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First karyotype description of the species of *Adenomera* Steindachner, 1867 (Anura, Leptodactylidae) in the "*thomei*" clade

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Abstract

The genus *Adenomera* Steindachner, 1867 currently comprises 29 nominal species, some of which are suggested to be cryptic species complexes. The present study was carried out with specimens of the "*thomei*" clade that encompasses three taxa distributed in the Atlantic Forest biome: *Adenomera thomei* Almeida et Angulo, 2006, *Adenomera* sp. L., and *Adenomera* sp. M. We used classical cytogenetics to describe the diploid number and karyomorphology of these three species collected in two different locations in the state of Bahia, Brazil. Our results revealed the diploid number 2n = 24 (FN = 34) with two pairs of metacentric chromosomes (pairs 1 and 5), three pairs of submetacentric chromosomes (pairs 2, 3, and 4), and seven pairs of telocentric chromosomes (pairs 6, 7, 8, 9, 10, 11, and 12). Further morphological, bioacoustic, and cytogenetic data (C-banding and AgNor) are needed to better delineate the lineages within the "*thomei*" clade.

Keywords
Chromosomes, cryptic species, cytogenetics, Giemsa, taxonomy

Introduction

The genus *Adenomera* Steindachner, 1867 currently comprises 29 described species that are distributed from tropical South America to the east of the Andean region (Carvalho et al. 2021). Due to the history of systematic reviews and the complex taxonomy of this group, taxonomic knowledge has not kept pace with the knowledge on its phylogeny (Duellman 2005; Menin et al. 2008; Fouquet et al. 2014). Out of the several hurdles for taxonomic studies on this genus, we highlight the high intra and interspecific similarities and the presence of cryptic species complexes (Fouquet et al. 2014). The difficulty increases when studies use only molecular data, disregarding other characteristics and making the interpretation of results less accurate (Pyron and Wiens 2011; De Sá et al. 2014).

Cytogenetic studies on the genus *Adenomera* date from the 1970s (Bogart 1970, 1974) when the karyotypes of *Adenomera andreae* (Müller, 1923), *Adenomera hylaedactyla* (Cope, 1868), *Adenomera lutzi* Heyer, 1975, and *Adenomera marmorata* (Steindachner, 1867) were described. However, the volume of cytogenetic information for the genus has not significantly advanced over these five decades. Campos et al. (2009) described the karyotypes of individuals from western São Paulo associating them with the nominal species *Adenomera* aff. *bokermanni* Heyer, 1973, *A. hylaedactyla*, and *A. marmorata*. Additionally, the karyotype of *Adenomera diptyx* (Boettger, 1885) was described by Zaracho and Hernando (2011). Thus, there is cytogenetic information for only five species among the 29 species described for this genus. Therefore, the small number of described karyotypes makes it difficult to both understand the chromosomal evolution of the genus and to better delimit species (Campos et al. 2009; Zaracho and Hernando 2011).

Among the clades within the genus *Adenomera*, the species of the *thomei* clade, *Adenomera thomei* Almeida and Angulo (2006), *Adenomera* sp. L, and *Adenomera* sp. M, are restricted to the Atlantic Forest in Brazil. *Adenomera thomei* was described from specimens collected in a cocoa plantation in the municipality of Linhares in the state of Espírito Santo (Almeida and Angulo 2006). Currently, there are records of this species also in the states of Rio de Janeiro, São Paulo, Minas Gerais, and Bahia (Almeida and Angulo 2006; Fouquet et al. 2014). The specific boundaries among these lineages are unclear mainly due to the lack of information on *Adenomera* sp. L and *Adenomera* sp. M, both of which are found only in the southern region of the state of Bahia (Fouquet et al. 2014). Knowledge on the bioacoustics, morphology, and cytogenetics for representatives of this clade is scarce (Angulo et al. 2003; Angulo 2004; Duellman 2005) and thus far it has not been used to distinguish between these two lineages.

Karyotypic information associated to DNA sequence data has helped clarify the taxonomy and systematics of some Brazilian anuran groups (Lourenço et al. 2008; Targueta et al. 2010; Suárez et al. 2013; Lourenço et al. 2015; Ferro et al. 2018; Marciano-Jr et al. 2021). To date, all information available regarding cytogenetic data within *Adenomera*

populations is taxonomically inconclusive (e.g., Campos 2009). Nevertheless, these chromosome data provided support on taxonomic decisions on a broad study of species delimitation of *Adenomera marmorata*, which included DNA sequence, morphological, and bioacoustic data (Cassini et al. 2020). Thus, it is clear that further cytogenetic studies on the genus *Adenomera* will allow more robust conclusions regarding this taxonomically challenging group. The objective of this study was to describe for the first time the karyotype of *Adenomera* species of the "*thomei*" clade from different locations in southern Bahia and compare the chromosomal patterns among the specimens.

Material and methods

Cytogenetic analysis was performed using 12 specimens of two species in the "*thomei*" clade collected in three sites in the state of Bahia (BA) (Table 1) under the SISBIO license 62181. The specimens were taken to the Tropical Herpetology Laboratory at the Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia, Brazil. We identified the specimens collected in the municipality of Ilhéus as *Adenomera* cf. *thomei*, since the bioacoustic data showed the same pattern as that recorded for populations in the "*thomei*" clade.

Table 1. Information on *Adenomera* specimens in the "*thomei*" clade used in this study.

Voucher	Genus	Species	Sex	Locality	Coordinates
MZUESC 22146	*Adenomera*	cf. *thomei*	Juvenile	Ilhéus - BA	-14.800189, -39.154594
MZUESC 22147	*Adenomera*	cf. *thomei*	Juvenile	Ilhéus - BA	-14.800189, -39.154594
MZUESC 22148	*Adenomera*	cf. *thomei*	Juvenile	Ilhéus - BA	-14.795269, -39.037339
MZUESC 22149	*Adenomera*	cf. *thomei*	Male	Ilhéus - BA	-14.795269, -39.037339
MZUESC 22150	*Adenomera*	cf. *thomei*	Male	Ilhéus - BA	-14.795269, -39.037339
MZUESC 22151	*Adenomera*	sp. L	Male	Igrapiúna - BA	-13.821933, -39.171175
MZUESC 22152	*Adenomera*	sp. L	Juvenile	Igrapiúna - BA	-13.821933, -39.171175
MZUESC 22153	*Adenomera*	sp. L	Male	Igrapiúna - BA	-13.821933, -39.171175
MZUESC 22154	*Adenomera*	sp. L	Female	Igrapiúna - BA	-13.821933, -39.171175
MZUESC 22155	*Adenomera*	sp. L	-	Igrapiúna - BA	-13.821933, -39.171175
MZUESC 22156	*Adenomera*	sp. L	-	Igrapiúna - BA	-13.821933, -39.171175
MZUESC 22157	*Adenomera*	sp. L	Juvenile	Igrapiúna - BA	-13.821933, -39.171175

We followed the protocol of Schmid (1978) with modifications. In the present study, a 2% colchicine solution (0.1 ml/10 g of weight) was used during 4–6 h. Subsequently, the specimens were sacrificed with lidocaine gel at a concentration of 5% spread over the entire body. The vouchers were fixed in 10% formaldehyde for 24 hours, kept in 70% alcohol, and deposited at the UESC Herpetological collection.

Chromosomal preparations were obtained from intestinal cells. The intestinal epithelium was kept in a hypotonic solution (0.075 M KCL) for 40 minutes and fixed in CARNOY solution (3:1 methanol: acetic acid). Then, the cell suspensions were placed on the surface of a slide and dried at room temperature in the dark. To determine chromosome composition and the fundamental number (FN), cells were stained with 3% Giemsa for 10 minutes. Chromosomes were classified according to Green and Sessions (1991) as metacentric (M), submetacentric (SM), subtelocentric (ST), and telocentric (T) (Table 2).

Results obtained were compared with cytogenetic data available in the literature. The images were captured and analyzed using an Olympus BX-51 microscope, a Q-Capture Pro image capture camera, and the Image Pro Plus software. We used Adobe Photoshop CC 2019 for the analysis and arrangement of the karyotype in descending order.

Table 2. *Adenomera* species with described karyotype, fundamental number and bibliographic references. Species Identification followed the taxon name used in the original contribution.

Species	Karyomorphology	Diploid number	Fundamental number	References
A. diptyx	1M+3SM+ 9T	26	FN = 34	Zaracho and Hernando 2011
A. andreae	1M+4SM+2ST, 6T	26	FN = 40	Bogart 1974
A. lutzi	-	26	NA	Bogart 1970 apud Kuramoto, 1990
A. hylaedactyla	1M+ 3SM+ 9T	26	FN = 34	Campos et al. 2009
A. hylaedactyla	1M+1SM+2ST+9T	26	FN = 36	Bogart 1974
A. marmorata	2M+1SM+2ST+7T	24	FN = 34	Bogart 1974
A. cf. *marmorata*	3M+3SM+6T	24	FN = 34	Campos et al. 2009
*A.*cf. *marmorata*	2M+3SM+7T	24	FN = 34	Campos et al. 2009
Adenomera sp. L	2M+3SM+7T	24	FN = 34	Present Study
A. cf. *thomei*	2M+3SM+7T	24	FN = 34	Present Study
A. cf. *bokermanni*	2M+3SM+1ST+4T+3NP (1M + 2T)	23	FN = 34	Campos et al. 2009

Results

We analyzed metaphases of 12 individuals of the lineages *Adenomera* sp. L (n = 6) and *Adenomera* cf. *thomei* (n = 6; sex of specimens is shown in Table 1). The karyotype of all analyzed specimens showed 2n = 24 (FN = 34) and no heteromorphic sex chromosomes. All individuals showed the karyotype 2n = 24 with a karyotypic formula of 4M + 6SM + 14T (metacentric pairs 1 and 5; submetacentric pairs 2, 3, and 4; telocentric pairs 6, 7, 8, 9, 10, 11, and 12) (Fig. 1).

The karyotypes obtained in this study and those already published for the genus *Adenomera* are shown in Table 2 with their respective diploid number, fundamental number, and karyomorphology.

