakloushinskaya et al. 2012, 2013). In total, we studied chromosomes for 114 specimens of *E. alaicus*, *E. tancrei* and *E. talpinus*.

Images were captured using VideoTesT-FISH 2.0. and VideoTesT-Karyo 3.1. (Imicrotec) or Case Data Manager 6.0 (Applied Spectral Imaging Inc., ASI) software with either ProgRes CCD (Jenoptik) or ASI CCD camera, respectively, mounted on an Axioskop 2 plus (Zeiss) microscope with filter sets for DAPI, FITC, and rhodamine. Hybridization signals were assigned to specific chromosome regions defined by GTG-banding patterns previously captured with the CCD camera. Routine and G-banded plates were captured with a CMOS camera, mounted on an Axioskop 40 (Zeiss) microscope. Images were processed using Paint Shop Pro X2 (Corel).

The suspensions and spreads of spermatocytes of two *E. alaicus* males (27024, 27025) were made as described by Kolomiets et al. (2010). Immunostaining was de-

signed as in our previous studies (Kolomiets et al. 2010, Matveevsky et al. 2016). Synaptonemal complexes (SC) and centromeres in pachytene spermatocytes were detected using antibodies to axial SC elements – SYCP3 (Abcam, UK) and the kinetochores (CREST, Fitzgerald Industries International, USA). The slides were analyzed with an Axioimager D1 microscope (Carl Zeiss, Jena, Germany). Images were processed using Adobe Photoshop CS3 Extended.

cytb sequencing

Total DNA was isolated by phenol-chloroform deproteinisation after treatment of shredded tissues with proteinase K (Sambrook et al. 1989). The primers used for amplification and sequencing of the complete *cytb* gene (1143 bp) in species of the *Ellobius* subgenus are listed in Table 2. Polymerase chain reaction (PCR) was carried out in a mixture containing 25–50 ng DNA, 2 μl 10×Taq-buffer, 1.6 μl 2.5 mM dNTP solution, 4 pM of each primer, 1 unit of Taq-polymerase, and deionized water to a final volume of 20 μL. Amplification was as follows: preheating at 94 °C for 3 min, then 35 cycles in a sequential mode of 30 s at 94 °C, 1 min at 55 or 57 °C depending on the applied pair of primers, and 1 min at 72 °C; the reaction was completed by a single final elongation of PCR products at 72 °C for 6 min. Automatic sequencing was carried out using a PRISM BigDye TM Terminator v. 3.1 kit (ABI, United States) with ABI 3500 genetic analyzer at the Core Centrum of the Koltzov Institute of Developmental Biology RAS.

A total of 53 samples of the subgenus *Ellobius* mole voles were used for mitochondrial *cytb* gene sequencing; all sequences have been deposited in GenBank, accession numbers MG264318–MG264347, MG264351–MG264354, MG264359, and MK544900-MK544917 (http://www.ncbi.nlm.nih.gov/genbank/) are listed in the Table 1.

XIST (X-inactive specific transcript) and *Rspo1* (R-spondin 1) sequencing

Fragments of *XIST* gene (449 bp including deletions/insertions) and one exon and one intron of *Rspo1* gene (816 bp) were sequenced for nine animals (Table 1). PCR was carried out in a mixture containing 35–50 ng DNA, 2 μl 10×Taq-buffer, 1.6 μl 2.5 mM dNTP solution, 4 pM of each primer, 1 unit of Taq-polymerase, and deionized water to a final volume of 20 μL. Amplification was as follows: preheating at 94 °C for 3 min, then 35 cycles in a sequential mode of 30 s at 94 °C, 1 min at 63 °C (in case of *XIST*) or 67 °C (*Rspo1*), and 1 min at 72 °C; the reaction was completed by a single final elongation of PCR products at 72 °C for 6 min. For *Rspo1* gene analysis, we conducted second PCR with a PRISM®BigDye TM Terminator v. 3.1 kit using two internal primers to the PCR product obtained by first amplification. All primers are listed in Table 3. GenBank accession numbers: 26493 MK544918; 26910 MK544919; 26802 MK544920; 24913 MK544921; 25159 MK544922; 27017 MK544923; 25602 MK544924; 25605 MK544925; 27025 MK544926.

Table 1. List of studied specimens, species, origin/locality, sex, 2n, *cytb* accession numbers.

No	Species	2n	Voucher #	Sex	Loc. #	Locality	Coordinates	Year	GenBank #
1	*E. alaicus*	–	S132131*	♂	1	Kyrgyzstan. The Alay Valley, 10 km to the North from the Sary-Tash, the Taldyk pass, 3500 m above sea level	39°46'N 73°10'E	1983	MG264319
2	*E. alaicus*	–	S132133*	♀	1	Kyrgyzstan. The Alay Valley, 10 km to the North from the Sary-Tash, the Taldyk pass, 3500 m above sea level	39°46'N 73°10'E	1983	MG264320
3	*E. alaicus*	–	S132135*	♀	1	Kyrgyzstan. The Alay Valley, 10 km to the North from the Sary-Tash, the Taldyk pass, 3500 m above sea level	39°46'N 73°10'E	1983	MG264321
4	*E. alaicus*	–	S132130*	♂	2	Kyrgyzstan. The Alay Valley, close to Daraut-Korgon settlement, 2160 m above sea level	39°33'N 72°15'E	1983	MG264318
5	*E. alaicus* × *E. tancrei* hybrid	53	20757	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
6	*E. alaicus*	52	20758	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
7	*E. alaicus* × *E. tancrei* hybrid	53	20759	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
8	*E. alaicus*	52	20760	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
9	*E. alaicus*	52	20764	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
10	*E. alaicus*	52	20765	♀	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
11	*E. alaicus*	52	20766	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
12	*E. alaicus* × *E. tancrei* hybrid	53	20778	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
13	*E. alaicus*	52	20779	♀	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
14	*E. alaicus*	52	20780	♀	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–

No	Species	2n	Voucher #	Sex	Loc. #	Locality	Coordinates	Year	GenBank #
15	*E. alaicus*	52	20788	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
16	*E. alaicus*	52	20789	♀	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
17	*E. alaicus*	52	20790	♀	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
18	*E. alaicus*	52	20791	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
19	*E. alaicus*	52	20792	♂	3	Kyrgyzstan. Pamir Highway, Osh – Gul'cha. 20 km to Gul'cha, the beginning of the ascent to the pass, 1500 m above sea level	40°15'N 73°20'E	1983	–
20	*E. alaicus*	52	21054	♂	4	Kyrgyzstan. Close to the lake Chatyr-Kel', the 522 km from Bishkek city	40°33'N 75°17'E	1983	–
21	*E. alaicus*	51	21055	♀	4	Kyrgyzstan. Close to the lake Chatyr-Kel', the 522 km from Bishkek city	40°33'N 75°17'E	1983	–
22	*E. alaicus*	52	21056	♂	4	Kyrgyzstan. Close to the lake Chatyr-Kel', the 522 km from Bishkek city	40°33'N 75°17'E	1983	–
23	*E. alaicus*	52	21057	♂	4	Kyrgyzstan. Close to the lake Chatyr-Kel', the 522 km from Bishkek city	40°33'N 75°17'E	1983	–
24	*E. alaicus*	52	21058	♀	4	Kyrgyzstan. Close to the lake Chatyr-Kel', the 522 km from Bishkek city	40°33'N 75°17'E	1983	–
25	*E. alaicus*	51	21084	♂	4	Kyrgyzstan. Close to the lake Chatyr-Kel', the 522 km from Bishkek city	40°33'N 75°17'E	1983	–
26	*E. alaicus*	52	21085	♂	4	Kyrgyzstan. Close to the lake Chatyr-Kel', the 522 km from Bishkek city	40°33'N 75°17'E	1983	–
27	*E. alaicus*	51	21086	♀	4	Kyrgyzstan. Close to the lake Chatyr-Kel', the 522 km from Bishkek city	40°33'N 75°17'E	1983	–
28	*E. alaicus*	52	21066	♀	5	Kyrgyzstan. The Aksay River Valley, 4 km to the south-west from the Aksay settlement	40°14'N 73°20'E	1983	–
29	*E. alaicus*	52	21067	♀	5	Kyrgyzstan. The Aksay River Valley, 4 km to the south-west from the Aksay settlement	40°14'N 73°20'E	1983	–
30	*E. alaicus*	52	21083	♀	5	Kyrgyzstan. The Aksay River Valley, 4 km to the south-west from the Aksay settlement	40°14'N 73°20'E	1983	–
31	*E. alaicus*	52	21049	♂	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
32	*E. alaicus*	52	21050	♀	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
33	*E. alaicus*	52	21051	♀	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
34	*E. alaicus*	52	21052	♂	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
35	*E. alaicus*	51	21053	♀	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–

No	Species	2n	Voucher #	Sex	Loc. #	Locality	Coordinates	Year	GenBank #
36	*E. alaicus*	52	21069	♀	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
37	*E. alaicus*	51	21070	♀	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
38	*E. alaicus*	52	21071	♂	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
39	*E. alaicus*	50	21087	♂	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
40	*E. alaicus*	51	21088	♂	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
41	*E. alaicus*	50	21089	♂	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
42	*E. alaicus*	52	21090	♀	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
43	*E. alaicus*	50	21091	♂	6	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 362 km	41°21'N 75°59'E	1983	–
44	*E. alaicus* × *E. tancrei* hybrid	53	21059	♀	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
45	*E. tancrei*	54	21060	♂	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
46	*E. alaicus* × *E. tancrei* hybrid	53	21061	♂	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
47	*E. tancrei*	54	21062	♂	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
48	*E. alaicus* × *E. tancrei* hybrid	53	21063	♀	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
49	*E. tancrei*	54	21064	♂	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
50	*E. tancrei*	54	21065	♀	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
51	*E. tancrei*	54	21072	♂	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
52	*E. tancrei*	54	21073	♀	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
53	*E. tancrei*	54	21074	♂	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
54	*E. tancrei*	54	21075	♂	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
55	*E. tancrei*	54	21076	♀	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–

No	Species	2n	Voucher #	Sex	Loc. #	Locality	Coordinates	Year	GenBank #
56	*E. tancrei*	54	21077	♀	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
57	*E. tancrei*	54	21078	♀	7	Kyrgyzstan. Highway Bishkek - Chatyr-Kel', 270 km, 4 km after Sary-Bulak settlement	41°55'N 75°43'E	1983	–
58	*E. alaicus*	48	25600	♂	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	2010	–
59	*E. alaicus*	48	25605	♀	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	2010	MG264322
60	*E. alaicus*	48	25610	♀	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	2010	MG264323
61	*E. alaicus*	48	25611	♂	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	2010	MG264324
62	*E. alaicus*	48	25612	♀	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	2010	MG264325
63	*E. alaicus*	48	25622	♀	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	2010	–
64	*E. alaicus*	50	20054	♀	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	1981	–
65	*E. alaicus*	50–51	20053	♂	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	1981	–
66	*E. alaicus*	50	20050	♂	8	Tajikistan. The right bank of the Kyzyl-Suu River, 4 km to the East from the Achek-Alma settlement, 2160 m above sea level	39°22.73'N 71°40.68'E	1981	–
67	*E. alaicus*	48	25602	♀	9	Tajikistan. The left bank of the Kyzyl-Suu River, in front of the Duvana settlement, 2000 m above sea level	39°20.7'N 71°34.73'E	2010	MG264326
68	*E. alaicus*	48	27023	♀	9'	Tajikistan. The left bank of the Kyzyl-Suu River, in front of the Duvana settlement, 2000 m above sea level	39°20.588'N 71°34.528'E	2018	–
69	*E. alaicus*	48	27024	♂	9'	Tajikistan. The left bank of the Kyzyl-Suu River, in front of the Duvana settlement, 2000 m above sea level	39°20.588'N 71°34.528'E	2018	–
70	*E. alaicus*	48	27025	♂	10	Tajikistan. The left bank of the Kyzyl-Suu River, close to Dzhailgan settlement	39°19.277'N 71°32.772'E	2018	MK544910

No	Species	2n	Voucher #	Sex	Loc. #	Locality	Coordinates	Year	GenBank #
71	*E. alaicus*	48	27026	♂	10	Tajikistan. The left bank of the Kyzyl-Suu River, close to Dzhailgan settlement	39°19.277'N 71°32.772'E	2018	MK544911
72	*E. alaicus*	48	27028	♀	11	Tajikistan. The left bank of the Kyzyl-Suu River, 3 km to the East from the bridge to Kashat settlement	39°18.449'N 71°28.480'E	2018	MK544913
73	*E. alaicus*	48	27029	♀	11	Tajikistan. The left bank of the Kyzyl-Suu River, 3 km to the East from the bridge to Kashat settlement	39°18.449'N 71°28.480'E	2018	MK544914
74	*E. alaicus*	48	27030	♀	12	Tajikistan. The left bank of the Muksu River, close to Sary-Tala settlement	39°14.748'N 71°25.000'E	2018	MK544915
75	*E. alaicus*	48	27031	♀	12	Tajikistan. The left bank of the Muksu River, close to Sary-Tala settlement	39°14.748'N 71°25.000'E	2018	–
76	*E. alaicus*	48	27032	♀	12	Tajikistan. The left bank of the Muksu River, close to Sary-Tala settlement	39°14.748'N 71°25.000'E	2018	MK544916
77	*E. alaicus*	48	27033	♂	12	Tajikistan. The left bank of the Muksu River, close to Sary-Tala settlement	39°14.748'N 71°25.000'E	2018	MK544917
78	*E. tancrei*	54	27019	♂	13	Tajikistan. Pamir-Alay, close to Utol Poyon settlement, the southern bank of the Surkhob River	39°9.737'N 71°7.374'E	2018	MK544906
79	*E. tancrei*	54	27020	♀	13	Tajikistan. Pamir-Alay, close to Utol Poyon settlement, the southern bank of the Surkhob River	39°9.737'N 71°7.374'E	2018	MK544907
80	*E. tancrei*	54	27021	♂	13	Tajikistan. Pamir-Alay, close to Utol Poyon settlement, the southern bank of the Surkhob River	39°9.737'N 71°7.374'E	2018	MK544908
81	*E. tancrei*	54	27022	♀	13	Tajikistan. Pamir-Alay, close to Utol Poyon settlement, the southern bank of the Surkhob River	39°9.737'N 71°7.374'E	2018	MK544909
82	*E. tancrei*	54	27017	♂	14	Tajikistan. Pamir-Alay, between settlements Kichikzy – Utol Poyon, the southern bank of the Surkhob River	39°7.625'N 70°59.762'E	2018	MK544904
83	*E. tancrei*	54	27018	♀	14	Tajikistan. Pamir-Alay, between settlements Kichikzy – Utol Poyon, the southern bank of the Surkhob River	39°7.625'N 70°59.762'E	2018	MK544905
84	*E. tancrei*	54	27027	♂	14	Tajikistan. Pamir-Alay, between settlements Kichikzy – Utol Poyon, the southern bank of the Surkhob River	39°7.625'N 70°59.762'E	2018	MK544912
85	*E. tancrei*	52	24898	♂	15	Tajikistan. Pamir-Alay, close to Kichikzy settlement, the southern bank of the Surkhob River	39°8.23'N 70°57.33'E	2008	MK544900
86	*E. tancrei*	51	24899	♀	15	Tajikistan. Pamir-Alay, close to Kichikzy settlement, the southern bank of the Surkhob River	39°8.23'N 70°57.33'E	2008	
87	*E. tancrei*	30	25601	♀	16	Tajikistan. Pamir-Alay, close to the settlement Shilbili, the northern bank of the Surkhob River, 1900 m above sea level	39°15.37'N, 71°20.59'E	2010	MG264327

No	Species	2n	Voucher #	Sex	Loc. #	Locality	Coordinates	Year	GenBank #
88	*E. tancrei*	30	25618	♀	16	Tajikistan. Pamir-Alay, close to the settlement Shilbili, the northern bank of the Surkhob River, 1900 m above sea level	39°15.37'N 71°20.59'E	2010	MG264328
89	*E. tancrei*	30	25625	♂	16	Tajikistan. Pamir-Alay, close to the settlement Shilbili, the northern bank of the Surkhob River, 1900 m above sea level	39°15.37'N 71°20.59'E	2010	MG264329
90	*E. tancrei*	30	25626	♀	16	Tajikistan. Pamir-Alay, close to the settlement Shilbili, the northern bank of the Surkhob River, 1900 m above sea level	39°15.37'N 71°20.59'E	2010	MG264330
91	*E. tancrei*	48	24872	♀	17	Tajikistan. Pamir-Alay, the right bank of the Surkhob River, close to the airport Garm, 1310 m above sea level	39°0.28'N 70°17.77'E	2008	MG264331
92	*E. tancrei*	48	24873	♀	17	Tajikistan. Pamir-Alay, the right bank of the Surkhob River, close to the airport Garm, 1310 m above sea level	39°0.28'N 70°17.77'E	2008	MG264332
93	*E. tancrei*	48	24874	♂	17	Tajikistan. Pamir-Alay, the right bank of the Surkhob River, close to the airport Garm, 1310 m above sea level	39°0.28'N 70°17.77'E	2008	MG264333
94	*E. tancrei*	48	24876	♂	17	Tajikistan. Pamir-Alay, the right bank of the Surkhob River, close to the airport Garm, 1310 m above sea level	39°0.28'N 70°17.77'E	2008	MG264334
95	*E. tancrei*	48	24914	♀	17	Tajikistan. Pamir-Alay, the right bank of the Surkhob River, close to the airport Garm, 1310 m above sea level	39°0.28'N 70°17.77'E	2008	MG264335
96	*E. tancrei*	48	24915	♂	17	Tajikistan. Pamir-Alay, the right bank of the Surkhob River, close to the airport Garm, 1310 m above sea level	39°0.28'N 70°17.77'E	2008	MG264336
97	*E. tancrei*	50	24904	♀	18	Tajikistan. Pamir-Alay, the left bank of the Surkhob River near the Shulonak, on the way to Voidara settlement, 1300 m above sea level	38°59.3'N 70°16.1'E	2008	MG264337
98	*E. tancrei*	50	24911	♂	19	Tajikistan. Pamir-Alay, the left bank of the Surkhob River near the Voydara settlement, 1440 m above sea level	38°58.9'N 70°14.71'E	2008	–
99	*E. tancrei*	50	24907	♀	19	Tajikistan. Pamir-Alay, the left bank of the Surkhob River near the Voydara settlement, 1440 m above sea level	38°58.9'N 70°14.71'E	2008	MG264338
100	*E. tancrei*	50	24910	♂	19	Tajikistan. Pamir-Alay, the left bank of the Surkhob River near the Voydara settlement, 1440 m above sea level	38°58.9'N 70°14.71'E	2008	MG264339
101	*E. tancrei*	54	20769	♂	20	Uzbekistan. Close to Sokh settlement, 11 km to the west	39°58'N 70°58'E	1983	–
102	*E. tancrei*	54	20770	♀	20	Uzbekistan. Close to Sokh settlement, 11 km to the west	39°58'N 70°58'E	1983	–
103	*E. tancrei*	54	20772	♂	20	Uzbekistan. Close to Sokh settlement, 11 km to the west	39°58'N 70°58'E	1983	–
104	*E. tancrei*	54	20773	♀	20	Uzbekistan. Close to Sokh settlement, 11 km to the west	39°58'N 70°58'E	1983	–

No	Species	2n	Voucher #	Sex	Loc. #	Locality	Coordinates	Year	GenBank #
105	*E. tancrei*	54	25159	♂	21	Uzbekistan. Tashkent city	41°20.49'N 70°18.71'E	2009	MG264346
106	*E. tancrei*	54	20561	♀	22	Kyrgyzstan. The Southern bank of the Issyk-Kel' Lake, 16 km to the South from the Barskaun settlement, Lake Barskaun canyon	42°00'N 77°37'E	1982	–
107	*E. tancrei*	54	20562	♂	22	Kyrgyzstan. The Southern bank of the Issyk-Kel' Lake, 16 km to the South from the Barskaun settlement, Lake Barskaun canyon	42°00'N 77°37'E	1982	–
108	*E. tancrei*	54	24912	♂	23	Tajikistan. The northern bank of the Vakhsh River, Miskinobod, 1780 m above sea level	38°39.78'N 69°33.29'E	2008	MG264344
109	*E. tancrei*	54	24913	♂	24	Tajikistan. Panchkotan gorge, left bank of the Sorbo River, close to Romit reserve, 1265 m above sea level	38°45.27'N 69°17.6'E	2008	MG264345
110	*E. tancrei*	50	24905	♂	25	Tajikistan. The Varzob Valley, near the Khodzha-Obi-Garm settlement, 2000 m above sea level	38°53.53'N 68°46.52'E	2008	MG264340
111	*E. tancrei*	50	24906	♂	25	Tajikistan. the Varzob Valley, near the Khodzha-Obi-Garm settlement, 2000 m above sea level	38°53.53'N 68°46.52'E	2008	MG264341
112	*E. tancrei*	50	24916	♀	25	Tajikistan. the Varzob Valley, near the Khodzha-Obi-Garm settlement, 2000 m above sea level	38°53.53'N 68°46.52'E	2008	MG264342
113	*E. tancrei*	50	24917	♂	25	Tajikistan. the Varzob Valley, near the Khodzha-Obi-Garm settlement, 2000 m above sea level	38°53.53'N 68°46.52'E	2008	MG264343
114	*E. tancrei*	54	27016	♂	26	Tajikistan. Khatlon district, close to Sovetabad settlement	37°28.479'N 68°15.568'E	2018	MK544903
115	*E. tancrei*	54	27013	♂	27	Tajikistan. Khatlon district, close to Aivadj settlement	36°58.168'N 68°0.791'E	2018	MK544901
116	*E. tancrei*	54	27014	♂	27	Tajikistan. Khatlon district, close to Aivadj settlement	36°58.168'N 68°0.791'E	2018	MK544902
117	*E. talpinus*	54	24736	♀	28	Russia. Orenburg oblast, Belyaevsky district, about 15 km southeast of the Belyaevka village	51°14'N 56°38'E	2005	MG264347
118	*E. talpinus*	54	26910	♂	29	Russia. Samara oblast, Stavropolsky rayon, Samarskaya Luka	53°9.98'N 49°35.35'E	2016	MG264354
119	*E. talpinus*	–	26491	♀	30	Russia. Crimea, Bakhchisaraysky district, 2 km south of the Sevastyanovka village	44°47.82'N 33°55.95'E	2013	MG264359
120	*E. talpinus*	–	26493	♀	30	Russia. Crimea, Bakhchisaraysky district, 2 km south of the Sevastyanovka village	44°47.82'N 33°55.95'E	2013	*cytb* mitotype is identical to MG264359
121	*E. talpinus*	54	26800	♀	31	Russia. Omsk oblast, Tavrichesky district, near the Novouralsky railway station, about 16 km south-east of the Novouralsky village	54°14.586'N 74°17.66'E	2014	MG264351
122	*E. talpinus*	54	26802	♀	32	Russia. Novosibirsk oblast, Tatarsky district, near the Novopervomayskoe village and Lagunaka railway station	55°8.64'N 75°21.94'E	2014	MG264352
123	*E. talpinus*	54	26850	♂	33	Russia. Omsk oblast, Cherlaksky district, approximately 3.5 km northeast of the Irtysh village	54°30.59'N 74°25.95'E	2015	MG264353

Molecular evolutionary analyses

DNA sequences were aligned using the MUSCLE algorithm (Edgar 2004) in MEGA X software (Kumar et al. 2018). Maximum likelihood analyses and calculation of genetic distances (D) were carried out in MEGA X software using the TN93+G model of DNA substitution (Tamura-Nei model, Gamma distributed) for *cytb* and Jukes-Cantor model (Jukes, Cantor 1969) for concatenated sequences of *XIST* and *Rspo1* genes according to modeltest, with statistical support for internodes tested by bootstrapping in 1,000 replications.

Bayesian inference for *cytb* sequences was additionally evaluated in MrBayes ver. 3.2 (Ronquist et al. 2012); analyses were run for 1 million generations with Markov chains sampled every 1000 generations, 25% trees were discarded ('burn-in') and node support was assessed with posterior probabilities. Final images of phylogenetic trees were rendered in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkspace (https://inkscape.org/).

Results

Karyotyping

The main result was a discovery of specific chromosome variability in *E. alaicus*, with 2n varying from 52 to 48 chromosomes. For mole voles from the Alay Ridge, the Naryn Valley, and the Aksai River Valley (localities # 3, 5, 6, Fig. 1, Table 1) we described 2n = 52 with two homozygous Robertsonian translocations, which was counted as 2.11 [2 Rb(2.11)] according to *E. tancrei* chromosome nomenclature (Bakloushinskaya et al. 2012 2013) (Fig. 2a). The northern side of the Alay Ridge slopes down to the Ferghana Valley, where *E. tancrei*, 2n = 54, exists (# 20). Hybrids with 2n = 53, heterozygous by the same translocation [1 Rb(2.11)] (Fig. 3a), were found at the northern slopes of the Alay Ridge (# 3), which marks the species contact zone. The Ferghana Ridge separates the Alay Valley from the Chatyr-Kel' Lake Basin, the Aksai River Valley, and the Naryn Valley, one of the largest within the Inner Tien Shan. Fascinating data were obtained for animals inhabiting the Chatyr-Kel' Lake surrounds and the Naryn district (localities # 4 and 6, Table 1, Fig. 1), where we found Alay mole voles with 2n = 50, and heterozygotes with 2n = 51, which are presumed hybrids with typical *E. alaicus*, 2n = 52 (Figs 2b, 3c). Chromosomal number in animals with 2n = 50 was decreased because of another translocation, the Rb(1.3). Nevertheless, in the Aksai River Valley, the typical *E. alaicus* with 2n = 52 [2 Rb(2.11)] were found (locality # 5, Table 1, Fig. 1).

Two heterozygous karyotypes with 2n = 53 due to the presence of different Rb metacentrics were found. In point # 3, we found animals with 2n = 53 and 1 Rb(2.11), which are hybrids of *E. alaicus* and *E. tancrei* (Fig. 3a). Mole voles with 2n = 53 from the Naryn district (#7, Fig. 1, Table 1) had another translocation, 1 Rb(1.3) (Fig. 3b).

Table 2. Primers, which were used for amplification and sequencing of *cytb* gene in mole voles of the *Ellobius* subgenus. Primers Eta_CytbF1, and VOLE14 were used to amplify the full *cytb* gene with flanked fragments of mtDNA; all other primers correspond to various internal areas of *cytb* gene, the position of their 5'-end nucleotide from the start of *cytb* gene is in parentheses.