Discussion

The number of cytogenetic studies on anurans has grown in recent years (e.g., Ferro et al. 2018; Gazoni et al. 2018; Marciano-Jr et al. 2021); however, information for some families and/or genera is still scarce. The genus *Adenomera* comprises common and abundant species, some of which often occur syntopically (Cassini et al. 2020), but cytogenetic data for the entire genus are still scant compared to other anuran genera. So far, only six of the 29 described species have been karyotyped. Campos (2009) analyzed four populations of *Adenomera* from the state of São Paulo and identified two species, *Adenomera marmorata* and *Adenomera* aff. *bokermanni* with distinct karyotypes. *Adenomera marmorata* shows a variation in chromosome pair 12, which is

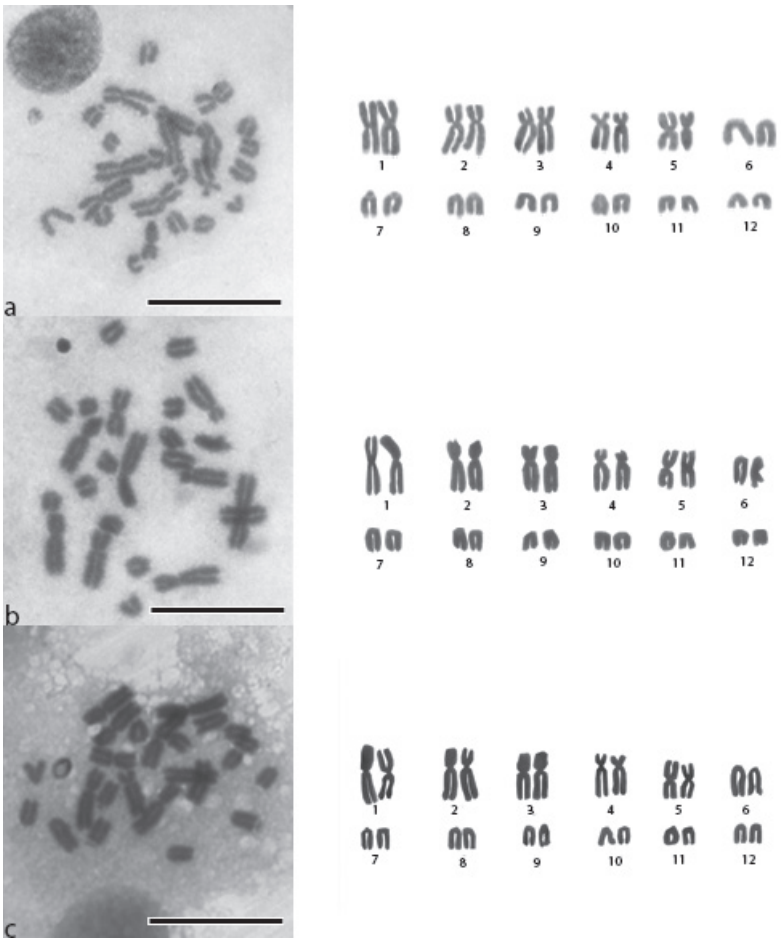

Figure 1. Karyotype of "*thomei*" clade specimens with conventional Giemsa staining **a** *Adenomera* cf. *thomei* from Ilhéus, Bahia, Brazil **b, c** *Adenomera* sp. L from Igrapiúna, Bahia, Brazil. All specimens showed the following karyomorphology: pairs 1 and 5 metacentric, 2–4 submetacentric, and 7–2 telocentric. Scale bar: 5 μm.

metacentric in the populations of the state of São Paulo. Thus, Campos et al. (2009) hypothesized that it is an interpopulation variation, which was later confirmed by Cassini et al. (2020) in a taxonomic study on the group that integrated DNA sequences, morphology, and bioacoustics. The specimen identified by Campos et al. (2009) as *A.* aff *bokermanni* was collected in the municipality of Santa Branca in the state of São Paulo, which is outside the current distribution of *A. bokermanni*, which is restricted to the southern region of the state of Paraná (Cassini et al. 2020).

The specimens analyzed in the present study were cytogenetically similar to those of *A. marmorata* and *A.* aff. *bokermanni* (Campos et al. 2009). Campos et al. (2009) found an unusual diploid number (2n = 23) when they described the karyotype of *A.* aff. *bokermanni*, Voucher - CFBH 11531, and concluded that it was most likely an indicative of a centric fusion involving the telocentric chromosome pairs 7 and 9. The authors

stated that it is not possible to determine with certainty whether the differences in chromosome pairs 7 and 9 correspond to a variation restricted to the specimen analyzed. Therefore, chromosome pairs 7 and 9 will not be used for comparison in our analyses. The chromosomes of pair 8 in the specimens analyzed in the present study are telocentric, whereas those of *A.* aff. *bokermanni* are subtelocentric (Campos et al. 2009).

Furthermore, the specimens of *Adenomera* cf. *thomei* (Ilhéus, BA) and *Adenomera* sp. L (Igrapiúna, BA) in the present study showed a karyotype (2n = 24 – FN = 34) identical to that of the specimen CFBH1512 from Santa Branca (SP) and the specimen CFBH 1713 (*Adenomera* sp. J). Moreover, no bioacoustic, molecular (DNA), or morphological data are available for the Ilhéus population and a taxonomic review including all species within the clade is needed to shed light on their specific limits.

Comparative cytogenetics can be considered an important tool for recovering phylogenetic relationships and confirming taxonomic identity (e.g., Baker 1970; Silva et al. 2004; Aguiar Jr et al. 2007; Urdampilleta et al. 2013; Cassini et al. 2020). The results presented here will contribute to expand the information on the taxonomy and phylogeny of the "*thomei*" clade and consequently lead to the delimitation of its taxa.

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References

Aguiar Jr O, Bacci Jr M, Lima AP, Rossa-Feres DDC, Haddad CFB, Recco-Pimentel SM (2007) Phylogenetic relationships of *Pseudis* and *Lysapsus* (Anura, Hylidae, Hylinae) inferred from mitochondrial and nuclear gene sequences. Cladistics 23(5): 455–463. https://doi.org/10.1111/j.1096-0031.2007.00154.x

Almeida AP, Angulo A (2006) A new species of *Leptodactylus* (Anura: Leptodactylidae) from the state of Espírito Santo, Brazil, with remarks on the systematics of associated populations. Zootaxa 1334(1): 1–25. https://doi.org/10.11646/zootaxa.1334.1.1

Angulo A (2004) The evolution of the acoustic communication system in members of the genus *Adenomera* (Anura: Leptodactylidae): A comparative approach. Doctoral thesis. University of Toronto, Toronto, 232 pp.

Angulo A, Cocroft RB, Reichle S (2003) Species identity in the genus *Adenomera* (Anura: Leptodactylidae) in southeastern Peru. Herpetologica 59(4): 490–504. https://doi.org/10.1655/20-104

Baker RJ (1970) Karyotypic trends in bats. In: Wimsatt WA (Ed.) Biology of bats. Academic Press Inc, New York, 65–97. https://doi.org/10.1016/B978-0-12-758001-2.50007-1

Bogart JP (1970) Systematic problems in the amphibian family Leptodactylidae (Anura) as indicated by karyotypic analysis. Cytogenetic and Genome Research 9(5): 369–383. https://doi.org/10.1159/000130106

Bogart JP (1974) A karyosystematic study of frogs in the genus *Leptodactylus* (Anura: Leptodactylidae). Copeia 3: 728–737. https://doi.org/10.2307/1442686

Campos RC, Ananias JF, Aguirre BC, Yamamoto M, Haddad CFB, Kasahara S (2009) Chromosome evolution in three Brazilian *Leptodactylus* species (Anura, Leptodactylidae), with phylogenetic considerations. Hereditas 146(2): 104–111. https://doi.org/10.1111/j.1601-5223.2009.02100.x

Carvalho TR, Moraes LJ, Lima AP, Fouquet A, Peloso PL, Pavan D, Drummond LO, Rodrigues MT, Giaretta AA, Gordo A, Neckel-Oliveira S, Haddad CF (2021) Systematics and historical biogeography of Neotropical foam-nesting frogs of the *Adenomera heyeri* clade (Leptodactylidae), with the description of six new Amazonian species. Zoological Journal of the Linnean Society 191(2): 395–433. https://doi.org/10.1093/zoolinnean/zlaa051

Cassini CS, Taucce PP, Carvalho TR, Fouquet A, Solé M, Haddad CF, Garcia PC (2020) One step beyond a broad molecular phylogenetic analysis: Species delimitation of *Adenomera marmorata* Steindachner, 1867 (Anura: Leptodactylidae). PloS ONE 15(2): e0229324. https://doi.org/10.1371/journal.pone.0229324

De Sá RO, Grant T, Camargo A, Heyer WR, Ponssa ML, Stanley E (2014) Systematics of the neotropical genus *Leptodactylus* Fitzinger, 1826 (Anura: Leptodactylidae): phylogeny, the relevance of non-molecular evidence, and species accounts. South American Journal of Herpetology 9(s1). https://doi.org/10.2994/SAJH-D-13-00022.1

Duellman WE (2005) Cusco Amazónico. The Lives of Amphibians and Reptiles in an Amazonian Rainforest. Cornell University Press, Ithaca, [xv +] 433 pp.

Ferro JM, Taffarel A, Cardozo D, Grosso J, Puig MP, Suárez P, Akmentins MS, Baldo D (2016) Cytogenetic characterization and B chromosome diversity in direct-developing frogs of the genus *Oreobates* (Brachycephaloidea, Craugastoridae). Comparative Cytogenetics 10(1): 141–156. https://doi.org/10.3897/CompCytogen.v10i1.5718

Fouquet A, Cassini SC, Haddad FBC, Pech N, Trefaut RM (2014) Species delimitation, patterns of diversification and historical biogeography of the neotropical frog genus *Adenomera* (Anura, Leptodactylidae). Journal of Biogeography 41(5): 855–870. https://doi.org/10.1111/jbi.12250

Gazoni T, Haddad CFB, Narimatsu H, Cabral-De-Mello DC, Lyra ML, Parise-Maltempi PP (2018) More sex chromosomes than autosomes in the Amazonian frog *Leptodactylus pentadactylus*. Chromosoma 127(2): 269–278. https://doi.org/10.1007/s00412-018-0663-z

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

Kuramoto M (1990) A list of chromosome numbers of anuran amphibians. Bulletin of Fukuoka University of Education 39: 83–127.

Lourenço LB, Bacci-Júnior M, Martins VG, Recco-Pimentel SM, Haddad CFB (2008) Molecular phylogeny and karyotype differentiation in *Paratelmatobius* and *Scythrophrys* (Anura, Leptodactylidae). Genetica 132(3): 255–266. https://doi.org/10.1007/s10709-007-9169-y

Lourenço LB, Targueta CP, Baldo D, Nascimento J, Garcia PC, Andrade GV, Haddad CFB, Recco-Pimentel SM (2015) Phylogeny of frogs from the genus *Physalaemus* (Anura, Leptodactylidae) inferred from mitochondrial and nuclear gene sequences. Molecular Phylogenetics and Evolution 92: 204–216. https://doi.org/10.1016/j.ympev.2015.06.011

Marciano-Jr E, Tanajura G, Costa MA, Orrico VG, Dias IR (2021) Advertisement call and cytogenetics of *Xenohyla eugenioi* Caramaschi, 1998 (Amphibia; Anura; Hylidae), an enigmatic frog from northeastern Brazil. South American Journal of Herpetology 20(1): 17–23. https://doi.org/10.2994/SAJH-D-18-00035.1

Menin M, Waldez F, Lima AP (2008) Temporal variation in the abundance and number of species of frogs in 10,000 ha of a forest in Central Amazonia, Brazil. South American Journal of Herpetology 3(1): 68–81. https://doi.org/10.2994/1808-9798(2008)3[68:TVITAA]2.0.CO;2

Pyron RA, Wiens JJ (2011) A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Molecular phylogenetics and evolution 61(2): 543–583. https://doi.org/10.1016/j.ympev.2011.06.012

Silva APZ, Garcia P, Martins V, Bacci M, Kasahara S (2004) Chromosomal and molecular analyses of *Leptodactylus gracilis gracilis*, *L. gracilis delattini*, and *L. plaumanni* (Anura, Leptodactylidae): taxonomic implications. Amphibia-Reptilia 25(2): 185–196. https://doi.org/10.1163/1568538041231193

Schmid M (1978) Chromosome banding in Amphibia. Chromosoma 66(4): 361–388. https://doi.org/10.1007/BF00328536

Suárez P, Cardozo D, Baldo D, Pereyra MO, Faivovich J, Orrico VGD, Catroli GF, Grabiele M, Bernarde PS, Nagamachi CY, Haddad CFB, Pieczarka JC (2013) Chromosome evolution in Dendropsophini (Amphibia, Anura, Hylinae). Cytogenetic and Genome Research 141(4): 295–308. https://doi.org/10.1159/000354997

Targueta CP, Rivera M, Souza MB, Recco-Pimentel SM, Lourenço LB (2010) Cytogenetic contributions for the study of the Amazonian *Engystomops* (Anura; Leiuperidae) assessed in the light of phylogenetic relationships. Molecular Phylogenetics and Evolution 54(3): 709–725. https://doi.org/10.1016/j.ympev.2009.10.018

Urdampilleta JD, Coulleri JP, Ferrucci MS, Forni-Martins ER (2013) Karyotype evolution and phylogenetic analyses in the genus *Cardiospermum* L. (Paullinieae, Sapindaceae). Plant Biology 15(5): 868–881. https://doi.org/10.1111/j.1438-8677.2012.00679.x

Zaracho VH, Hernando AB (2011) The karyotype of *Adenomera diptyx* (Boettger 1885) (Anura, Leptodactylidae) from northeastern Argentina. Genetics and Molecular Biology 34(1): 84–87. https://doi.org/10.1590/S1415-47572010005000116

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RESEARCH ARTICLE

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Karyotype and COI gene sequences of *Chironomus melanotus* Keyl, 1961 from the Yaroslavl region, Russia, and the difficulties with its identification using GenBank and BOLD systems

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Abstract

Karyotype and *COI* gene sequences of *Chironomus melanotus* Keyl, 1961 from the Yaroslavl region (Russia) were analyzed. A low level of chromosomal polymorphism has been confirmed, eventually eight banding sequences were found: melA1, melB1, melC1, melD1, melE1, melF1, and melG1; only melD2 was found in two larvae from the Sunoga river. Analysis of phylogenetic tree and estimated genetic distances has shown not all *COI* gene sequences of *Ch. melanotus* in GenBank and BOLD to belong to this species. The lower distance of 0.4% was observed between two sequences from the Yaroslavl region and Finland, apparently these are true *Ch. melanotus* sequences. The distances between true *Ch. melanotus* and other sequences from Finland were 9.5% and 12.4%, and from Sweden it was 11%. The average genetic distance between studied sequences of 9.1% is out of the range of the 3% threshold previously determined for chironomids. According to our estimates, there are two sequences with a distance of 2.9% that may belong to *Ch. annularius* Meigen, 1818, and one sequence with a genetic distance of 2.1%, may belonging to *Ch. cingulatus* Meigen, 1830, which has been confirmed karyologically. Another two sequences form a separate cluster. We suggest that they either belong to a known species, but are not present in the databases, or belong to a distinct, undescribed species.

Keywords

Chironomidae, *Chironomus melanotus*, *COI*, dark taxa, DNA-barcode, karyotype

Introduction

Chironomus melanotus Keyl, 1961 is one of the most widespread and well-known species. It does not belong to any sibling species group (Kiknadze et al. 2010). The first finding and description was in Germany (Keyl 1961, 1962; Degelmann et al. 1979). *Ch. melanotus* is in demand in classical hydrobiology (Fjellheim, Raddum 1996) and in toxicology (Grebenjuk 1994; Grebenjuk and Tomilina 2014). The main problem in the investigation of *Chironomus* Meigen, 1803 is the difficulties with the species identification by larval morphology. Due to the presence of the giant chromosomes in the salivary gland of *Chironomus* larvae, it is more convenient to identify cytogenetically (Kiknadze et al. 1991, 2016). The karyotype of *Ch. melanotus* was described by Keyl (1961) as a "cytospecies" that belongs to "thummi" cytocomplex and mapped chromosomal arms A and F (Keyl, 1962). It has been shown that the level of polymorphism in *Ch. melanotus* is very low (Wülker 1973; Kiknadze et al. 1991; Jabłońska-Barna et al. 2013). Only in polluted water bodies a high spectrum of somatic rearrangements and a case of trisomy were observed (Jabłońska-Barna et al. 2013). Finally, due to the development of new techniques in molecular biology, for species identification/delimitation the fast and cost-effective technology DNA barcoding is commonly used and for massive analysis in biomonitoring metabarcoding is used. In recent years, many works on this theme have been published. This is a barcoding of invertebrates, including chironomids from Canada (Hebert et al. 2016), Germany (Morinière et al. 2019), Finland (Roslin et al. 2022), South Korea (Kang et al. 2022), Montenegro/Albania (Gadawski et al. 2022) and others. The disadvantage of this approach is the presence in the databases of genetic information (GenBank and BOLD) from unidentified or incorrectly identified specimens, so-called "dark taxa" (Morinière et al. 2019). The next problem is the understanding that a sequence divergence threshold is not suitable for all *Chironomus* species and depends on intraspecific and interspecific sequence divergences. Interspecific - varied for *COI* gene sequences in most cases from 9 to 20% and in rare cases from 1 to 4% (Proulx et al. 2013). Due to the fact that we cannot fully estimate intra- and inter-specific sequence divergences, here we will use the average value of this parameter – 3% (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016).

We could not find any studies of *Ch. melanotus* involving approaches of morphology, cytogenetics and DNA barcoding published in one article. In the GenBank and BOLD databases were found five and one *COI* gene sequences, respectively. These sequences were obtained from individuals collected in Finland and Sweden, and deposited during the preparation of this paper (Roslin et al. 2022). Preliminary examination has shown that not all of these sequences belong to *Ch. melanotus*.

The present study aims to calculate and compare the genetic distances between *COI* gene sequences of *Ch. melanotus* from Yaroslavl region identified by morphology and cytogenetics and the sequences obtained from GenBank and BOLD of *Ch. melanotus* from different populations identified by morphology or molecular-genetics (barcode), and additional sequences from GenBank and BOLD of several *Chironomus* identified by cytogenetics.

Materials and methods

Fourth instar larvae of *Ch. melanotus* were collected from a few places in the Yaroslavl region, Russia. Thirty-one larvae were found in a puddle on the Shumarovka river shore (58°02'25.5"N, 38°15'33.2"E) in October 2018. The depth is 0.5 m, and the bottom is black silt. Seven larvae were collected in the Sunoga river (58°03'20.3"N, 38°14'04.2"E) in August 2018. The depth is 0.1 – 0.2 m, and the bottom is gray silt with sand. Four larvae were collected in a small stream (brook) in the shore zone of the Kotorosl' river (57°22'41.6"N, 39°50'08.5"E) in June 2016. The depth is 0.5 m, and the bottom is black silt and rotting wood.

The age was determined by the standard method (Ilyinskaya, 1983). All larvae were taken for karyotype analysis using the ethanol-orcein technique (Dyomin 1989). A Micromed-6C (LOMO, St. Petersburg) light microscope equipped with a standard (kit) oil objective ×100 and a camera ToupCam5.1 (China) were used for microscopy analysis. To identify chromosome banding sequences, the cytomaps by Kiknadze et al. (1991, 2016), Keyl (1961, 1962), Hirvenoja and Michailova (1991) were used. Preparations of *Ch. melanotus* have been deposited at IBIW RAS.

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

For alignment of *COI* nucleotide sequences, we used MUSCLE in the MEGA6 software (Tamura et al. 2013). The MEGA6 was used to calculate pairwise genetic distances (p-distance) with codon position preferences: 1st, 2nd, 3rd and noncoding sites. The Bayesian analysis was performed using the program MrBayes v.3.2.6 (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) with settings suggested by Karmokov (2019; Bolshakov, Prokin 2021), for 1 000 000 iterations and 1000 iterations of burn-in, nst = 6 (GTR + I + G). The phylogenetic trees resulting in Bayesian inference analyses were visualized and edited using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

In addition, thirty-four *COI* gene sequences of the genus *Chironomus* from "GenBank" and "Barcode of Life Data Systems" (BOLD) were analyzed. Accession numbers of used sequences in GenBank and BOLD: *Ch. acutiventris* Wülker et al., 1983 (AF192200.1), *Ch. annularius* Meigen, 1818 (AF192189.1), *Ch. anthracinus*

Zetterstedt, 1860 (KF278222), *Ch. balatonicus* Devai et al., 1983 (JN016826.1), *Ch. bernensis* Wülker et Klötzli, 1973 (AF192188.1), *Ch. borokensis* Kerkis et al., 1988 (AB740261), *Ch. cingulatus* Meigen, 1830 (AF192191.1), *Ch. commutatus* Keyl, 1960 (AF192187.1), *Ch. curabilis* et al., 1990 (JN016810.1), *Ch. dilutus* et al., 1999 (KF278335.1), *Ch. entis* Shobanov, 1989 (KM571024.1), *Ch. heterodentatus* Konstantinov, 1956 (AF192199.1), *Ch. heteropilicornis* Wülker, 1996 (MK795770.1), *Ch. maturus* Johannsen, 1908 (DQ648204.1), *Ch. melanotus* (MZ659620, MZ657748, MZ658877, MZ657558, MZ658420, BSCHI737-17), *Ch. nipponensis* Tokunaga, 1940 (DQ648206), *Ch. novosibiricus* Kiknadze et al., 1993 (AF192197.1), *Ch. nuditarsis* Keyl, 1961 (KY225345.1), *Ch. obtusidens* Goetghebuer, 1921 (CHMNO207-15*), *Ch. piger* Strenzke, 1959 (AF192202.1), *Ch. pilicornis* Fabricius, 1787 (HM860166.1), *Ch. plumosus* Linnaeus, 1758 (KF278217.1), *Ch. riparius* Meigen, 1804 (KR756187.1), *Ch. sokolovae* Istomina et al., 1999 (MW471100), *Ch. sororius* Wulker, 1973 (MZ324811), *Ch. tentans* Fabricius, 1805 (AF110157.1), *Ch. tuvanicus* Kiknadze et al., 1993 (AF192196.1), *Ch. usenicus* Loginova et Belyanina, 1994 (JN016820.1), *Ch. whitseli* Sublette et Sublette, 1974 (KR683438.1). The *COI* sequence of *Drosophila melanogaster* Meigen, 1830 (HQ551913) was used as outgroup in phylogenetic analysis.

Results and discussion

The karyotype of *Chironomus melanotus* Keyl, 1961 from the Yaroslavl region, Russia

The species has a 2n = 8 set of chromosomes. By the chromosome arm combination – AB, CD, EF and G, the species belongs to *Chironomus* "thummi" cytocomplex. The chromosomes AB and CD are metacentric, EF is submetacentric, and G is telocentric. The nucleus and Balbiani ring were found in arm G. The peculiarity of the karyotype of *Ch. melanotus* is a heterochromatinized centromeric region that forms an unstable chromocenter (Fig. 1), also observed only in *Ch. cucini* Webb, 1969, *Ch. pilicornis*, *Ch. athalassicus* Cannings 1975, *Ch. magnus* White et Ramsey, 2015 and *Ch. hyperboreus* Staeger, 1845 (Wülker and Butler 1983; Int Panis et al. 1994; Kiknadze and Istomina 2000; Wülker and Martin 2000; Kiknadze et al. 2010).