Species	Primer designation	Nucleotide sequence of primer (5'–3') and its localization within the full gene *cytb*	Citation
E. talpinus	Forward primers		
	Eta_CytbF1	GAAACACCTAATGACAATCATACG	Bogdanov et al. 2015
	L15095-Ell	(370)-ATAGCCACAGCATTCATA	Bogdanov et al. 2015
	L15473-Ell	(748)-CTCGGAGACCCAGATAACTAC	Bogdanov et al. 2015
	Reverse primers		
	MVZ04m	(431)-GTGGCCCCTCAAAATGATATTTGTCCTC	Bogdanov et al. 2015
	CLETH16m	(824)-AGGAAGTACCATTCTGGTTTAAT	Bogdanov et al. 2015
	VOLE14	TTTCATTACTGGTTTACAAGAC	Conroy and Cook 1999
E. tancrei, *E. alaicus*	Forward primers		
	Eta_CytbF1	GAAACACCTAATGACAATCATACG	Bogdanov et al. 2015
	L15095-Ell	(370)-ATAGCCACAGCATTCATA	Bogdanov et al. 2015
	Vole23m	(590)-TCCTGTTCCTTCACGAAACAGGTTC	Bogdanov et al. 2015
	L15473-Elal	(748)-CTTGGAGACCCAGACAATTTC	Our design
	Reverse primers		
	MVZ04m	(431)-GTGGCCCCTCAAAATGATATTTGTCCTC	Bogdanov et al. 2015
	CLETH16m	(824)-AGGAAGTACCATTCTGGTTTAAT	Bogdanov et al. 2015
	VOLE14	TTTCATTACTGGTTTACAAGAC	Conroy, Cook 1999

Table 3. Primers, which were used for amplification and sequencing of *XIST* and *Rspo1* genes in the mole voles of the *Ellobius* subgenus.

Nuclear gene	Primer designation	Nucleotide sequence of primer (5'–3')	Source
XIST	Xist1-L11841	GGGGTCTCTGGGAACATTTT	Our design
	Xist1-R12504 or Xist1-Rint	TGCAATAACTCACAAAACCAAC AAGCAGGTAAGTATCCACAGC	Our design
Rspo1	Primers used for first amplification		
	Rspo1F-Ell	CACTGTACACTTCCGGGTCTCTTT	Our design
	Rspo1R-Ell	AGAAGTCAACGGCTGCCTCAAGTG	Our design
	Primers used for second PCR with a PRISM®BigDye TM Terminator v. 3.1 kit		
	Rspo1-5intF-Ell	CAGGCACGCACACTAGGTTGTAA	Our design
	Rspo1-1intR-Ell	GTCTAGACTCCCAACACCTG	Our design

We were not able to find animals with 2n = 52 and 2 Rb(1.3), but probably they inhabit an extensive unstudied area in the Naryn Valley, between points #6 and 7.

The most surprising data we revealed for animals from the Pamir-Alay mountains, Tajikistan, (# 8, Fig. 1, Table 1). In 1981, we got Alay mole voles from there for breeding and karyotyping; two animals had 2n = 50, and one was a somatic mosaic, 2n = 50-51. Their karyotypes included 2 Rb(2.11) and 1–2 Rb(4.9); the last one was heterozygous in the mosaic specimen (Fig. 2c). After almost 30 years (in 2010) we caught animals with 2n = 48 at the same locality, and one mole vole with the same karyotype at the opposite bank of the Kyzyl-Suu River (locality # 9, Fig. 1, Table 1). Their karyotypes contained one more pair of Rb metacentrics, Rb(3.10). The entire set of Rbs was 2 Rb(2.11), 2 Rb(4.9),

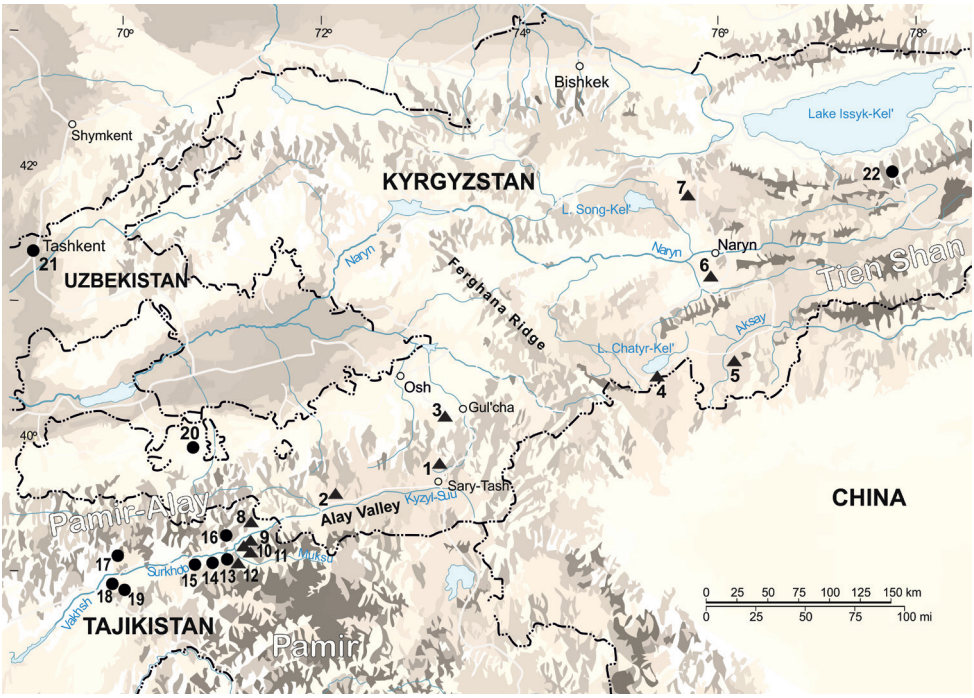

Figure 1. The geographic location of studied populations of the mole voles *E. alaicus* (dark triangles) and *E. tancrei* (dark spots). Localities are numbered as in Table 1. Localities 23–27 are outside the map.

2 Rb(3.10), all of which were confirmed by chromosome painting for specimens 25610, 25611, 25612 (Figs 4, 5). The 21 MAG (*Microtus agrestis*) autosomal probes revealed 35 conserved segments in the mole voles' genome, which corresponds to the genome composition of the typical *E. tancrei*, 2n = 54 (Bakloushinskaya et al. 2012), and its form with the lowest chromosome number, 2n = 30 (Bakloushinskaya et al. 2013). The MAG X chromosome probe produced signals on both male and female X chromosomes; the MAG Y probes did not demonstrate any specific signal. Therefore, we suppose that *E. alaicus* has the same isomorphic sex chromosomes, XX in both sexes, as *E. talpinus* and *E. tancrei*.

In 2018 we checked chromosome sets for Alay mole voles from the Kyzyl-Suu River Valley, the Kyzyl-Suu and Muksu Rivers interfluve, and the left bank of the Muksu River (localities # 9–12, Fig. 1, Table 1). All 10 studied animals have 2n = 48 [2 Rb(2.11), 2 Rb(4.9), 2 Rb(3.10)].

In total we described seven variants of karyotypes for *E. alaicus* (Table 1, Figs 2, 3, 5): 2n = 48, 50 (two forms), 51, 52, 53 (two variants) with four different Rb translocations Rb(2.11), Rb(1.3), Rb(4.9), Rb(3.10) in different combinations. We assumed, by comparing our data on G-banded karyotypes and chromosomal painting, that the Rb(2.11) is typical for *E. alaicus.* This translocation was revealed in all specimens of the species (Table 1, Figs 2, 3a,c), excluding interspecific hybrids of *E. tancrei* and *E. alaicus* from the Naryn district 2n = 53, 1 Rb(1.3) (Table 1, Fig. 3b), see Discussion.

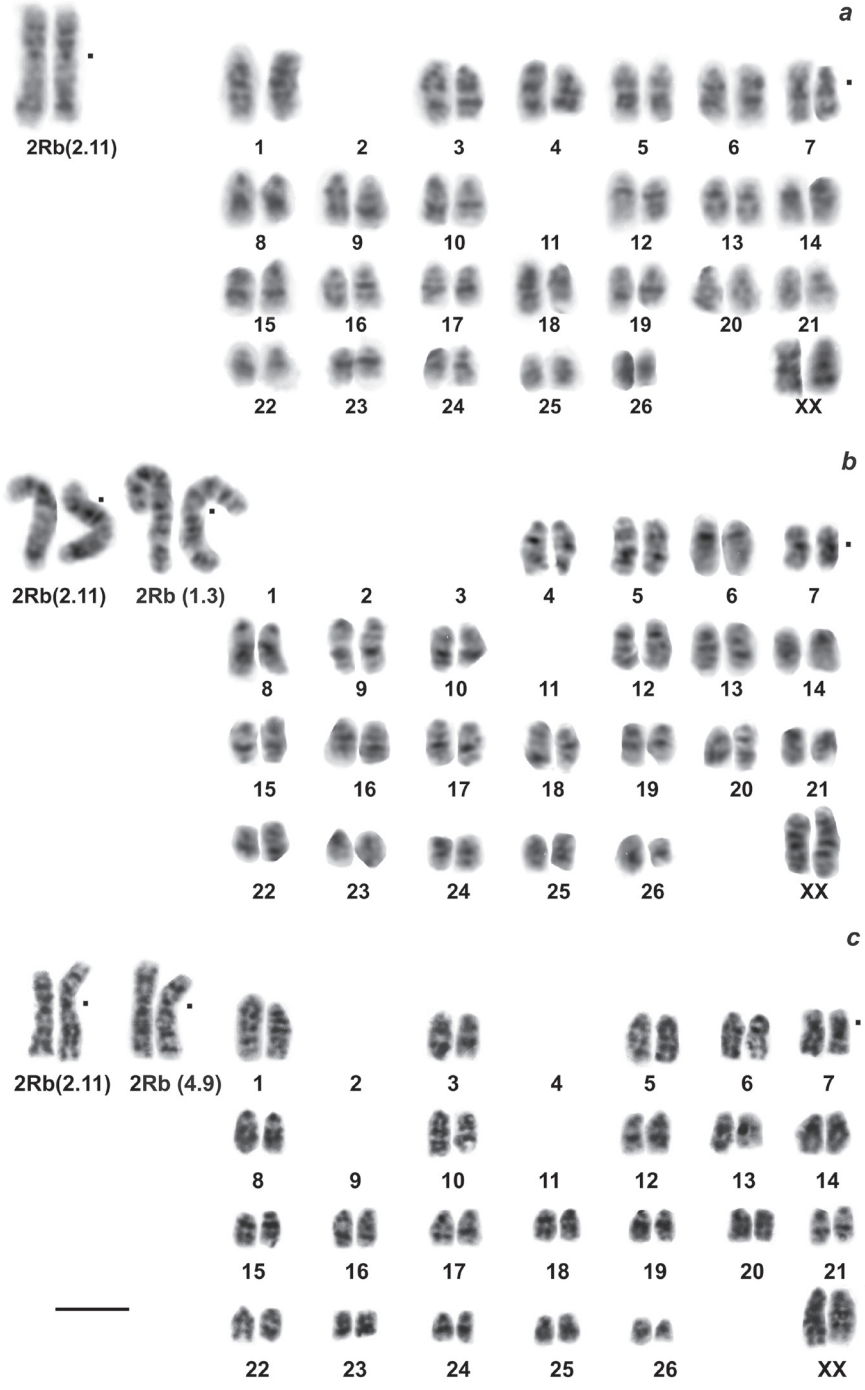

Figure 2. G-banded karyotypes of *E. alaicus* **a** 2n = 52, 21071, ♂, locality #6 **b** 2n = 50, 21089, ♂, locality #6 **c** 2n = 50 20054, ♀, locality #8. The chromosome nomenclature follows Bakloushinskaya et al. (2012, 2013). Black dots mark the positions of centromeres in bi-armed chromosomes. Scale bar: 10 μm.

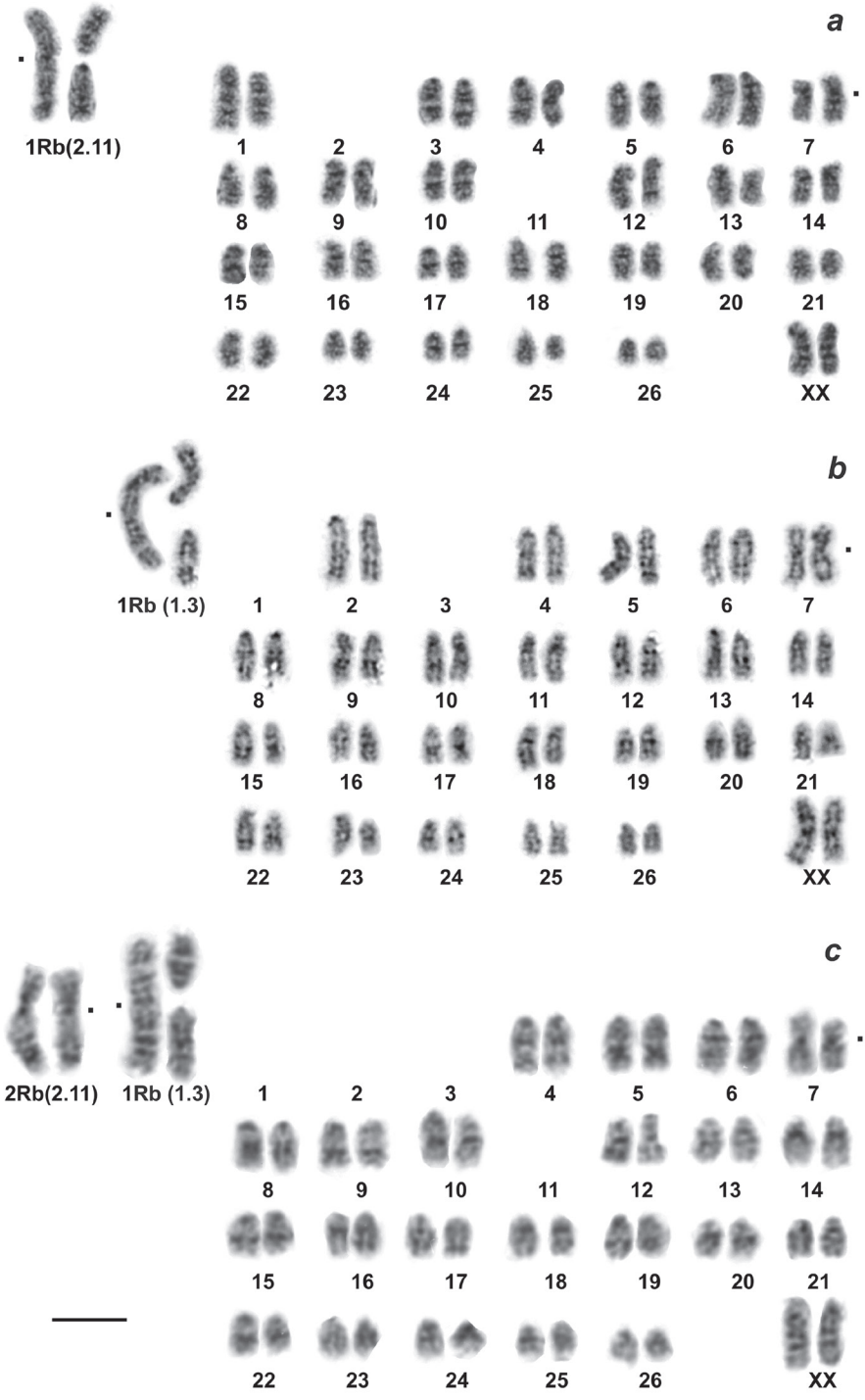

Figure 3. G-banded karyotypes of heterozygous mole voles **a** 2n = 53 20778, ♂, locality #3 **b** 2n = 53, 21059, ♀, locality #7 **c** 2n = 51, 21070, ♀, locality #6. Scale bar: 10 μm.