We found two zygotic combinations: melA1.1. B1.1. C1.1. D1.1. E1.1. F1.1. G1.1, and melA1.1. B1.1. C1.1. D1.2. E1.1. F1.1. G1.1, which was found only in two larvae from the Sunoga river.

All eight banding sequences coincide with banding sequences in Keyl et al. (1961, 1962), Hirvenoja and Michailova (1991) and Kiknadze et al. (1991, 2016).

Arm A. One banding sequence: melA1 1a-2c 10a-12c 3i-2h 4d-9e 2g-d 4c-a 13a-19f C.

Arm B. One banding sequence: melB1 28-27-26-25-24-23-22-21-20-19 C (mapped according to Hirvenoja, Michailova 1991). Different from *Ch. plumosus* by four inversion steps.

Arm C. One banding sequence: melC1 1a-2e 15c-e 11c-8a 6b-2f 15b-11d 6gh 17a-16a 7d-a 6f-c 17b-22g C.

Arm D. Two banding sequences: melD1 1a-3g 11a-13a 16a-e 8a-10a 13b-15e 10e-b 4a-7g 17a-24g C in heterozygous state with melD2 1a-3g 11a-13a 16a-e 8a-9e 7g-4a 10b-e 15e-13b 10a 17a-24g C.

Arm E. One banding sequence: melE1 1a-3e 5a-10b 4h-3f 10c-13g C.

Arm F. One banding sequence: melF1 1a-8f 9a-11i 12a-15i 16a-23f C.

Arm G. One banding sequence: melG1. Not mapped.

The level of polymorphism in *Ch. melanotus* is known to be very low (Kiknadze et al. 1991; Jabłońska-Barna et al. 2013). At the moment, we know of two alternative banding sequences, melB2 and melD2, and the sequence melD2 is more typical for Western Siberia populations (Kiknadze et al. 2016) and a Finnish population (Hirvenoja et Michailova 1991). We found melD2 in a heterozygous state in two larvae from the Sunoga river. Any deviations in the karyotype characteristics like a trisomy, rearrangements, insertions and deletions, more typical for polluted water bodies (Jabłońska-Barna et al. 2013) were not found.

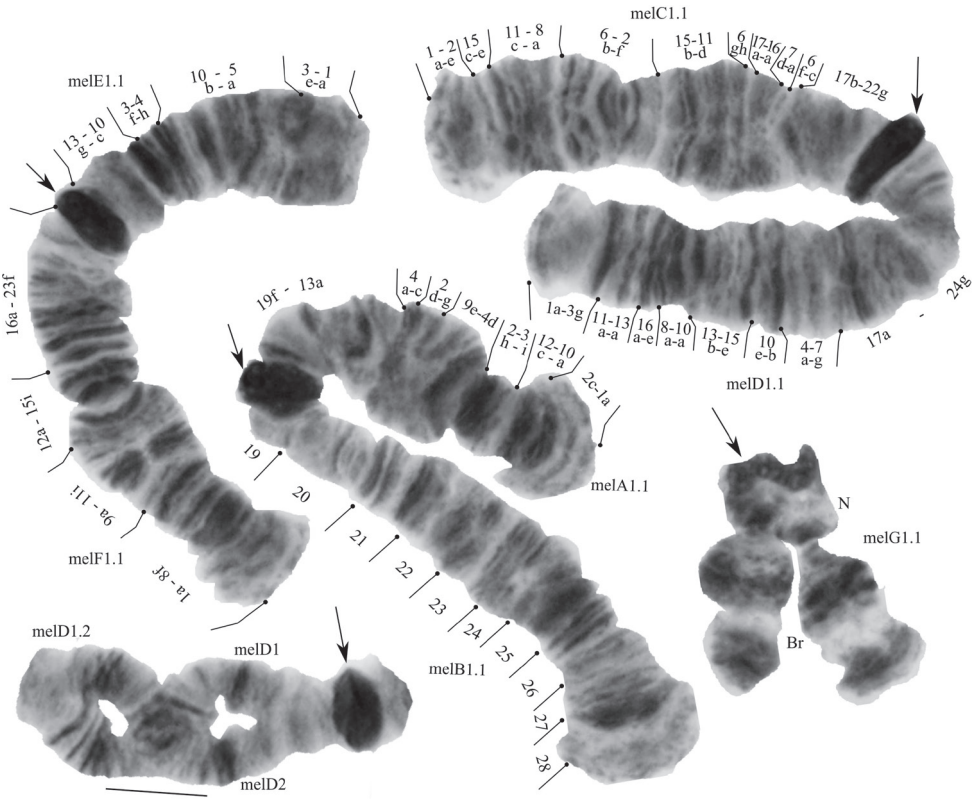

Figure 1. Karyotype of *Chironomus melanotus* from the Sunoga river, Yaroslavl, Russia. Arrows indicate centromeric bands, melA1, melB1 and etc. – genotypic combinations of banding sequences in chromosome arms, BR – Balbiani rings, N – nucleous.

DNA-barcoding and phylogenetic analysis

The obtained *COI* gene sequence for *Ch. melanotus* from the Yaroslavl region was deposited in the GenBank with accession number OL546775; the length of the sequence is 658 bp (percentage of nucleotides A: 25; T: 38; G: 17; C: 19).

More interesting was the analysis of *COI* gene sequences. As was said previously, for the species name *Ch. melanotus* in the databases match six sequences of the *COI* gene from Finland (MZ659620, MZ657748, MZ658877, MZ657558, MZ658420) identified by molecular-genetics and Sweden (BSCHI737-17) identified by imago characters, and the average genetic distance between them of 9.1% is out of the range of 3% distances previously determined for chironomids (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016). Low chromosomal variability of *Ch. melanotus* does not allow us to talk about a high level of genetic diversity. We can conclude not all sequences belong to *Ch. melanotus* species (Table 1). According to our estimation, the lower distance, about 0.4%, was between *Ch. melanotus* (MZ659620) from Finland and Yaroslavl reg. (OL546775). The distance between sequences of *Ch. melanotus* (OL546775) from Yaroslavl reg. and sequences from Finland (MZ657748, MZ658877) - 9.5%, (MZ657558, MZ658420) - 12.4%, and Sweden (BSCHI737-17) - 11%. These values are greater than those between sequences from the Yaroslavl reg. (OL546775) and *Ch. anthracinus* (KF278222), identified karyologically (Proulx et al. 2013), with a distance of 4%. This still doesn't mean these species are really closely related, the analysis of one short segment of the *COI* gene is not enough to make such conclusions (DeSalle et al. 2005). However, a high similarity of their karyotypes has been noted, up to identity of some banding sequences (Keyl 1962; Kiknadze et al. 1991).

The distance between the two similar sequences (MZ657558 and MZ658420) from Finland and *Ch. annularius* (AF192189.1) confirmed karyologically (Guryev et al. 2001) was 2.9%; between sequences (BSCHI737-17) from Sweden and *Ch. cingulatus* (AF192191.1) confirmed karyologically (Guryev et al. 2001) - 2.1%, *Ch. nipponensis* (DQ648206) - 4.2%, identified by morphology and molecular-genetics (Kondo et al. 2016). Two similar sequences are particularly interesting (MZ657748 and MZ658877), the distances between of them and all the analyzed sequences varied from 6.5 to 10.5%, and the average was 12%. Unfortunately, we didn't find any matches in GenBank and BOLD.

On the phylogenetic tree constructed by Bayesian inference (Fig. 2), we see the same situation as with the genetic distances. The sequence of *Ch. melanotus* (OL546775) from Yaroslavl reg., and *Ch. melanotus* (MZ659620) from Finland combined into one cluster, while the other sequences spread out into different branches. Two similar sequences (MZ657558 and MZ658420) from Finland and *Ch. annularius* (AF192189.1) combined in one cluster, with a support value of 1.0. The sequence (BSCHI737-17) from Sweden and *Ch. cingulatus* (AF192191.1) formed another cluster, with a support value of 1.0. Two similar sequences from Finland (MZ657748 and MZ658877) have formed a separate cluster, without including any other specimens.

Table 1. Pairwise genetic distances (p-distances, %) between *COI* gene sequences of *Ch. melanotus* and closest sequences of *Chironomus* from GenBank and BOLD.

	Ch. melanotus OL546775 Yaroslavl, RUS	*Ch. melanotus* MZ659620 Finland	*Ch. melanotus* MZ657748 Finland	*Ch. melanotus* MZ658877 Finland	*Ch. melanotus* BSCHI737-17 Sweden	*Ch. melanotus* MZ657558 Finland	*Ch. melanotus* MZ658420 Finland	*Ch. anthracinus* KF278222	*Ch. annularius* AF192189.1	*Ch. cingulatus* AF192191.1
Ch. melanotus MZ659620 Finland	0,4									
Ch. melanotus MZ657748 Finland	9,5	9,7								
Ch. melanotus MZ658877 Finland	9,5	9,7	0,0							
Ch. melanotus BSCHI737-17 Sweden	11,0	11,4	7,0	7,0						
Ch. melanotus MZ657558 Finland	12,4	12,6	10,7	10,7	11,2					
Ch. melanotus MZ658420 Finland	12,4	12,6	10,7	10,7	11,2	0,4				
Ch. anthracinus KF278222	4,0	3,6	10,5	10,5	11,6	12,0	12,4			
Ch. annularius AF192189.1	12,6	12,8	9,7	9,7	10,1	2,9	2,9	12,6		
Ch. cingulatus AF192191.1	10,9	11,2	7,2	7,2	2,1	11,0	11,0	11,8	10,1	
Ch. nipponensis DQ648206	10,3	10,7	6,5	6,5	4,2	11,4	11,4	11,2	9,9	4,8

All the obtained data show that several species are hidden in GenBank and BOLD under the name "*Chironomus melanotus*". First, there is a true *Ch. melanotus* cluster (Fig. 2) (MZ659620 and OL546775) the reliability of which is confirmed by karyotype analysis. Probably, two similar sequences (MZ657558 and MZ658420) belong to *Ch. annularius* (AF192189.1), and the genetic distance of 2.9% is very close to 3% accepted interspecific threshold (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016), but does not exceed it. Another sequence (BSCHI737-17), with a genetic distance of 2.1%, likely belongs to *Ch. cingulatus* (AF192191.1).

Two similar sequences (MZ657748 and MZ658877) need special attention. The samples of *Ch. melanotus* from Finland were investigated during the project of FinBOL (Finnish Barcode Of Life), in the framework of which the authors tested the system FinPROTAX (Probabilistic Taxonomic Assignment Tool) (Roslin et al. 2022). As the authors report, the accuracy of taxonomic assignments at the level of species reached 88.5% (Roslin et al. 2022). Such precision is still insufficient, especially in a group rich in sibling species. This approach does not consider estimate intra- and inter-specific sequence divergences. For the *COI* gene, the estimated interspecific sequence divergences in most cases varied from 9 to 20%, but in a few cases with well-identified by cytogenetics species, this

parameter varied from 1 to 4%, which overlapped between intraspecific and interspecific sequence divergences (Proulx et al. 2013). According to Morinière et al. (2019), the database of genetic information contains about 65% of sequences without species-level assignment, so-called "dark taxa" of all Chironomidae recorded from Germany. But we think that "superficial taxonomic impediment" (species are so poorly and unreliably named, they will need to be redescribed before they can be used) (Meier et al. 2022) is better applicable in this case. Thus, we can conclude that two sequences (MZ657748 and MZ658877) belong to well-known species that are absent in databases, or they can be considered as distinct species. A similar case was with Japanese *Ch. nipponensis*. At first, Yamamoto (2010, cited by Kondo et al. 2016) proposed dividing the "highland" and "lowland" populations of *Ch. nipponensis* by morphology, then Kondo et al. (2016) revealed the genetic distances between them at 9.1%, and confirmed the presence of two separate species.

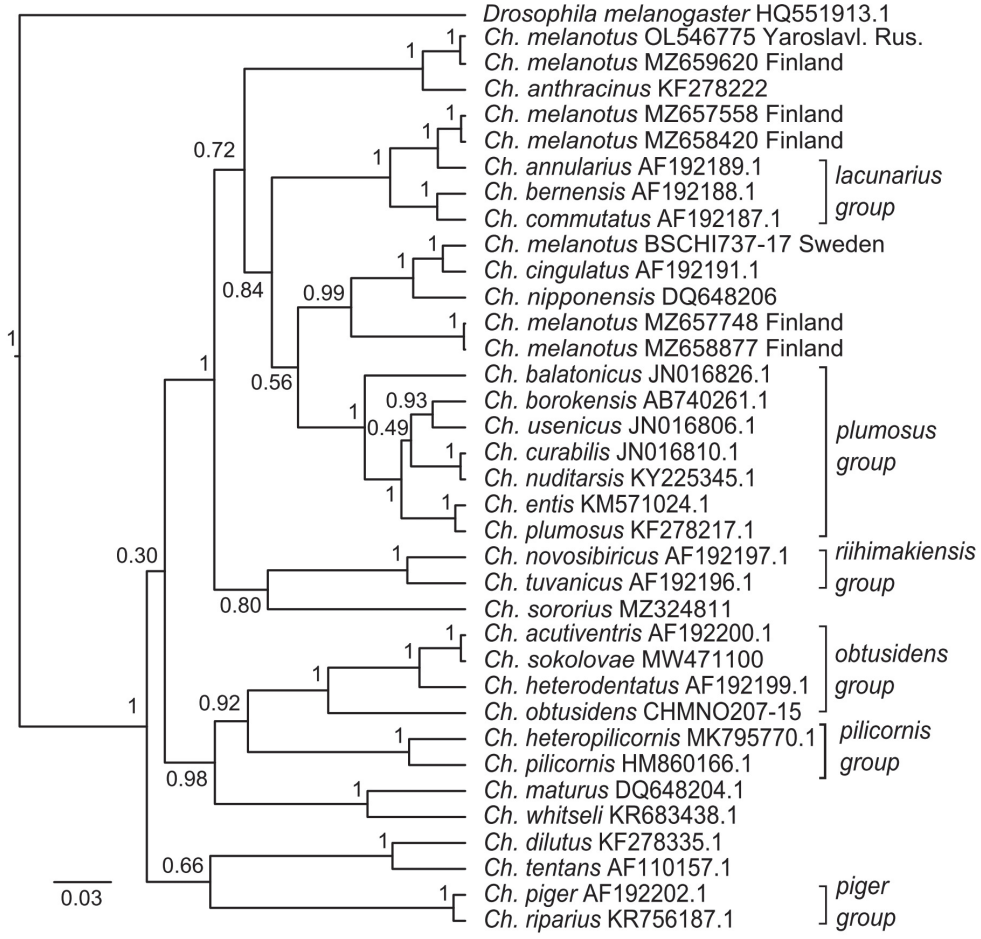

Figure 2. Bayesian tree of the analyzed samples of *Chironomus* spp. inferred from COI sequences. Species name, GenBank accession numbers and group name are shown to the right of the branches. Support values are given if they exceed 0.3. The numbers at the nodes indicate posterior probabilities.

Conclusions

On the example of *Ch. melanotus*, we confirmed that in *Chironomus* species identification we must use all available comprehensive approaches, involving morphological, cytogenetic and molecular genetic studies (mitochondrial and nuclear genes) (DeSalle et al. 2005; Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016).

At least four species of *Chironomus* could be in the databases under the name "*Ch. melanotus*" from Finland and Sweden. This suggests that at the present stage of the collection of genetic data, it is impossible to trust only a computer algorithm. We agree with Zamani et al. (2022) that the use of DNA-based analyses for an initial sorting of new and known species is extremely useful as a first step, which significantly narrows the range of search before precise species identification. Nevertheless, despite the difficulties, the species identification of *Chironomus* greatly enriches our understanding of ecosystem functioning because this is an important part of it.

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References

Bolshakov VV, Prokin AA (2021) Karyotype and COI sequences of *Chironomus sokolovae* Istomina, Kiknadze et Siirin, 1999 (Diptera, Chironomidae) from the bay of Orkhon River, Mongolia. Comparative Cytogenetics 15(2): 149–157. https://doi.org/10.3897/CompCytogen.v15.i2.66549

Degelmann A, Roye HD, Hollenberg CP (1979) The organization of the ribosomal RNA genes of *Chironomus tentans* and some closely related species. Chromosoma 71(3): 263–281. https://doi.org/10.1007/BF00287136

DeSalle R, Egan MG, Siddall M (2005) The unholy trinity: taxonomy, species delimitation and DNA barcoding. Philosophical Transactions of the Royal Society B: Biological Sciences 360: 1905–1916. https://doi.org/10.1098/rstb.2005.1722

Dyomin SYu (1989) Variability of the degree of condensation of polytene chromosomes in the cells of different organs of *Chironomus plumosus* larvae from nature. PhD Thesis, Institute of Cytology of the USSR Academy of Sciences, Leningrad, 25 pp. [In Russian]

Ekrem T, Willassen E, Stur E (2007) A comprehensive DNA sequence library is essential for identification with DNA barcodes. Molecular Phylogenetics and Evolution 43(2): 530–542. https://doi.org/10.1016/j.ympev.2006.11.021

Fjellheim A, Raddum GG (1996) Weir building in a regulated west Norwegian river: Long-term dynamics of invertebrates and fish. Regulated Rivers: Research and Management 12(4–5): 501–508. https://doi.org/10.1002/(sici)1099-1646(199607)12:4/5<501::aid-rrr414>3.0.co;2-f

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

Gadawski P, Montagna M, Rossaro B, Giłka W, Pešić V, Grabowski M, Magoga G (2022) DNA barcoding of Chironomidae from the Lake Skadar region: Reference library and a comparative analysis of the European fauna. Diversity and Distributions: 1–20. https://doi.org/10.1111/ddi.13504

Grebenjuk LP (1994) Qualitative and quantitative composition of chironomids of the Latka River, a small tributary of the Rybinsk Reservoir. Biologiya vnutrennikh vod: Informatsionnyi byulleten 96: 40–46. [In Russian]

Grebenjuk LP, Tomilina II (2014) Morphological deformations of hard-chitinized mouthpart structures in larvae of the genus *Chironomus* (Diptera, Chironomidae) as the index of organic pollution in freshwater ecosystems. Inland Water Biology 7(3): 273–285. https://doi.org/10.1134/S1995082914030092

Guryev V, Makarevitch I, Blinov A, Martin J (2001) Phylogeny of the genus *Chironomus* (Diptera) inferred from DNA sequences of mitochondrial Cytochrome b and Cytochrome oxidase I. Molecular Phylogenetics and Evolution 19: 9–21. https://doi.org/10.1006/mpev.2001.0898

Hebert PDN, Ratnasingham S, Zakharov EV, Telfer AC, Levesque-Beaudin V, Milton MA, Pedersen S, Jannetta P, DeWaard JR (2016) Counting animal species with DNA barcodes: Canadian insects. Philosophical Transactions of the Royal Society B: Biological Sciences 371: 20150333. https://doi.org/10.1098/rstb.2015.0333

Hirvenoja M, Michailova P (1991) *Chironomus melanotus* Keyl and its taxonomical and ecological relations to *C. riihimakiensis* Wülker (Diptera, Chironomidae). Spixiana 14(3): 309–320.

Ilyinskaya NB (1983) The development of 4th instar larvae and diapause. In: Sokolova NYu (Ed.) *Chironomus plumosus* L. (Diptera, Chironomidae). Systematics, Morphology, Ecology, Production. Nauka publishers, Moscow, 167–188. [In Russian]

Int Panis L, Kiknadze II, Bervoets L, Aimanova K (1994) Karyological identification of some species of the genus *Chironomus* Meigen 1803 from Belgium. Bulletin & Annales de la Société Royale d'Entomologie de Belgique 130: 135–142.

Jabłońska-Barna I, Szarek-Gwiazda E, Michailova P (2013) Environmental agents in Lake Łuknajno (Poland) affecting the genome of *Chironomus melanotus* Keyl, 1961 (Diptera, Chironomidae) - A new species of Polish fauna. Oceanological and Hydrobiological Studies 42(2): 164–172. https://doi.org/10.2478/s13545-013-0070-2

Kang HJ, Baek MJ, Kang JH, Bae YJ (2022) Diversity and DNA Barcode Analysis of Chironomids (Diptera: Chironomidae) from Large Rivers in South Korea. Insects 13: 1–14. https://doi.org/10.3390/insects13040346

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

Keyl H-G (1961) Die cytologische Diagnostik der Chironomiden III. Diagnose yon *Chironomus parathummi* n. sp. und Ergfinzungen zur Bestimmungstabelle. Archiv für Hydrobiologie 58: 1–6.

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

Kiknadze II, Shilova AI, Kerkis IE, Shobanov NA, Zelentsov NI, Grebenyuk LP, Istomina AG, Prasolov VA (1991) Karyoypes and larval morphology in tribe Chironomini. Atlas. Nauka Publish, Novosibirsk, 115 pp. [In Russian]

Kiknadze II, Butler MG, Gunderina LI, Istomina AG, Gusev VD, Nemytikova LA (2010) Chromosomal Evolution of Nearctic and Palearctic *Chironomus* Species (Diptera: Chironomidae). Proceedings of the XV international Symposium on Chironomidae, Chironomidae Research Group, University of Minnesota, Saint Paul, Minnesota, 203–221.