Synaptonemal complexes

In the 48-chromosomal form of *E. alaicus* the 23 bivalents (including 19 acrocentric SC, four bi-armed SC), and sex (XX) bivalent were formed in spermatocytes at the pachytene stage (Fig. 6). Male XX bivalent was shifted to the periphery of the meiotic nucleus and had two short distal synaptic segments and an extended asynaptic region, which is typical for mammals.

Phylogenetic analyses

We inferred phylogenies on the complete *cytb* (1143 bp) of *E. talpinus*, *E. tancrei* and *E. alaicus*, N = 53 (Table 1), including *E. tancrei* with 2n = 54, 30, and its forms with the same chromosome numbers, as we found in *E. alaicus*, 2n = 48–50, but with other Rb translocations. The analysis, which was carried out by Maximum Likelihood (ML) and Bayesian inference (BI) approaches, revealed the specific clustering for all *Ellobius* species (Fig. 7a, 7b). Samples from the Alay Valley (# 1, 2, Fig. 1, Table 1), the *terra typica* for *E. alaicus*, and 5 points from the Pamir-Alay mountains (# 8–12, Fig. 1, Table 1) were clustered together, in accordance with chromosome data. These results supported our assumption that the populations of mole voles from the Pamir-Alay mountains, localities numbers 8–12 (Tajikistan) belong to *E. alaicus* (Fig. 1).

Our data on *cytb* revealed a significant range of interspecies genetic distances, which are moderate for *E. alaicus* – *E. tancrei* (D = 0.0256), and quite high for *E. alaicus* – *E. talpinus*

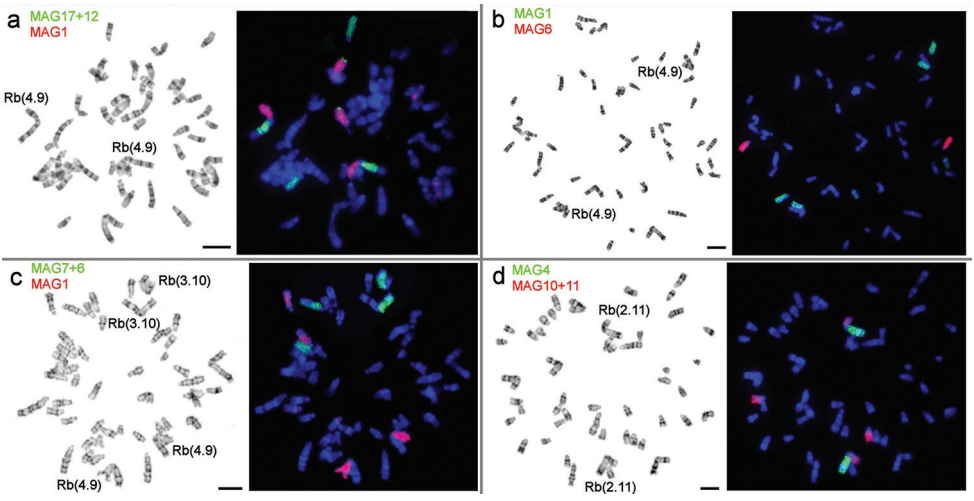

Figure 4. Fluorescent *in situ* hybridization of *M. agrestis* (MAG) probes on *E. alaicus* metaphase chromosomes, 2n = 48 (locality #8): **a** MAG 1 (red) and MAG 17+12 (green), 25610 ♀, locality #8; **b** MAG 1 (green) and MAG 6 (red), 25610 ♀, locality #8; **c** MAG 1 (red) and MAG 7+6 (green), 25612 ♀, locality #8; **d** MAG 4 (green) and MAG 10+11 (red), 25612 ♀, locality #8. Scale bar: 10 μm.

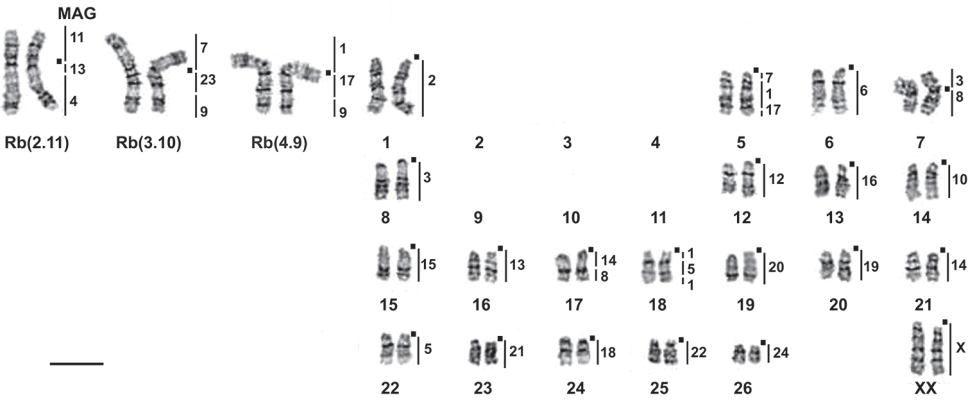

Figure 5. G-banded karyotype of a new form of *E. alaicus*, 2n = 48, 2 Rb (2.11), 2 Rb (4.9), 2 Rb (3.10), 25610 ♀, locality #8. The chromosome nomenclature follows Bakloushinskaya et al. (2012, 2013). Black squares mark the positions of centromeres. Vertical black bars and the numbers beside them mark the localization of *M. agrestis* (MAG) chromosome segments. Scale bar: 10 μm.

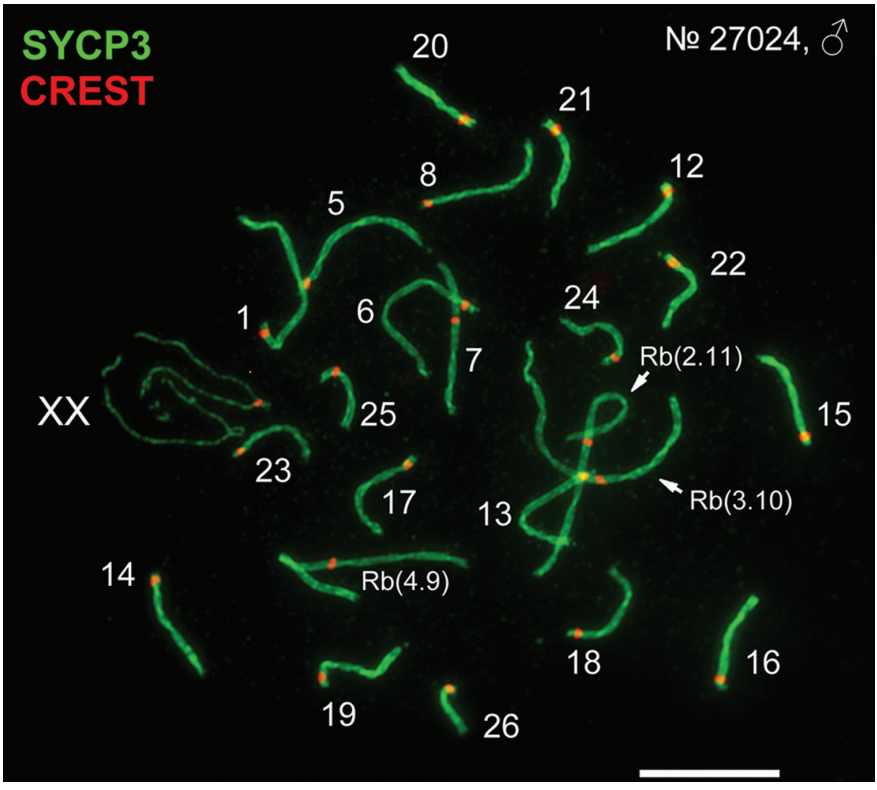

Figure 6. Chromosome synapsis in pachytene spermatocytes of *E. alaicus*, 27024, ♂ (2n = 48, NF = 56), locality #9'. Axial SC elements were identified using anti-SYCP3 antibodies (green), anti-CREST for kinetochores (red). Numbers of SC correspond to chromosome numbers in the karyotype (see Fig. 5). Scale bar: 10 μm.

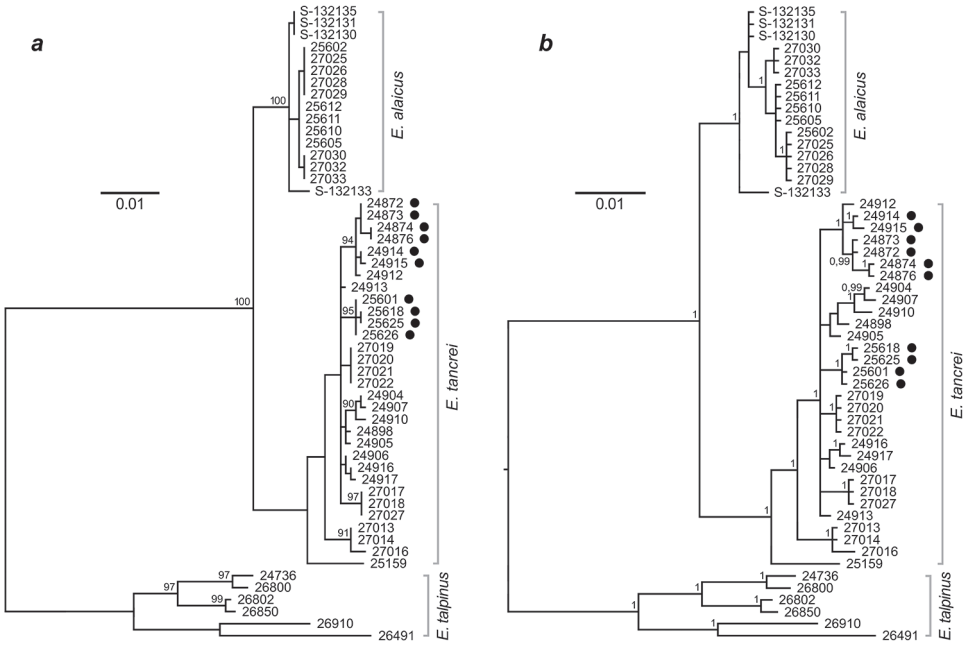

Figure 7. Trees of the subgenus *Ellobius* inferred from complete mitochondrial *cytb* gene sequences (1143 bp) of 53 specimens **a** a tree was got by using the Maximum Likelihood method based on the Tamura-Nei model, bootstrap support is listed above main branches. Only values greater than 70 percent are shown **b** Bayesian inference tree was made in MrBayes ver. 3.2 (Ronquist et al. 2012), posterior probabilities >0.75 are given above nodes. *E. tancrei* with 2Rb(2.11) were marked by black spots in both trees.

(D = 0.0799), *E. tancrei* – *E. talpinus* (D = 0.0839). Thus, *E. alaicus* and *E. tancrei* are the most closely related species that coincides with results of chromosomal analysis too. *E. talpinus* demonstrated high intraspecific differentiation (D value averages 0.033), as we described earlier (Bogdanov et al. 2015). We also evaluated the physical differences between sequences using uncorrected so-called *p* distances: *E. alaicus* – *E. tancrei p* = 0.0243, *E. alaicus* – *E. talpinus p* = 0.0688, *E. tancrei* – *E. talpinus p* = 0.0715. *P* distances were lower if compare with genetic distances (D), calculated using the TN93+G model, but even in the case of *E. alaicus* – *E. tancrei*, *p* distance was more than 2%. It had a high probability of being indicative of valid species (Bradley and Baker 2001).