Kiknadze II, Istomina AG (2000) Karyotypes and Chromosomal Polymorphisms in Siberian Chironomids (Diptera, Chironomidae). Sibirskiy ekologicheskiy zhurnal 4: 445–460. [In Russian]

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

Kondo NI, Ueno R, Ohbayashi K, Golygina VV, Takamura K (2016) DNA barcoding supports reclassification of Japanese *Chironomus* species (Diptera: Chironomidae). Entomological Science 19(4): 337–350. https://doi.org/10.1111/ens.12212

Meier R, Blaimer BB, Buenaventura E, Hartop E, Rintelen T, Srivathsan A, Yeo D (2022) A re-analysis of the data in Sharkey et al.'s (2021) minimalist revision reveals that BINs do not deserve names, but BOLD Systems needs a stronger commitment to open science. Cladistics 38: 264–275. https://doi.org/10.1111/cla.12489

Morinière J, Balke M, Doczkal D, Geiger MF, Hardulak LA, Haszprunar G, Hausmann A, Hendrich L, Regalado L, Rulik B, Schmidt S, Wägele J-W, Hebert PDN (2019) A DNA barcode library for 5,200 German flies and midges (Insecta: Diptera) and its implications for metabarcoding-based biomonitoring. Molecular Ecology Resources 19(4): 900–928. https://doi.org/10.1111/1755-0998.13022

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

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

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

Roslin T, Somervuo P, Pentinsaari M, Hebert PDN, Agda J, Ahlroth P, Anttonen P, Aspi J, Blagoev G, Blanco S, Chan D, Clayhills T, deWaard J, deWaard S, Elliot T, Elo R, Haapala S, Helve E, Ilmonen J, Hirvonen P, Ho C, Itämies J, IvanovV, Jakovlev J, Juslén A, Jussila R, Kahanpää J, Kaila L, Kaitila J-P, Kakko A, Kakko I, Karhu A, Karjalainen S, Kjaerandsen J, Koskinen J, Laasonen EM, Laasonen L, Laine E, Lampila P, Levesque-Beaudin V, Lu L, Lähteenaro M, Majuri P, Malmberg S, Manjunath R, Martikainen P, Mattila J, McKeown J, Metsälä P, Miklasevskaja M, Miller M, Miskie R, Muinonen A, Mukkala V-M, Naik S, Nikolova N, Nupponen K, Ovaskainen O, Österblad I, Paasivirta L, Pajunen T, Parkko P, Paukkunen J, Penttinen R, Perez K, Pohjoismäki J, Prosser S, Raekunnas M, Rahulan M, Rannisto M, Ratnasingham S, Raukko P, Rinne A, Rintala T, Romo SM, Salmela J, Salokannel J, Savolainen R, Schulman L, Sihvonen P, Soliman D,Sones J, Steinke C, Ståhls G,Tabell J, Tiusanen M,Várkonyi G, Vesterinen EJ, Viitanen E, Vikberg V, Viitasaari M, Vilen J, Warne C, Wei C, Winqvist K, Zakharov E, Mutanen M (2022) A molecular-based identification resource for the arthropods of Finland. Molecular Ecology Resources 22(2): 803–822. https://doi.org/10.1111/1755-0998.13510

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

Zamani A, Fric ZF, Gante HF, Hopkins T, Orfinger AB, Scherz MD, Bartoňová AS, Pos DD (2022) DNA barcodes on their own are not enough to describe a species. Systematic Entomology 1–5. https://doi.org/10.1111/syen.12538

Wülker WF (1973) Revision der Gattung *Chironomus* Meıg. III. Europaische arten des thummi-Komplexes. Archiv für Hydrobiologie 72: 356–374.

Wülker WF, Butler MG (1983) Karyosystematics and morphology of Northern *Chironomus* (Diptera: Chironomidae): Freshwater species with larvae of the salinarius-type. Entomologica Scandinavica 14: 121–136. https://doi.org/10.1163/187631283X00010

Wülker W, Martin J (2000) Northernmost *Chironomus* karyotypes (Insecta, Diptera, Chironomidae). Spixiana 23: 151–156.

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The new highest number of B chromosomes (Bs) in Leisler's bat *Nyctalus leisleri* (Kuhl, 1817)

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Abstract

B chromosomes (Bs) are supernumerary to the standard chromosome set, from which they prevalently derive. Variation in numbers both among individuals or populations and among cells within individuals is their constant feature. Leisler's bat *Nyctalus leisleri* (Kuhl, 1817) is one of only four species of Chiroptera with detected Bs. Four males of *N. leisleri* were collected from two localities on the territory of Serbia and cytogenetically analysed. All animals had Bs with interindividual variability ranging from two to five heterochromatic micro Bs. The highest number of Bs was detected in this species. Among mammals, Rodentia and Chiroptera are orders with the largest number of species, but Bs frequently appear in rodents and rarely in chiropterans. Possible explanations for this difference are offered.

Keywords

B chromosomes, Chiroptera, *Nyctalus leisleri*

Introduction

B chromosomes (Bs) are supernumerary but dispensable karyotype components of standard karyotypes (A chromosomes). Although their appearance has been known for more than a century, many questions related to them still seek answers. These additional elements are frequently present in different species of animals, plants, and fungi.

It is estimated that 3% of all analysed species contain Bs (D'Abrosio 2017). Why they are frequently present in some species but not in others, and why are they absent or rare in specific taxa of animals and plants are among these riddles. Usually, Bs originate from A chromosomes of the same species, but also through hybridization between two closely related species (reviewed in Camacho et al. 2000; Jones and Houben 2003; Houben et al. 2014; Valente et al. 2016). They show significant variability in morphology, size, and number in which they appear in some species, populations, and even in different tissues of an individual. Usually, they do not follow Mendelian segregation law rules and also do not recombine with chromosomes of the A set, thus following their own evolutionary destiny (Jones 2018). Although dispensable chromosomes are often heterochromatic, many recent studies have shown that they are transcriptionally active and, most likely, contribute to the phenotypes of their carriers (summarized for mammals in Vujošević et al. 2018).

B chromosomes have been detected in 85 mammalian species (Vujošević et al. 2018), and recently another bat species was added to this list – *Megaderma spasma* (Linnaeus, 1758) (Volleth et al. 2021). With more than 1440 species (Simmons and Cirranello 2022), bats represent the second-largest mammalian group. To date, extra chromosomes were detected in only four bat species, three vespertilionids *Pipistrellus tenuis* (Temminck, 1840) (Bhatnagar and Srivastava 1974), *Myotis macrodactylus* (Temminck, 1840) (Obara 1976) *Nyctalus leisleri* (Volleth 1992), and one megadermatid *Megaderma spasma* (Volleth et al. 2021). Compared to rodents, which are the largest mammalian order and have 61 species with B chromosomes detected, the presence of B chromosomes in bats seems to be far less frequent event. Here we will present possible reasons for this occurrence.

Leisler's bat *Nyctalus leisleri* (Kuhl, 1817) is a medium-sized bat distributed throughout Europe up to 57°N (Dietz and Kiefer 2016). Although a widespread species, it is considered rare almost everywhere except in Ireland (Boston et al. 2015). It is a typical woodland bat, and it shows a clear preference for mature forests in most of its distribution area. *N. leisleri* roosts mainly in tree holes, and it forages over the canopy, along forest trails, and over water bodies (Dietz and Kiefer 2016). Nursery colonies are usually in tree holes and contain 20–50 females. Females of this species give birth to 1–2 young during June, in Great Britain and Ireland only one, but in the rest of the areal usually two (Dietz and Kiefer 2016). This species hibernates in tree holes as well, and occasionally in buildings or underground sites (Dietz and Kiefer 2016; Juste and Paunović 2016). Leisler's bat migrates over longer distances with regular seasonal movements between summer and winter habitats (Hutterer et al. 2005). *N. leisleri* has been recorded at seven localities in Serbia. Records consist of single individuals (mainly males) captured using mist-nets at species' foraging grounds from July to September. There are no known roosts of this species in Serbia, and there is a lack of information on habitat use. Additionally, there is no evidence of the reproduction of Leisler's bat in Serbia (Paunović et al. 2020).

Volleth (1992) analysed the karyotypes of *N. leisleri* and found 1, 2, and 3 B chromosomes (2n=44, NFa=50, NF=54 + 1-3Bs) in three males originating from Turkey,

Germany, and Greece, respectively. Additionally, karyotypes of one more specimen, from Poland (Fedyk and Fedyk 1970) was conventionally stained and analysed, and probably contained 2n = 46, with two microchromosomes in the karyotype.

The modern view on Bs highlights their role in genome evolution as an extra genomic compartment with huge potential and still unknown biological significance, making Bs very interesting for research on different levels. Here we studied the presence of Bs and cytogenetic characteristics of karyotypes in *Nyctalus leisleri* in Serbia, for the first time.

Materials and methods

Ethics statement

Capturing and sampling was carried out under the permit provided by the Ministry of Environmental Protection of the Republic of Serbia (nos. 353-01-2814/2019-04; 353-01-195/2020-04). Animals were safely released immediately after sampling.

Sampling

Bats were captured at two localities in Serbia (Fig. 1): Bebića Luka (44.1963, 19.6962) in Western Serbia on 12.6.2020. and Zlot (44.0288, 21.9627) in Eastern Serbia on 1.9.2020. A total of four males of *Nyctalus leisleri* were captured.

Mist-nets were mounted over water bodies (river Jablanica and Lazareva river) before the sunset and remained open for 4 hours. All captured individuals were identified to the species level following Dietz and Kiefer (2016), sexed and age-determined based on the degree of ossification of the epiphyseal plates on finger bones (Brunet-Rossinni and Wilkinson 2009). Four adult males were captured (three in Bebića Luka and one in Zlot). Two tissue samples of plagiopatagium were taken from each individual using 3-mm sterile biopsy punch following Worthington et al. (1996) and immersed in physiological solution in the presence of antibiotics (penicillin 500000 U/l and kanamycin 500 mg/l) and antimycotic (amphotericin B 12,5 mg/l). Tissue samples were stored at 4 °C and transported to the laboratory within 24h from the moment of sampling.

Cell culture

Primary fibroblast cell cultures were established using the protocol by Stanyon and Galleni (1991) and modified as in Romanenko et al. (2015). Cell passages were done each time when cells covered the flask surface completely. Dissociation of affixed cells was done by 0.25% trypsin, 0.2% EDTA. After a few passages the quantity of cells was sufficient for chromosome preparation.

Cells were kept in CO_2 controlled incubator at 37 °C after adding colchicine (0.04 μg/ml) overnight and Ethidium bromide (EtBr 1.5 μg/ml) for three hours before cell picking. Cells were treated with hypotonic solution (33.5 mM KCl, 7.75 mM

Figure 1. Map of sampling localities: 1. Bebića Luka, Western Serbia; 2. Zlot, Eastern Serbia.

sodium citrate) and incubated for 55 minutes at 37 °C. Chromosomes were prefixed and fixed with fresh ice-cold fixative (methanol and glacial acetic acid in ratio 3:1). Slides for preparation were previously cleaned in chromic acid and well washed and preserved at 4 °C in distilled water.

Chromosome preparations

Fibroblast cells grown in cell culture were used for chromosome preparations following the protocol described by Rajičić et al. (2017). One drop of chromosome suspension was spread on a slide and stained by Giemsa. The number of chromosomes was determined from at least 20 analysed metaphase plates per animal using Axias 2 plus (Zeiss) microscope. The standard chromosome complement of *N. leisleri* counts 44 chromosomes, and animals with more than 44 were considered to have Bs.

G-banding of metaphase chromosomes was performed according to the standard protocol (Graphodatsky and Radjabali 1988). Constitutive heterochromatin was detected by the modified techniques of C-banding (Sumner 1972). The position and number of nucleolus organizer regions (NORs) were identified using silver staining (Howell and Black 1980).

Results

A total of four *N. leisleri* males were captured and their karyotypes were analysed by different cytogenetic methods for the first time in the territory of the Republic of Serbia. In three of the samples collected in Bebića Luka locality we detected the following karyotypes: one with 2n=44 +1-2Bs, two with 2n=44+3-5Bs, while the karyotype of the bat from Zlot had 2n=44+2-4Bs (Table 1).