The evolutionary history of the subgenus *Ellobius* was also inferred by using the concatenated sequences of nuclear *XIST* (449 bp) and *Rspo1* (1203 bp) genes, 1652 bp in total. The analysis showed the existence of "fixed" nucleotide substitutions and the species-specific clustering for three *Ellobius* species despite the genetic distances were rather low: D = 0.003 for *E. alaicus* – *E. tancrei*, D = 0.006 for *E. alaicus* – *E. talpinus* and D = 0.004 for *E. tancrei* – *E. talpinus*. As a result, the species relationships were proven by analyses of mitochondrial and nuclear DNA markers. It is noticeable that nuclear genes variability indicates more significant intraspecific differentiation for *E. tancrei* compared with results of *cytb* analysis. Thus, differences between the specimen from

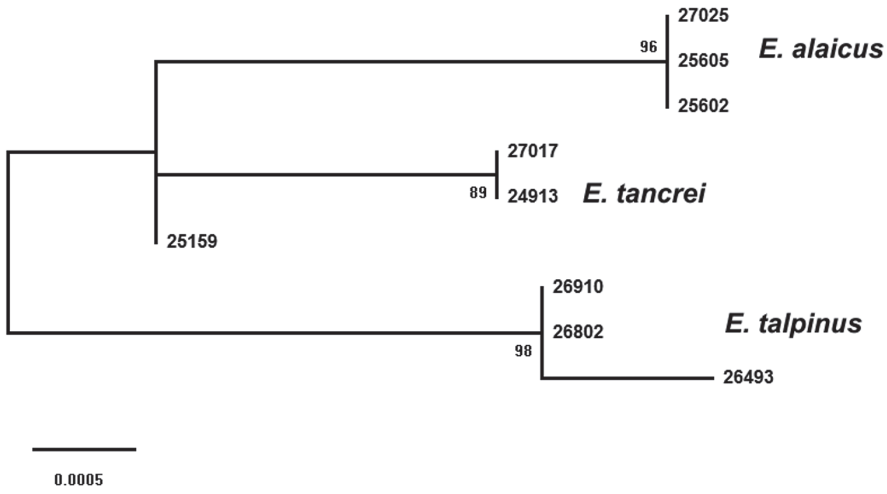

Figure 8. Molecular phylogenetic analysis of three *Ellobius* species based on variability of *XIST* and *Rspo1* genes fragments (1652 bp in total) and constructed by using the Maximum Likelihood method and the Jukes-Cantor model. Bootstrap support is listed for main branches. Only values over 70 percent are shown.

Tashkent vicinities, which could not be assigned to any of the two clades in this analysis, and Tajikistan eastern mole voles (D = 0.002) reach up to a half and even more of interspecific distance in *E. tancrei* – *E. talpinus* and *E. alaicus* – *E. tancrei* (Fig. 8).

Discussion

A few studies dealt with *Ellobius* molecular phylogeny before. Conroy and Cook (1999) studied *cytb* of two species, *E. fuscocapillus* and *E. tancrei*, and their position in the Arvicolinae tree appeared to be unstable in different models. Data on variations of short fragments of nuclear genes (partial *LCAT* and exon 10 *GHR*) in *E. talpinus* and *E. tancrei* contradicted the conventional view that *Ellobius* is an ancient group because of simplicity of rooted molars and the peculiar structure of the skull (Abramson et al. 2009). Fabre et al. (2012) re-analyzed these data among others for comparative meta-analyses of the rodent diversity and phylogeny without special attention to Ellobiini. Nevertheless, the genus *Ellobius* appears to be a young group; its morphological characters indicate adaptation to subterranean life and provide no phylogenetic signal. *E. talpinus* and *E. tancrei* separated not earlier than the latest Pliocene and Early Pleistocene between ca. 2.1–1.0 Ma (Abramson et al. 2009). The phyletic lineage leading to the recent *E. talpinus* includes at least two chronospecies: Late Pliocene-Early Pleistocene *E. kujalnikensis* and early Middle Pleistocene *E. melitopoliensis*; *E. talpinus* was recognized from the late Middle Pleistocene (Tesakov 2009). There are no such data for *E. tancrei* and *E. alaicus*.

Here, for the first time, we demonstrated data on molecular, mitochondrial (*cytb*) and nuclear (*XIST* and *Rspo1* fragments) specificity of *E. alaicus*. The *cytb* variability

in the subgenus *Ellobius*, which we demonstrated here, is comparable and even higher than in *Ctenomys*, subterranean rodents with numerous species-specific chromosome changes (Buschiazzo et al. 2018). In *Ctenomys* genetic distances, calculated on *cytb* gene, range from 0 to 2.28%, whereas 2n varies from 41 to 70, and autosomal fundamental numbers (NFa) from 72 to 84. Nevertheless, *cytb* appears to be more informative for phylogenetic reconstructions compared to nuclear markers. Published data on partial sequences of *XIST* and *Sox9* revealed no differences for *E. talpinus* and *E. tancrei* (Just et al. 2007, Bagheri-Fam et al. 2012). Our data on fragments of *Eif2s3x* and *Eif2s3y* for *E. talpinus*, *E. tancrei*, and *E. alaicus* also reveal no changes in the exonic part of the genes (Matveevsky et al. 2017). The cryptic *Ellobius* species are rather young ones, so this may be why nuclear DNA markers were insufficient. However, our new data on *XIST* and *Rspo1* variability demonstrated apparent clustering for all species of the *Ellobius* subgenus despite interspecific genetic distances were rather low and relatively high difference of *E. tancrei* specimens from Tajikistan and Uzbekistan, as nuclear markers of the latest (specimen 25159) could not be assigned to any of the two clades.

Originally, *E. alaicus* was described as a species with specific karyotype structure, including a pair of very large bi-armed chromosomes (Vorontsov et al. 1969, Lyapunova, Vorontsov 1978). Now we proved, that this Rb(2.11) metacentric is the same as in the *E. tancrei* forms with 2n = 30 and 2n = 48 from the northern bank of the Surkhob River (Bakloushinskaya et al. 2010 2013), but not the Rb(2.18) as in the form with 2n = 50 from the opposite bank of the river. Moreover, translocations Rb(1.3), Rb(4.9), and Rb(3.10) were revealed in the Alay mole voles only. Thus, the Alay mole vole generated a distinctive Robertsonian variability with special structure that highlights genetic distinctness of this species compared to *E. tancrei*. No specimens with 2n = 52 and a single pair of Rb(2.11) were found among over 400 studied *E. tancrei* with Rb translocations (Bakloushinskaya, Lyapunova 2003). Probably, the translocation Rb(2.11) originated independently in *E. alaicus* and *E. tancrei*. The results of the phylogenetic analyses support this assumption because both ML and BI trees demonstrated distant positions for *E. alaicus* and *E. tancrei* specimens carrying Rb(2.11). Their relationships were established indirectly through Uzbekistan and South-West Tajikistan populations of *E. tancrei*, which have no any Robertsonian translocations (Fig. 7).

Earlier (Lyapunova et al. 1990) we obtained the experimental hybrids of *E. alaicus*, 2n = 52, 2 Rb(2.11) (#3, Fig. 1, Table 1) and *E. tancrei* with 2n = 50, 2 Rb(2.18), 2 Rb(5.9) from the left bank of the Surkhob River (#18, Fig. 1, Table 1). In meiosis, during pachytene I, chains of chromosomes were described (Lyapunova et al. 1990). Now we can explain the results by the partial, monobrachial homology of Rbs involved in the meiotic chains: Rb(2.11) of *E. alaicus* and Rb(2.18) of *E. tancrei*, 2n = 50. Complex chains in meiotic prophase I led to the reduction of fertility in hybrids or even sterility. It might be a possible post-copulation mechanism for reproductive isolation. Here, we demonstrated, that the synapsis and behaviour of *E. alaicus* (2n = 48) meiotic chromosomes were very similar to *E. tancrei* and *E. talpinus* ones (Kolomiets et al.

Figure 9. *Ellobius alaicus*, locality #8. Photo by I. Bakloushinskaya.

Figure 10. Habitat of *E. alaicus*, the Kyzyl-Suu River Valley, locality # 8. Photo by I. Bakloushinskaya.

1991, 2010, Bakloushinskaya et al. 2012, Matveevsky et al. 2016, 2017). Isomorphic sex chromosomes exhibit a functional heteromorphism in the meiotic prophase I in all three species, that is a unique case for mammals.

Therefore, characteristic nucleotide substitutions in mitochondrial and nuclear genes, distinct Rbs variability and independent origin of typical for *E. alaicus* translocation Rb(2.11) support the species status of the Alay mole vole notwithstanding the closeness to *E. tancrei*.

The discovery of different heterozygous animals with 2n = 53 and two different Rb translocations raised the question of natural hybridization and mechanisms of genome stability. Animals that carried 1 Rb(2.11) with a high probability were hybrids of *E. alaicus*, 2n = 52 and *E. tancrei*, 2n = 54. For the second variant, 2n = 53 and 1 Rb(1.3), two scenarios are possible. The first is the existence of an unknown form (or species) with 2n = 52, 2 Rb(1.3), which hybridized with *E. tancrei*, 2n = 52, so hybrids of the first generation or backcrosses had 2n = 53, 1 Rb(1.3). Another possibility is that they were remote hybrids of *E. alaicus* with 2n = 50, 2 Rb(2.11), 2 Rb(1.3) (as animals from the Lake Chatyr-Kel' vicinities, #4 or Naryn district, #6) and *E. tancrei*, 2n = 54. In that case, hybrids might have lost the Rb(2.11) in numerous generations under meiotic drive (de Villena and Sapienza 2001, Lindholm et al. 2016). Sociality described in mole voles (Smorkatcheva and Lukhtanov 2014, Smorkatcheva and Kuprina 2018) and underground lifestyle could accelerate the fixation of mutations in disjunct populations.

As we mentioned previously (Bogdanov et al. 2015), the differentiation of wide-ranging steppe species *E. talpinus* has occurred because of isolation due to geographic barriers, for example, large rivers such as the Volga River and the Irtysh River. *E. tancrei* and *E. alaicus* inhabit mountainous steppes and alpine meadows. Mountain ranges might be the most important geographic barriers for the spreading of mole voles because the animals do not inhabit mountains higher than 3500–4000 m above sea level. In the Tien Shan, the Pamir and the Pamir-Alay a distribution of mole voles should be sporadic because suitable habitats are mosaic. The complex orography of the regions may be a main source for geographical separation and ensuing fixation of the chromosomal forms (Bush et al. 1977). The situation is further complicated by the rapid change in the landscape due to neotectonic activity. The Alay Valley is an asymmetric intra-montane sedimentary basin with an average elevation of 2700 m, which formed in response to the convergence between India and Eurasia during the late Cenozoic (Coutand et al. 2002). The Pamir continues to move northward with a large fraction absorbed near the Alay Valley. The highest observed rate of the North-South convergence is between 10 and 15 mm/year as derived from Global Positioning System (GPS) measurements (Zubovich et al. 2016). The Pamir-Tien Shan region accommodates a high deformation over a short distance and is capable of producing magnitude 7 earthquakes in nearly decadal repeat times (Storchak et al. 2013). The last large seismic event was the 2008 magnitude 6.6 Nura earthquake with an epicenter just east of the Alay Valley (Sippl et al. 2014). Large earthquakes, which appeared to be in the Tien Shan and the Pamir, can trigger landslides (Havenith et al. 2003). Mudflows and landslides may quickly separate habitats of subterranean mole voles (Vorontsov and Lyapunova 1984). All three *E. alaicus* forms (2n = 52, 50 and 48) live in valleys, which

are bordered by the mountain ranges. The evident pathways for mole voles spreading are the river banks in canyons crossing the ridges. Mole voles have a complex system of burrows, with at least three horizontal levels and numerous vertical connecting tunnels. But sometimes, most often at night, the animals run out onto the surface and move quickly over the ground. They probably can use human-made bridges, which are often destroyed by flows; new bridges may open a new route for mole voles. The suggestion was inspired when bursts of variations in chromosome numbers in mole voles from the opposite banks of the Vakhsh River were discovered at places close to bridges (Lyapunova et al. 1980 1984). In some cases, as when mole voles inhabit opposite banks of the Kyzyl-Suu River in a deep canyon (localities # 8, 9, Figs 1, 9, 10), we can only explain how animals cross a mountain river if we assume that they use human-made bridges.

Despite a complex relief of the region, the geographical barriers are not as strong as genomic ones. We revealed no signs of hybridization in neighbor populations of *E. alaicus* and *E. tancrei* yet, i.e. between *E. alaicus* (2n = 48, locality #8, Fig. 1) and *E. tancrei* (2n = 30, locality # 16, Fig. 1) or *E. alaicus* (2n = 48, locality #12, Fig. 1) and *E. tancrei* (2n = 54, locality # 13, Fig. 1). There are no geographical barriers preventing active contact between these populations in about ten or even few kilometers. In such cases, the assumption that genomic (chromosomal) reorganization in mammals is often rapid (Vorontsov, Lyapunova 1989, Bakloushinskaya 2016, Dobigny et al. 2017) seems plausible, if one considers that polymorphism for isolation traits segregates within populations with different genetic compositions and ecological settings. If we assume that loci, which may contribute to a reproductive barrier, are dispersed throughout the genome, and intragenomic interactions that arise from genetic pathways can maintain species-specific differences (Lindtke and Buerkle 2015, Payseur and Rieseberg 2016), we can consider speciation starting with chromosome changes as a reliable and fast way of speciation.

Conclusion

The study of *E. alaicus* demonstrates that the difficulty of species delimitation due to lack of morphological differences might be resolved by using chromosomal and molecular markers.

We assumed, that the independent emergence of Robertsonian translocation Rb(2.11) was crucial for the divergence of ancestors of *E. alaicus* and *E. tancrei*, which both developed specific karyotypic variability, more extensive in *E. tancrei* (2n = 54-30) but distinct due to non-homological (except Rb(2.11)) translocations in *E. alaicus* (2n = 52–48). Notwithstanding, the closeness of species, which was demonstrated here by studying mitochondrial DNA (*cytb*) and fragments of two nuclear genes, determines the possibility of sporadic hybridization at the zones of species contacts. Using different cytogenetic methods, G-banding and chromosome painting, along with by *cytb*, *XIST*, and *Rspo1* genes sequencing allowed us to expand the range of *E. alaicus* from the terra typica, the Alay Valley (South Kyrgyzstan) up to the Ferghana Ridge and the Naryn Basin, Tien Shan at the north-east and to the Pamir-Alay Mountains (Tajikistan) at the west.