Analysed karyotypes of all specimens consist of 42 autosomes, pair of sex chromosomes (XY) and variable number of Bs (2–5). Among the autosomes, three pairs were large metacentrics, one pair was small submetacentric, and the remaining 17 pairs were acrocentrics. The X chromosome was a medium-sized metacentric, and the Y chromosome was a small acrocentric. All Bs were microchromosomes (Figs 2–5).

C-banding showed the presence of constitutive heterochromatin in centromeric regions of all autosomes, sex chromosomes, and Bs (Fig. 4).

Table 1. Intraindividual variability in number of B chromosomes in all studied samples. Number of cells with 0B, 1B, 2Bs, 3Bs, 4Bs, 5Bs and the total number of studied cells.

Sample	Locality	0B	1B	2Bs	3Bs	4Bs	5Bs	Total
1	Bebića Luka	0	**12**	**17**	2	0	0	31
2	Bebića Luka	0	1	3	6	**13**	6	29
3	Bebića Luka	0	1	0	0	7	**16**	24
4	Zlot	0	0	1	3	**16**	4	24

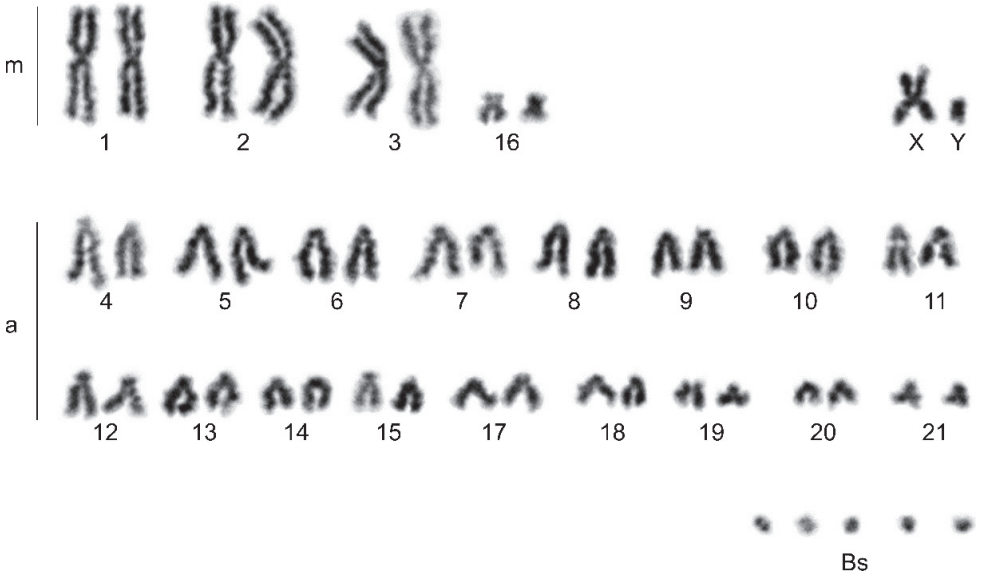

Figure 2. Conventionally stained karyotype of *N. leisleri* male with 5Bs (44+5Bs). m – metacentrics; a – acrocentrics; Bs – B chromosomes.

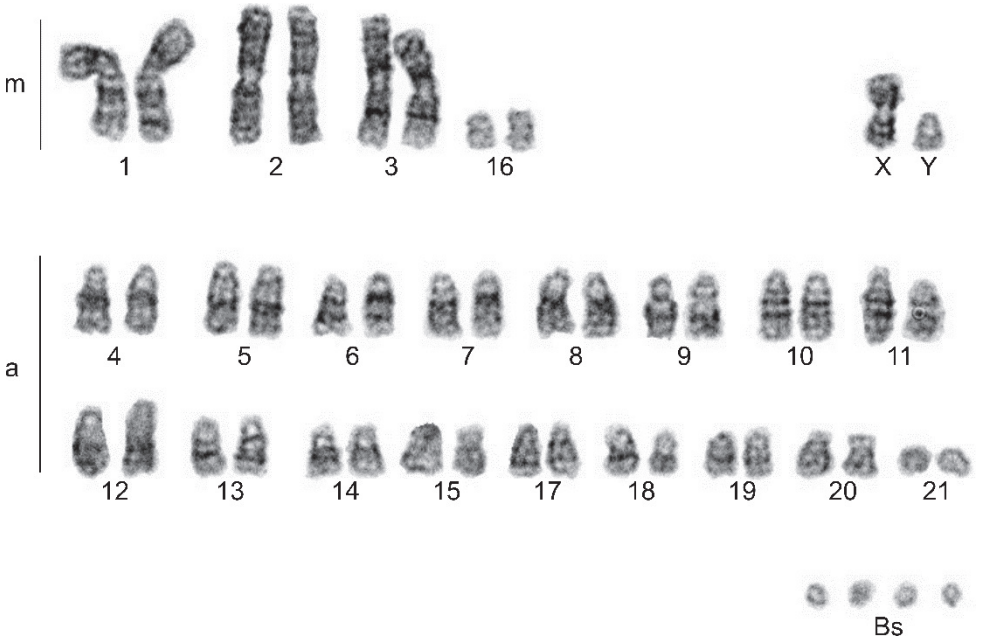

Figure 3. G-banded chromosomes of *N. leisleri* male with 4Bs (44+4Bs). m – metacentrics; a – acrocentrics; Bs – B chromosomes.

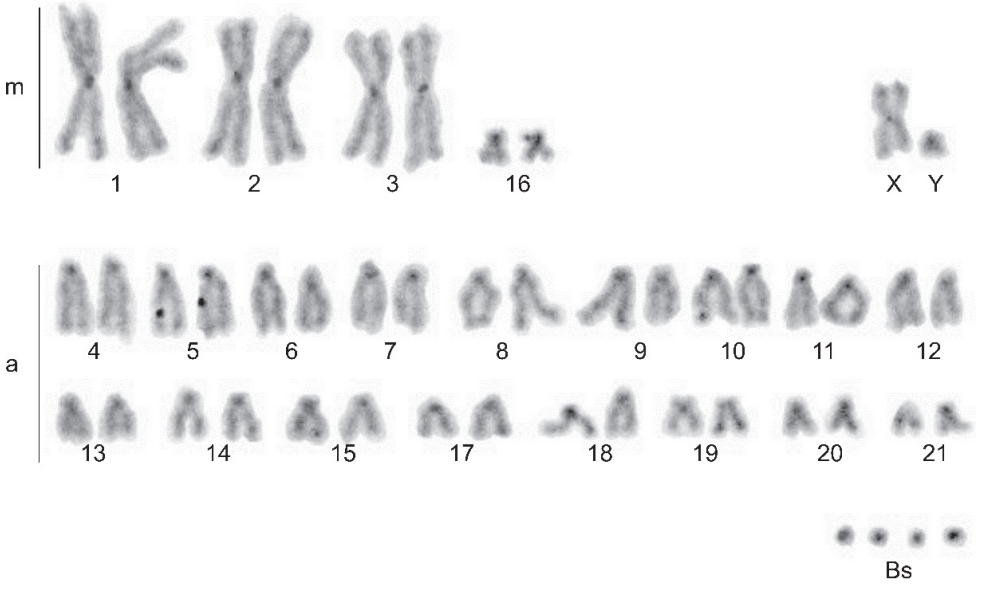

Figure 4. C-banded chromosomes of *N. leisleri* male with 4B (44+4Bs). m – metacentrics; a – acrocentrics; Bs – B chromosomes.

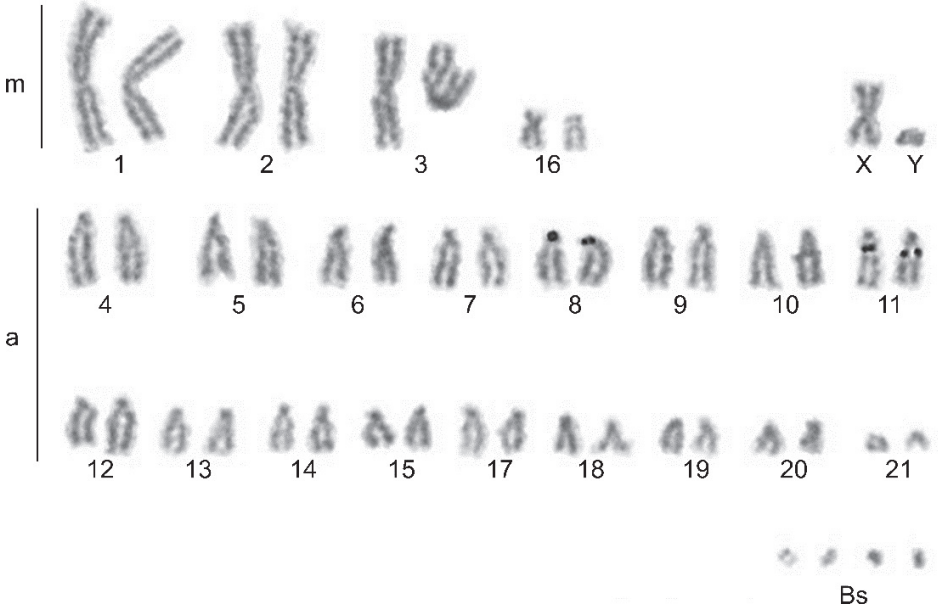

Figure 5. Nucleolus organiser regions (NORs) located on chromosome pairs no. 8 and 11 in *N. leisleri* male with 4Bs (44+4Bs). m – metacentrics; a – acrocentrics; Bs – B chromosomes.

NORs were detected on two pairs of chromosomes in all analysed metaphases of all specimens from Serbia. In one pair, the active NORs were located on the minute arm of a pair of acrocentric chromosomes, while on the other pair, they were intercalary positioned, at the place of secondary constriction. Chromosomes are arranged from left to right and numerated in decreasing order, so the acrocentric pair with NORs at minute arms was at chromosome pair no. 8, and intercalary NORs were at pair no. 11 in the karyotype (Fig. 5).

Discussion

After rodents, bats are the second most numerous group of mammals (Burgin et al. 2018). While rodents are the most frequent carriers of Bs, with 61 species possessing them, additional chromosomes are detected only in four bat species to date (Vujošević et al. 2018; Volleth et al. 2021). In vespertilionids, Bs are heterochromatic microchromosomes (reviewed Vujošević et al. 2018), while in *Megaderma spasma*, although among the smallest chromosomes, Bs are not microchromosomes and do not seem to be fully heterochromatic (Volleth et al. 2021). Least pipistrelle, *Pipistrelus tenuis* (previously *Pipistrellus mimus*) has two or four metacentric Bs (Bhatnagar and Srivastava 1974). Big-footed Myotis, *Myotis macrodactylus*, possess one micro B chromosome that

can be acrocentric or metacentric (Obara et al. 1976). It is known that the species *N. leisleri* contains heterochromatic micro B chromosomes in addition to the standard karyotype, but until now, the highest recorded Bs number was three (Volleth 1992). Our study is the first study of the *N. leisleri* karyotype in the territory of the Republic of Serbia. Previously published cytogenetic analyses (Vollteh 1987) showed the same number but different positions of active nucleolus organisers (NORs) in this species. We obtained NORs at chromosome pairs 8 and 11 in *N. leisleri*, while according to Vollteh (1987) they were on 8th and 15th chromosome pairs in specimens from Greece and Turkey. These differences could be a result of spatial diversity. However, we must not omit differences in the degree of chromosome condensation during preparation, which can be a problem when there are many acrocentrics of similar size in the karyotype. Furthermore, basic set in our samples consists of 44 chromosomes, 42 autosomes and pair of sex chromosomes, (2n=44, NFa=50, NF=54 + 2-5Bs) of the same morphology as it was previously described (Valleth 1992; Aslan and Zima 2014). Interestingly, all analysed *N. leisleri* samples were males and all of them got Bs in the karyotype (Volleth 1992; Aslan and Zima 2014). The only one female karyotype reported with 2n=46 from Poland (Fedyk and Fedyk 1970), seems to have two micro B chromosomes. Authors probably did not reported Bs since they analysed only one animal.