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Different behaviour of C-banded peri-centromeric heterochromatin between sex chromosomes and autosomes in Polyphagan beetles

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Abstract

Heterochromatin variation was studied after C-banding of male karyotypes with a XY sex formula from 224 species belonging to most of the main families of Coleoptera. The karyotypes were classified in relation with the ratio heterochromatin/euchromatin total amounts and the amounts of heterochromatin on autosomes and gonosomes were compared. The C-banded karyotypes of 19 species, representing characteristic profiles are presented. This analysis shows that there is a strong tendency for the homogenization of the size of the peri-centromeric C-banded heterochromatin on autosomes. The amount of heterochromatin on the X roughly follows the variations of autosomes. At contrast, the C-banded heterochromatin of the Y, most frequently absent or very small and rarely amplified, looks quite independent from that of other chromosomes. We conclude that the Xs and autosomes, but not the Y, possibly share some, but not all mechanisms of heterochromatin amplification/reduction. The theoretical models of heterochromatin expansion are discussed in the light of these data.

Keywords

Coleoptera, Polyphaga, karyotypes, heterochromatin, variation, sex chromosomes

Introduction

There is a consensus to consider that the ancestral karyotype of Polyphagan beetles was composed of 20 chromosomes, a number observed in living specimens from most families (Smith and Virkki 1978). The sex chromosomes, XX and XY in females and males,

respectively, are usually among the smallest chromosomes. Occasional size increases have recurrently been reported, but the origin of these increases has never been systematically investigated. Considering the large compilation of Smith and Virkki (1978), the rate of enlarged sex chromosomes, generally referred to as neo sex chromosomes, was estimated at 8.3% of species (Dutrillaux and Dutrillaux 2009), but the formal distinction between the sex chromosomes derived from a translocation with autosomal material and those having amplified their heterochromatin content could not be made in most ancient publications. On the whole, the use of chromosome banding remains limited in beetles. Their euchromatin does not contain large fragments of repetitive DNA sequences, such as LINES and SINES in mammals, which probably originate G and R bands, after appropriate treatments (Bickmore and Craig 1997). This explains the high compaction of the genome in beetles, in which the gene density is many-fold that of mammals (Dutrillaux 2016). Thus, beside techniques of molecular cytogenetics using satDNA probes, which were applied on some species (Petitpierre 1980, Pons et al. 2002, 2004), only C-banding can be regularly achieved for detecting heterochromatin, which harbors highly repeated DNA. However, it remains poorly efficient in some families, such as Cerambycidae, in which centromere regions often remain poorly or not C-banded (Dutrillaux and Dutrillaux 2018). Silver staining, generally used for the detection of nucleolar proteins at contact with the NOR (Nucleolus Organizer Region), frequently stains a portion of heterochromatin in beetles. DNA replication studies, which allow a differentiation between late replicating heterochromatin and early replicating euchromatin are difficult to apply in the absence of cell culture and remain exceptionally used (see below). In spite of these difficulties, we tried to find some rules governing heterochromatin variation in beetles, especially that of the sex chromosomes, in relation with that of autosomes. For this purpose, we analyzed male specimens of 344 species of Polyphagan beetles, for which C-banding was systematically applied. It will be shown that, as regard their heterochromatin content, the X and the Y have a very different behavior.

Material and methods

Insects

We collected most of the specimens from the 344 studied species in France, Greece and West Indies. Some specimens were also obtained from amateur breedings, Besançon insectarium, or kindly provided by colleagues and friends. The species studied here were distributed into 21 families, but most belonged to Cerambycidae (67 species), Chrysomelidae (40 species), Curculionidae (18 species), Lucanidae (11 species), Scarabaeidae (136 species) and Tenebrionidae (28 species). We established the karyotype of the 344 species, among which we selected the19 following species, as examples of the various situations observed:

Adalia bipunctata Linneaeus, 1758 (Coccinelidae, Coccinelinae) (France);
Amphimallon solstitiale Linnaeus, 1758 (Scarabaeida, Melolonthinae) (France);
Asida jurinei Solier, 1836 (Tenebrionidae, Pimeliinae) (France);

Crioceris asparagi Linnaeus, 1758 (Chrysomelidae, Criocerinae) France;
Cyclocephala picipes Olivier, 1789 (Scarabaeidae, Dynastinae) (French Guyana);
Disonycha latifrons Schaeffer, 1919 (Chrysomelidae, Alticinae) (Canada, Quebec);
Dorcadion (*Cribridorcadion*) *etruscum* Rossi, 1790 (Cerambycidae, Lamiinae) (Italy);
Lamprima adolphinae Gestro, 1875 (Lucanidae) (New Guinea);
Leucothyreus nolleti Paulian, 1947 (Scarabaeidae, Rutelinae) (Martinique);
Lilioceris lili Scopoli, 1763 (Chrysomelidae, Criocerinae) (France);
Lucanus cervus Linneaeus, 1753 (Lucanidae) (France);
Macraspis tristis Castelnau, 1840 (Scarabaeidae, Rutelinae) (Guadeloupe);
Melolontha melolontha Linnaeus, 1758 (Scarabaeidae, Melolonthinae) (France);
Melolontha hippocastani Fabricius, 1801 (Scarabaeidae, Melolonthinae) (France);
Morimus funereus Mulsant, 1862 (Cerambycidae; Lamiinae) (Greece);
Propomacrus davidi Deyrolle, 1874 (Scarabaeidae, Euchyrinae) (China);
Scarabaeus variolosus Fabricius, 1787 (Scarabaeidae, Scarabaeinae) (Greece);
Strategus syphax Fabricius, 1775 (Scarabaeidae, Dynastinae) (Guadeloupe);
Uloma retusa Fabricius, 1801 (Tenebrionidae, Tenebrioninae) (Guadeloupe).

Cytogenetic methods

After anaesthesia by ethyl acetate, testicular follicles were dropped into an aqueous solution of 0.88 M KCl where they remained for 15 min at room temperature. They were transferred into a micro-centrifuge tube (VWR International SAS, code 211-0033, Strasbourg, France) containing 0.5 ml of 0.55 M KCl (hypotonic) solution, where they were squashed and suspended using a piston pellet (VWR, code 045420) adjusted to the internal diameter of the tube. The volume of 0.55 M KCl was completed to 1.5 ml. After 10 min, they were centrifuged during 5 min at 800 g. The supernatant was replaced by Carnoy I fixative, in which the cells were suspended and left for at least 30 min. After one change of fixative, the cells were spread on wet and cold slides or conserved for a few days before use. Slides were stained by Giemsa, photographed and C-banded according to Angus (1982). Many studies were also performed on midgut cells, according to Angus (1988). In addition, a prolonged hypotonic shock was applied for pachytene stage obtaining (Dutrillaux et al. 2006). For DNA replication studies on *Crioceris asparagi,* BrdU (5-bromodeoxyuridine) was added to the 0.88 M KCl solution (final concentration 20 mg/l) for 4h before the hypotonic shock. Slides were stained by acridine orange (Dutrillaux et al. 1973) and observed in fluorescence. Staining by quinacrine mustard was performed according to Caspersson et al. (1970).

Evaluation of heterochromatin amplification

Not all heterochromatin is stainable by C-banding, but for technical reasons, only C-band positive heterochromatin will be considered. The usual intra- and inter-specific variation of heterochromatin makes it somewhat arbitrary to decipher its amplifica-

tion. At the level of the whole karyotype, we have visually considered that heterochromatin is not amplified (NAH) when its amount represents less than 10% of the total chromosome length (Fig. 1A, C, D). It was considered as mildly amplified (MAH) when its total length was comprised between 10% and 25% that of chromosomes (Fig. 1B, 2D) and highly amplified (HAH) above 25% (Fig. 3A, B, D). Physical measurements were performed for ambiguous evaluations only. At the level of individual chromosomes, heterochromatin will be considered as amplified when its length is twice that of the average of other chromosomes of the karyotype (chromosome X, Fig. 3C).

Results

For the above-mentioned species, this is the first report on C-banded karyotype, with the exception of *L. cervus*, *L. adolphinae*, *M. tristis*, *M. hippocastani*, *M. melolontha* and *S. syphax* (Giannoulis et al. 2011, Dutrillaux et al. 2007, 2012, Dutrillaux and Dutrillaux 2009). Some cytogenetic data, mainly chromosome counts, were also published for *A. solstitialis*, *A. jurinei*, *A. bipunctata*, *C. asparagi* and *L. lili* (John and Lewis 1960, Juan and Petitpierre 1991, Petitpierre 1980, Petitpierre et al. 1988, Virkki 1951).

Among the 344 male karyotypes studied, 25 (7.3%) without Y chromosome (X0 sex formula), 9 with a XYY formula (2.6%) and 35 (10.2%) with a gonosome-autosome translocation were excluded. Among the 275 remaining ones, the quality of the C-banding was considered to be sufficient for analysing both the size and the distribution of heterochromatin on chromosomes in 224 species. In this sample, a complete lack of C-banding on the Y chromosome was recorded in 134 instances (60%). At contrast, no C-banding was observed on the X chromosome in only 9 instances (4%). Among a large variety of profiles of heterochromatin distribution, some were particularly recurrent. They are listed below by order of decreasing occurrence.

a) ***Presence of clearly but not strongly amplified (NAH and MAH) C-banded heterochromatin on the centromere regions of all the chromosomes but the Y.*** It was observed in 86/224 instances (38.4%). Four examples are given in figure 1 in species from different families: *A. bipunctata* (Fig. 1A); *A. jurinei* (Fig. 1B); *M. funereus* (Fig. 1C) and *C. picipes* (Fig. 1D). In these species, the amount of centromeric heterochromatin varies from NHA , as in *M. funereus*, to MAH, as in *A. jurinei*, but is fairly similar, from chromosome to chromosome within each karyotype. Thus, there is a indisputable homogenization of the C-band size between autosomes and X. The lack or very small amount of C-banding on the Y shows that its heterochromatin dynamics is independent from that of both the X and the autosomes.

b) ***Presence of a clearly but not strongly amplified (NAH and MAH) C-banded heterochromatin on the centromere regions of all the chromosomes including the Y.*** It was observed in 60 instances (27%). Four examples are given in figure 2: *A. solstitiale*; *D. etruscum*; *L. lili* and *S. syphax*. Here again, there is some homogenization of the size of C-bands on both autosomes and X chromosome, but the

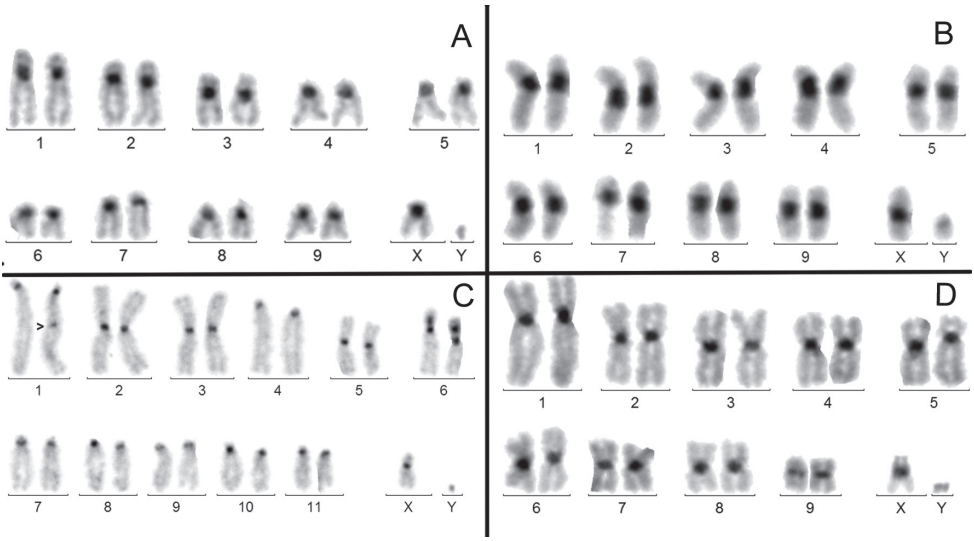

Figure 1. C-banded male karyotypes. **A** *Adalia bipunctata* **B** *Asida jurinei* **C** *Morimus funereus* **D** *Cyclocephala picipes*. The autosomes and the X chromosomes have similar amounts of C-banded heterochromatin, but the Y chromosomes remain unstained.

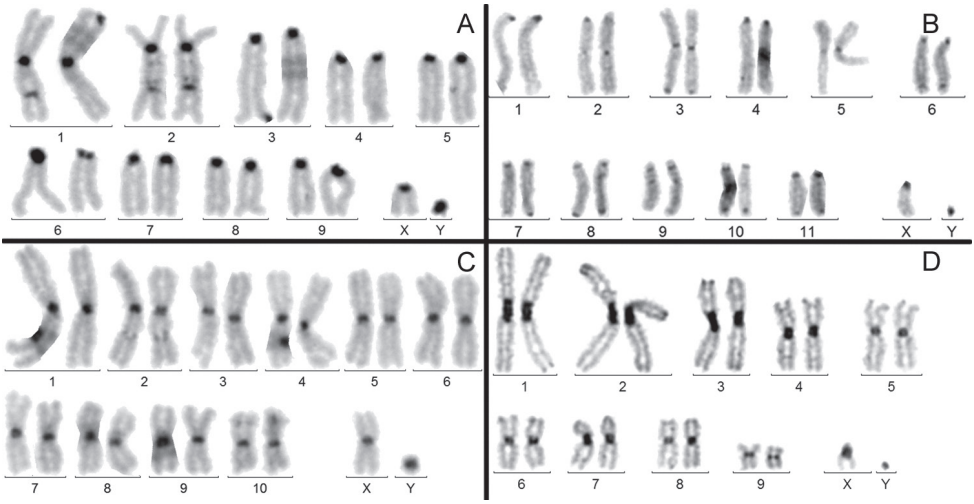

Figure 2. C-banded male karyotypes. **A** *Amphimallon solstitiale* **B** *Dorcadion etruscum* **C** *Lilioceris lili* **D** *Strategus syphax*. In each karyotype, all centromere regions are similarly C-banded, but that of the Y is more variable.

size of the C-band on the Y is more independent: large in *A. solstitiale* (Fig. 2A) and very, small in *S. syphax* (Fig. 2D) karyotypes.

c) ***Presence of large heterochromatic fragments (MAH and HAH) on both the autosomes and the X.*** It was observed in 28 instances (12.5%). In this condition, there is not a systematic homogenization of the heterochromatin size on the autosomes, as in *U. retusa* (Fig. 3A) and the X may exhibit a very large heterochromatic

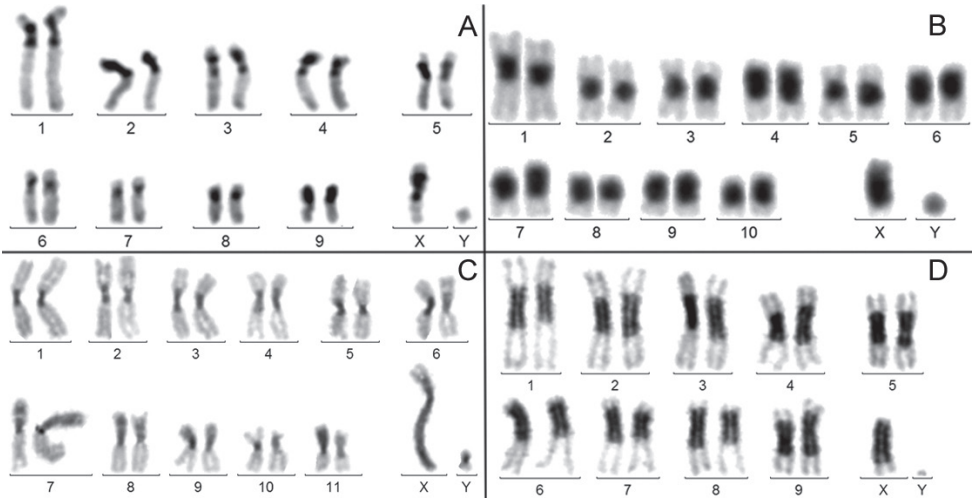

Figure 3. C-banded males karyotypes. **A** *Uloma retusa* **B** *Lucanus cervus* **C** *Disonycha latifrons* **D** *Melolontha hippocastani*. The level of heterochromatin amplification is often similar in the X and autosomes (**A, B, D**). The amplification may also be scattered, as in **C**, but it rarely involves the Y chromosome.

fragment, as in *D. latifrons* (Fig. 3C). In many species, however, the amplification of heterochromatin is roughly similar on the X and autosomes, as in *L. cervus* and *M. hippocastani* (Fig. 3B, D). The C-banding of the Y is poor or absent, thus completely independent from that of both the X and autosomes.

d) ***Presence of a large amplification of heterochromatin on the X chromosome but not on autosomes.*** It was observed in 25 species (11.2%). In these karyotypes, C-banded heterochromatin was either invisible on chromosome Y, as in *L. nolleti* and *P. davidi* (Fig. 4A, B), or present and even amplified, as in *L. adolphinae* (Fig. 4C).

e) ***Heterochromatin amplification on chromosome* Y.** It was noticed in 23 instances only (10.4%). Compared to both the X and autosomes, this amplification was almost always limited in size, some of the largest C-bands on the Y were observed in *S. variolosus* (Fig. 4D) and in species of Geotrupidae (not shown), as described by Wilson and Angus (2004). We recently found a very strong amplification of heterochromatin on both the X and Y in *Oxymirus cursor* (Cerambycidae, Lepturinae) but this species was not included in this study (Dutrillaux and Dutrillaux 2018).

Intra-specific variation of heterochromatin

The analysis of most species was generally limited to a few specimens, but short series could be studied for some species. The high variability of both location and amount of heterochromatin is a common place, which was verified here. However, it appeared that variations of heterochromatin are more important on autosomes than on gonosomes. For example, amongst 18 males of *M. melolontha*, the X was always and the Y never C-banded. At contrast, the C-banding of several autosomes was highly poly-

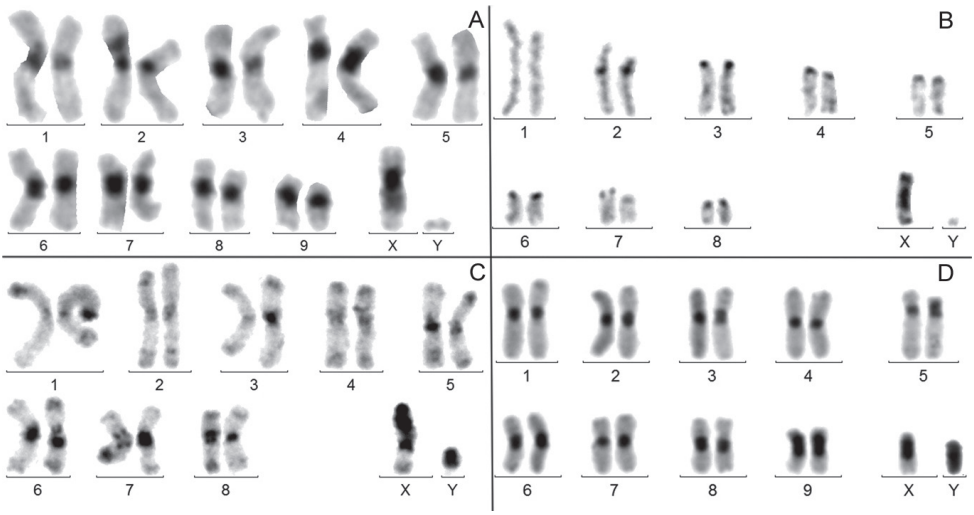

Figure 4. C-banded male karyotypes. **A** *Leucothyreus nolleti* **B** *Propomacrus davidi* **C** *Lamprima adolphinae* **D** *Scarabaeus variolosus*. Large heterochromatin amplification can involve the X alone (**A, B, C**) and more rarely the Y (**D**).

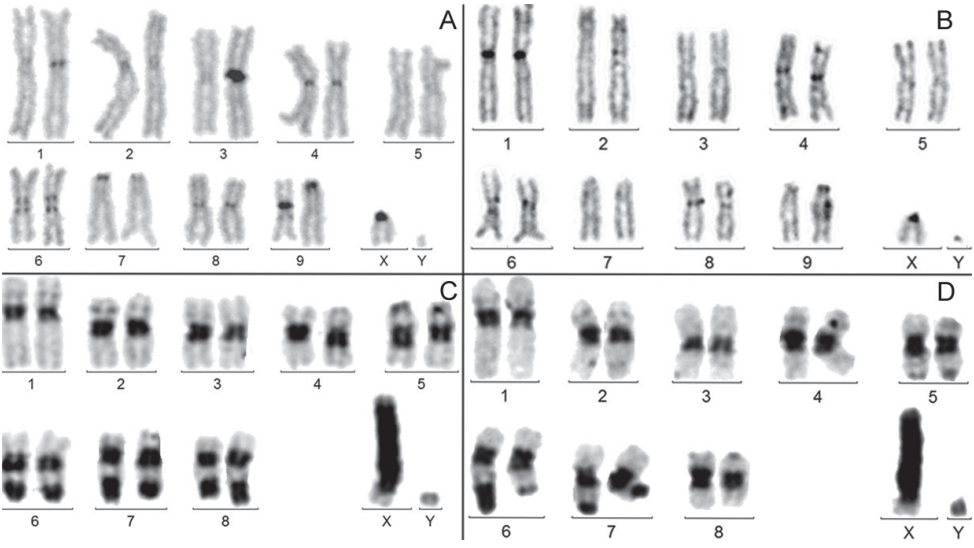

Figure 5. C-banded male karyotypes. **A, B** *Melolontha melolontha* **C, D** *Macraspis tristis*. At contrast with the high variability of autosomes, there is a remarquable stability of the amount of C-banded heterochromatin on the X (average in **A, B** and amplified in **C, D**).

morphic: it varied in size and could be either present or absent on a single or both homologs (Fig. 5A, B). The same variation of autosomes was observed in 12 females, in which the 2 Xs were always homogenously C-banded. A similar example is provided by the heterochromatin of *M. tristis*, whose heterochromatin is highly and constantly amplified on the X and variable on the autosomes (Fig. 5C, D).

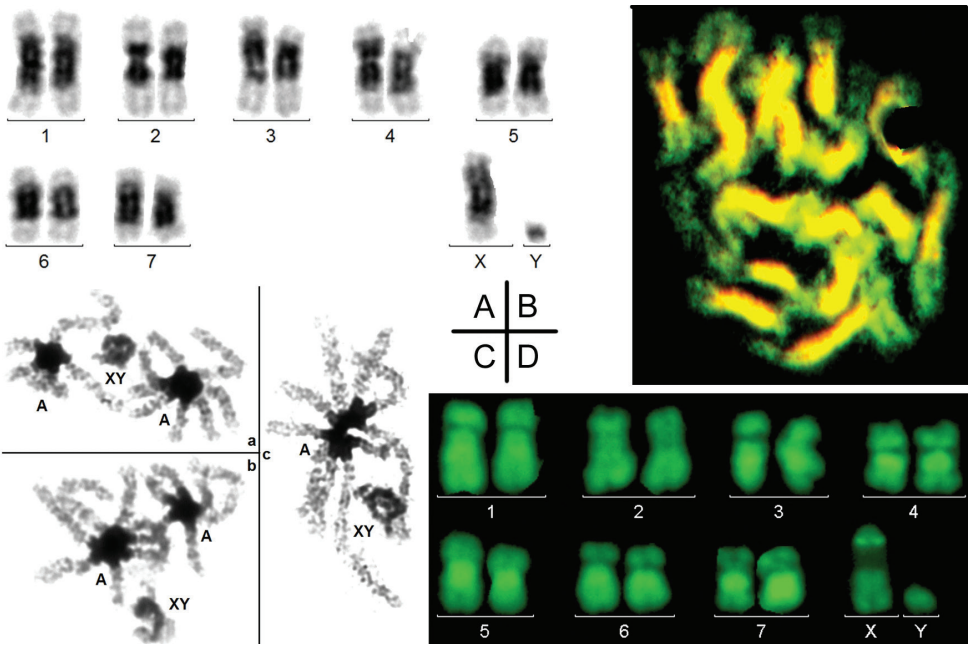

Figure 6. *Crioceris asparagi*. **A** C-banded male karyotype displaying a large heterochromatin amplification in all chromosomes but the Y. **B** Incorporation of BrdU during late S-phase in a female cell: all heterochromatin is homogeneously late replicating (orange staining). The distal fragments of all chromosomes are early replicating (green), which indirectly indicates that there is no Lyonisation of one X. **C** C-banding of 3 spermatocytes (**a, b, c**) at pachynema : autosomal bivalents are at contact and form rosettes after heterochromatin fusion. The sex bivalent is always separated. **D** Q-banded male karyotype: heterochromatin displays at least 3 levels of fluorescence.

Heterogeneity of C-banded heterochromatin

The possible heterogeneity of heterochromatin was investigated in the karyotype of *C. asparagi,* in which heterochromatin is strongly amplified on both the X and autosomes. As in most other species, its heterochromatin is homogenously stained after C-banding (Fig. 6A). As usual, compared to mitotic chromosomes, heterochromatin on bivalents at pachynema is much more compacted. Autosomal bivalents frequently form rosettes by fusion of their heterochromatin, while the sex bivalent remains alone (Fig. 6C a, b, c).

After BrdU incorporation during the late S-phase and acridine orange staining, heterochromatin homogenously fluoresces in orange, indicating its late replication, while early replicating euchromatin fluoresces in green (Fig. 6B). Finally, after staining by quinacrine mustard (Fig. 6D) heterochromatin displays very heterogeneous staining patterns, with at least 3 different levels of fluorescence. Autosomes 3 to 7 share the same fluorescence pattern: dull at centromeres, medium on proximal short arm and brilliant on proximal long arm. The Q-banding of the X is very different with a very large dull and a

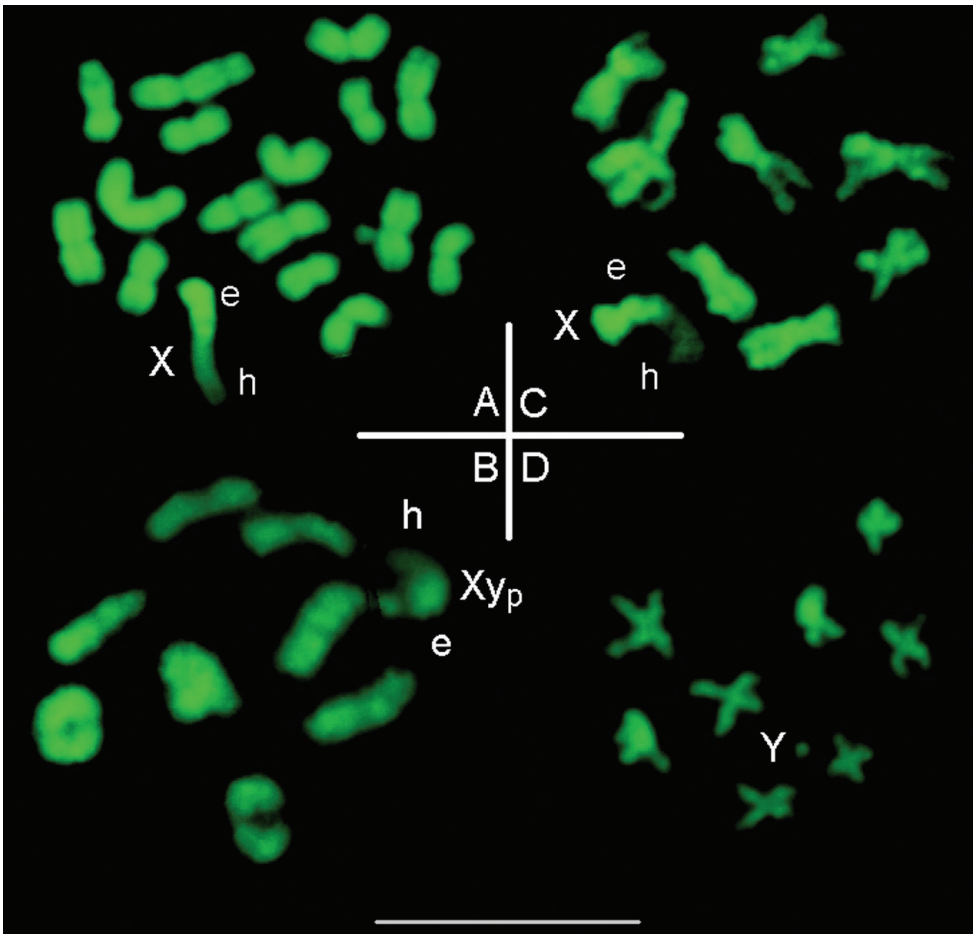

Figure 7. QM-staining of *Macraspis tristis* cells. **A** Spermatogonium **B, D** 9,X and 9,Y spermatocytes II **C** 8+Xyp spermatocyte I at diakinesis/metaphase. Heterochromatin, in particular that of the X, displays very different levels of fluorescence. e= euchromatin, h=heterochromatin.

small brilliant fragment. This relative homogenization of heterochromatin on autosomes, but not on the X is also evidenced in *M. tristis* after quinacrine mustard staining (Fig. 7): heterochromatin is brilliant on autosomes, while a large fragment is dull on the X.

Discussion

Structural chromosome rearrangements, such as reciprocal and Robertsonian translocations, fissions and intra-changes (inversions, translations, centromere shifts) recurrently occur and differentiate the karyotypes of related species. It seems that in beetles, in which most species possess 20 chromosomes, the karyotype diversification

is principally the consequence of intra-changes, but this category of chromosome rearrangements remains difficult to detect, as long as chromosome banding is limited to heterochromatin (Dutrillaux and Dutrillaux 2016). Chromosomal rearrangements create a gametic barrier, and once fixed in a species, they are clonally transmitted to the progeny and can be used for establishing phylogenies. This does not seem to be the case for heterochromatin changes, which are highly frequent within populations and without clear consequence on both reproduction and phenotype. This variation of heterochromatin often affects a variable number of chromosomes, and it is very difficult to decipher both the mechanism inducing these changes and the rules governing their trans-generational transmission. Nevertheless, multiple examples from mammals to insects show that most karyotypes are characterized by a certain heterochromatin pattern, more or less strictly maintained at the level of species, genus or family. This indicates that heterochromatin is not modified and transmitted by each chromosome independently, thus that some regulatory mechanisms exist.

Hypotheses about the mechanisms of peri-centromeric heterochromatin homogenisation and expansion

The origin of heterochromatin and its highly repeated DNA content, as well as the factors modulating its quantitative and qualitative variations, remain largely unknown, but two main mechanisms have been envisaged.

1) **The recombination process.** As in other animals, peri-centromeric heterochromatin of beetles harbours sequences of repetitive (satellite) DNA (Lorite et al. 2001, 2003, Pons et al. 2002, 2004). Thus, recombination in heterochromatin often consists in exchanges between homologous or pseudo-homologous repeated DNA (Schweizer and Loidl 1987). With time and generations, the repetition of such exchanges would lead to a statistical homogenization of heterochromatin, as regard both its total amount per chromosome and its molecular composition, conferring a characteristic pattern to the whole karyotype. It has been proposed that quantitative variations of heterochromatin could be dependant on external factors, such as altitude, thus would correspond to an adaptation to environmental constraints (Cassagnau 1974). But what kind of exchanges could be in cause? It is well established that meiotic recombination by crossing-over generally avoids heterochromatin and neighbouring regions, which are highly compacted (Fig. 6C). Exchanges (crossing-over) principally occur in euchromatin, which is under-condensed, around the synaptonemal complex (Heyting 1996). Supposing that rare exchanges by crossing-over occur in heterochromatic regions, the presence of repeated DNA would lead to a high probability of asymmetrical exchanges, leading to duplications/deficiencies originating the variation of the amount of heterochromatin between homologous chromosomes. But this would not directly explain the homogenization at the level of the whole karyotype, including the X in particular. For that, exchanges between similar sequences of non-homologous

chromosomes would be necessary. In the model of Schweizer and Loidl (1987), which was proposed for telomeric heterochromatin principally, it is supposed that the proximity of telomeres, at early prophase (bouquet stage), might facilitate such pseudo-homologous exchanges. Centromeric heterochromatin is not associated at early prophase, but tight associations recurrently occur later, during the pachytene stage (Dutrillaux et al. 2006 and Fig. 6C). This could also facilitate inter-chromosomal exchanges, but odd numbers of exchanges would lead to form deleterious reciprocal translocations, at difference with exchanges at telomeres. DNA hypo-methylation, particularly of satellite DNA located in heterochromatin, is a strong factor of chromosome instability, leading to breakages and exchanges between both homologous and non-homologous chromosomes (Almeida et al. 1993). Huge variations of DNA methylation, including deep hypo-methylations in heterochromatin, occur at various stages of gametogenesis (Coffigny et al. 1999 and Bernardino-Sgherri et al. 2002). This may favour DNA exchanges and homogenization of heterochromatin at long-term.

2) ***The ocean ridges model.*** This model was proposed to explain the expansion of centromeric repeated DNA (Rudd et al. 2006, Shepelev et al. 2009). It is assumed that centromeric repeated DNA expands by a mechanism recalling the ocean ridges process, with new satellite families appearing in the core centromere and displacing pre-existing satellites towards more distal regions. This process may involve similarly all chromosomes and lead to a fairly homogenous expansion of heterochromatin harbouring satellite DNA in peri-centromere regions of all chromosomes. Mutations could occur later and accumulate, modifying the sequence of the DNA repeats in proportion with their age, i.e., their distance to centromere. For example, a C to T transversion occurring during the expansion of a large DNA repeat would considerably decrease its resistance to denaturation, change the staining properties of the harbouring heterochromatin and even suppress the C-banding.

Heterochromatin variation in beetles partially supports these hypotheses

Most of the karyotypes of this report share the same tendency for heterochromatin homogenization. The more or less important heterochromatin or C-banding expansion is not totally independent from the systematic classification: for example, most Cerambycidae have small or inconsistent C-bands (Figs 1C, 2B); most Scarabaeidae have average C-bands (Figs 1D, 2A, D, 4A, D), while many Tenebrionidae have very large C-bands (Figs 1B, 3A). However, large heterochromatin amplification may also involve one or a few species only, as *M. hippocastani* in genus *Melolontha* (Fig. 3D), or a genus, as *Crioceris* amongst Criocerinae (personal data) (Fig. 5). According to the above-proposed criterion, amplified heterochromatin was observed in about 25% of species. It was generally similarly amplified on autosomes and the X, which suggests that common mechanisms were at work. However, this expectation, which fits with the result of C-banding only, is obviously over-simple, as shown by our data on *C. asparagi* and *M. tristis*, in which all chromosomes but the Y have amplified hetero-

chromatin in mitotic metaphases. This heterochromatin is homogeneously compacted (shortened) at pachynema, homogeneously C-banded and late replicating, but staining with quinacrine mustard (or DAPI, not shown), known to fluoresce in proportion to the AT richness of DNA, displays huge differences of fluorescence. This demonstrates the presence of different components in different amounts. In these 2 species, the dull fragment (AT-poor) is much larger and the brilliant fragment (AT-rich) much smaller on the X than on the average autosomes. Thus, there is a certain homogenization for the autosomes while the X has a unique fluorescence pattern. A plausible explanation is that heterochromatin amplification depends on an unique mechanism at the cell level, but exchanges occur between autosomes and not or more rarely between gonosomes and autosomes. This interpretation is in agreement with the bivalent behaviour at pachytene stage: all autosomes may form rosettes with tight associations of their heterochromatin, while the sex bivalent remains separated (Fig. 6D), but their eventual exchanges might not be of the same type as crossing-over between euchromatic regions. The small size of the heterochromatin of the Y may depend on an independent erosion mechanism, recalling that proposed for mammals.

In conclusion, there is a large variety of the heterochromatin patterns in the karyotypes of Polyphagan beetles. In spite of inter-individual variations, phylogenetically related taxa tend to share similar characteristics, but exceptions exist: huge amplifications of heterochromatin may affect only a single or all chromosomes of a karyotype and may characterize one or several species in a genus. Thus, heterochromatin constitutes a weak criterion for establishing phylogenetic relationships. A certain homogenisation of the heterochromatin amount and staining capacities exists between the autosomes of a same karyotype. The quantitative, but not qualitative, variations of the heterochromatin of the X grossly follow that of autosomes. At difference, the heterochromatin content of the Y is generally very limited and its variations look largely independent from those of other chromosomes. The concerted variations of autosomes, and the relative independence of the gonosomes, and the Y in particular, may be explained by the strong tendency for fusions of heterochromatin of autosomes, but not gonosomes, at male (and female?) meiotic prophase.

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LETTERS TO THE EDITOR

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Revisiting history. The memory of Prof. Hermann J. Muller (1890–1967) in Moscow revived by Helen Muller

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Introduction

Last autumn, Moscow geneticists had the pleasure to meet Helen Muller, daughter of Professor Hermann Joseph Muller, American geneticist, educator, and Nobel laureate, best known for his work on the physiological and genetic effects of radiation (X-ray mutagenesis). In search of materials for a book that she is writing about her father, Helen Muller visited the institutions of Russian Academy of Sciences, associated with the presence of H.J. Muller in Moscow in the 1930s.

The scientific career of Hermann J. Muller (1890–1967) began in Columbia University, New York under the supervision of one of the founders of cytogenetics, E.B. Wilson. Muller remained as a postgraduate in the same university through 1912–1916, and in 1918–1920 became an assistant of T.H. Morgan, whose theory of chromosomal heredity (Morgan et al. 1915) they explored together with A.H. Sturtevant and C.B. Bridges. During 1921–1931 Muller was based in University of Texas, where he became professor in 1925. Significant for Muller's future was his acquaintance with the

Figure 1. Prof. Helen Muller (left) in the conference hall situated close to the laboratory of her famous father in Moscow. With Dr. Tatjana B. Avrutskaya (N.I. Vavilov Institute) and Academician V.V. Rozhnov, Director of A.N. Severtsov Institute. Photo by N. Bulatova.

Russian scientist Nikolai I. Vavilov who came to USA in 1921 as a rising leader of the new Soviet genetics and organizer of the world collection of cultured plants organised according to his newly proposed scheme of homologous genetic series (Vavilov 1920). In 1922, H.J. Muller visited for the first time the institutions in Russia under Vavilov's influence. Muller brought with him his collections of fruit-fly mutants, introducing genetic studies on *Drosophila* Fallén, 1823 to Russia. In February 1933, H.J. Muller was elected foreign corresponding member of the USSR Academy of Sciences, succeeding the honorary membership of T.H. Morgan in 1932 (www.ras.ru), also at the instigation of N.I. Vavilov.

For four fruitful years Hermann J. Muller worked at the Institute of Genetics, founded by N.I. Vavilov in Leningrad, now St. Petersburg (restoring the traditional name for the city), and moved with his Laboratory of Genes and Mutagenesis to Moscow in 1934. His colleagues were M.L. Bel'govsky, A.A. Prokofieva-Bel'govskaya, Y.J. Kerkis, N.N. Medvedev, K.V. Kosikov and others, well-known Soviet geneticists. Together with his scientific successes, during the last two years of his work in the USSR, Muller devoted much effort to the public defence of the chromosome theory of heredity from the anti-genetic attacks of T. Lysenko. As a consequence of this opposition to Lysenko, H.J. Muller was forced to leave USSR in 1937.

Two places related to Hermann J. Muller's work in Moscow were visited by his daughter last Autumn. Muller's lab was situated in a building, which continues to be part of the Russian Academy of Sciences. The old building at number 33 Leninsky Avenue is that one from which academician Nikolai I. Vavilov departed for his last expedition in 1940 and never returned. In protest against the expulsion of N.I. Vavilov, Hermann Muller, already a Nobel laureate (1946), refused membership of the USSR Academy of Sciences. Muller's name was, however, added to the Academy (www.ras.ru) in recent times (1990). In the 1960s, the rehabilitation of the name of N.I. Vavilov should be proceeded in different ways, including the naming of the new academic Institute of General Genetics, situated close to where Vavilov used to work (www.vigg.ru).

Helen Muller, emeritus Professor of Sociology from the University of New Mexico, visited the N.I. Vavilov Institute of General Genetics and memorial museum with portraits of Vavilov's collaborators, including, of course, H.J. Muller (http://vigg.ru/istorija-instituta/muzei-ni-vavilova/). On 27 September 2018 Helen Muller gave a lecture at the A.N. Severtsov Institute of Ecology and Evolution, at Leninsky Avenue, 33 (http://sev-in.ru/ru/node/804) in the same conference hall familiar to both Vavilov and Muller (Fig. 1).

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