Bs are found in all major taxonomic groups of animals except birds (Vujošević and Blagojević 2004). However, recently, tissue-specific B-like chromosomes, restricted to germline cells (germline restricted chromosomes – GRCs), appeared to be widely present in songbird species (Torgasheva et al. 2019). As previously mentioned, Bs are found in only four species of bats. Small genome sizes characterize both birds and bats. Genome size in birds has a narrow range from 2 to 4 pg (Tiersch and Wachtel 1991). Furthermore, birds' content of repeated sequences is the lowest among vertebrates (15–20%). A similar situation is characteristic for bats whose genome size is even smaller, averaging 2.35 pg (ranging from 1.3 to 3.2 pg) of DNA (Teeling et al. 2018). Bats are the only mammals capable of active flight and, together with birds, one of the two only living vertebrate taxa possessing this highly specialized mode of locomotion. It has been hypothesized that flight may impose a constraint on genome size. Genome size may be reduced in vertebrate groups having extreme metabolic demands for flight based on the relationship between genome size, cell size, and mass-specific metabolic rate (Hughes and Hughes 1995; Gregory 2002; Organ and Shedlock 2009). Smaller cells that characterize small genomes have a higher surface area to volume ratio, allowing improved gas exchange to satisfy metabolic demands (Szarski 1983). Reduced genome size may be why both birds and bats cannot tolerate the presence of Bs. Additionally, in flowering plants, the presence of Bs positively correlates with total genome size, and Bs frequently do not feature species with small genomes (Trivers et al. 2004).

The unique life-history traits of bats can also contribute to this non-acceptance of Bs. Longevity, slow reproductive rates, and small litters (Racey and Entwhistle 1999) make a chance of establishing and maintaining Bs much less possible than in rodents, which are characterized by a short life span, fast reproductive rates, and large litters (Promislow and Harvey 1990). Also, one must take into account the frequency of bat karyotype studies, compared to the ones conducted on rodents.

Nyctalus leisleri is considered to be a migratory species in Europe, generally following the NE-SW direction between summer roosts in Northeastern Europe and hibernation sites in central and southwestern parts of Europe (Hutterer et al. 2005; Boston et al. 2020). In other parts of Europe (NW and SE) this species may be vagrant or sedentary (Bogdanowicz and Ruprecht 2004), while data on the migration of *N. leisleri* in Eastern Europe is scarce (Hutterer et al. 2005). The longest migratory distances (over 1500 km) were recorded in females that bred in Germany and hibernate on the Iberian Peninsula (Ohlendorf et al. 2000; Wohlgemuth et al. 2004). According to Ruczyński (2004), males of Leisler's bats occur sporadically in Poland and other northern regions but dominate in populations in Southern Europe. This is probably the reason why the only published karyotype of *N. leisleri* female is from Poland (Fedyk and Fedyk 1970). The vast majority of all captured Leisler's bats in the territory of Serbia were males (Paunović et al. 2018; Paunović et al. 2020; Budinski unpublished data). There is no information on whether this species breeds in Serbia (Paunović et al. 2020). Scarce records of *N. leisleri* females in Serbia could be explained also by relatively low sampling efforts during the migration period.

The low number of analysed samples, the highest detected number of Bs, lack of data on female karyotype, and scarce data on this species' ecology in the territory of Serbia, make *Nyctalus leisleri* very interesting model for further studies on Bs.

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References

Aslan A, Zima J (2014) Karyotypes of the mammals of Turkey and neighbouring regions: review. Folia Zoologica 63: 1–62. https://doi.org/10.25225/fozo.v63.i1.a1.2014

Bhatnagar VS, Srivastava MDL (1974) Somatic chromosomes of four common bats of Allahabad. Cytologia 39: 327–334. https://doi.org/10.1508/cytologia.39.327

Bogdanowicz W, Ruprecht AL (2004) *Nyctalus leisleri* – Kleinabendsegler. In: Niethammer J, Krapp F (Eds) Handbuch der Saugetiere Europas 4: 717–756.

Boston ESM, Dechmann DKN, Ruczynski I (2020) Leisler's Noctule *Nyctalus leisleri* (Kuhl, 1817). In: Hackländer K, Zachos FE (Eds) Handbook of the Mammals of Europe. Springer, Cham, 1–15. https://doi.org/10.1007/978-3-319-65038-8_64-1

Boston ESM, Montgomery IW, Hynes R, Prodohl PA (2015) New insights on postglacial colonization in western Europe: the pyhlogeography of the Leisler's bat (*Nyctalus leisleri*). Proceedings of the Royal Society B 282: 20142605. https://doi.org/10.1098/rspb.2014.2605

Brunet-Rossinni AK, Wilkinson GS (2009) Methods for age estimation and the study of senescence in bats. In: Kunz TH, Parsons S (Eds) Ecological and behavioral methods for the study of bats. Johns Hopkins University Press, 315–325.

Burgin CJ, Colella JP, Kahn PL, Upham NS (2018) How many species of mammals are there? Journal of Mammalogy 99(1): 1–4. https://doi.org/10.1093/jmammal/gyx147

Camacho JPM, Sharbel TF, Beukeboom LW (2000) B-chromosome evolution. Philosophical Transaction of the Royal Society B Biological Science 355: 163–178. https://doi.org/10.1098/rstb.2000.0556

D'Ambrosio U, Alonso-Lifante MP, Barros K, Kovařík A, Mas de Xaxars G, Garcia S (2017) B-chrom: a database on B-chromosomes of plants, animals and fungi. New Phytology 216: 635–642. https://doi.org/10.1111/nph.14723

Dietz C, Kiefer A (2016) Bats of Britain and Europe. Bloomsbury publishing, 302–305.

Fedyk A, Fedyk S (1970) Karyotypes of some species of Vespertilionid bats from Poland. Acta Ther XV(20): 295–302. https://doi.org/10.4098/AT.arch.70-20

Graphodatsky AS, Radjabli SI (1988) Chromosomes of agricultural and laboratory mammals. Atlas. Nauka Press, 127 pp.

Gregory TR (2002) A bird's-eye view of the C-value enigma: genome size, cell size, and metabolic rate in the class Aves. Evolution 56: 121–130. https://doi.org/10.1111/j.0014-3820.2002.tb00854.x

Houben A, Banaei-Moghaddam AM, Klemme S, Timmis JN (2014) Evolution and biology of supernumerary B chromosomes. Cellular and Molecular Life Sciences 71: 467–478. https://doi.org/10.1007/s00018-013-1437-7

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: A 1-step method. Experientia 36(8): 1014–1015. https://doi.org/10.1007/BF01953855

Hughes AL, Hughes MK (1995) Small genomes for better flyers. Nature 377: 391–391. https://doi.org/10.1038/377391a0

Hutterer R, Ivanova T, Meyer-Cord C, Rodrigues L (2005) Bat migrations in Europe. Naturschutz und Biol Vielfalt, 92–94.

Jones N, Houben A (2003) B chromosomes in plants: escapees from the A chromosome genome? Trends Plant Science 8: 417–423. https://doi.org/10.1016/S1360-1385(03)00187-0

Jones RN (2018) Transmission and drive involving parasitic B chromosomes. Genes 9(8): e388. https://doi.org/10.3390/genes9080388

Juste J, Paunović M (2016) *Nyctalus leisleri*. The IUCN Red List of Threatened Species 2016: e.T14919A22016159.

Obara Y, Tomyiasu T, Saitoh K (1976) Chromosome studies in the Japanese vespertilionid bats. I. Karyotypic variation in *Myotis macrodactylus* Temmink. The Japanese Journal of Genetics 51: 201–206. https://doi.org/10.1266/jjg.51.201

Ohlendorf B, Hecht B, Strasburg D, Agirre-Mendi PT (2000) Fernfund Kleinabendseglers (*Nyctalus leisleri*) in Spanien. Nyctalus (N.F.) 7: 239–242.

Organ CL, Shedlock AM (2009) Paleogenomics of pterosaurs and the evolution of small genome size in flying vertebrates. Biological Letters 5: 47–50. https://doi.org/10.1098/rsbl.2008.0491

Paunović M, Karapandža B, Budinski I, Pejić B, Josipović J (2018) Mali noćnik – *Nyctalus leisleri*, Leisler's Bat. In: Stanković D, Paunović M, Raković M (Eds) Atlas migratornih ptica i slepih miševa Srbije. Ministarstvo zaštite životne sredine Republike Srbije, Ministarstvo

kulture i informisanja Republike Srbije, Prirodnjački muzej u Beogradu, posebna izdanja 46: 239–241. [Beograd] [In Serbian]

Paunović M, Karapandža B, Budinski I, Stamenkovć S (2020) Bats (Mammalia, Chiroptera) of Serbia. Serbian Academy of Sciences and Arts and Natural History Museum Belgrade Monographs 693, book 13: 309–314. [In Serbian]

Promislow DEL, Harvey PH (1990) Living fast and dying young: A comparative analysis of life-history variation among mammals. Journal of Zoology 220(3): 417–437. https://doi.org/10.1111/j.1469-7998.1990.tb04316.x

Racey PA, Entwhistle AC (1999) Life history and reproductive strategies of bats. In: Krutzch PH, Crichton EG (Eds) Reproductive biology of bats. New York, NY: Academic Press, 364–366. https://doi.org/10.1016/B978-012195670-7/50010-2

Rajičić M, Romanenko SA, Karamysheva TV, Blagojević J, Adnađević T, Budinski I, Bogdanov AS, Trifonov VA, Rubtsov N, Vujošević M (2017) The origin of B chromosomes in yellow-necked mice (*Apodemus flavicollis*)—Break rules but keep playing the game. PLoS ONE 12: e0172704. https://doi.org/10.1371/journal.pone.0172704

Romanenko SA, Biltueva LS, Serdyukova NA, Kulemzina AI, Beklemisheva VR, Gladkikh OL, Lemskaya NA, Interesova EA, Korentovich MA, Vorobieva NV, Graphodatsky AS, Trifonov VA (2015) Segmental paleotetraploidy revealed in sterlet (*Acipenser ruthenus*) genome by chromosome painting. Molecular Cytogenetics 8: e90. https://doi.org/10.1186/s13039-015-0194-8

Ruczyński I (2004) Bats in trees. In: Jędrzejewska B, Wójcik JM (Eds) Essays on mammals of Białowieża forest. Mammal Research Institute, Polish Academy of Sciences, Białowieża, Poland, 121–128.

Simmons NB, Cirranello AL (2022) Bat Species of the World: A taxonomic and geographic database. https://batnames.org/ [Accessed on 18.04.2022]

Stanyon R, Galleni L (1991) A rapid fibroblast culture technique for high resolution karyotypes. Bollettino di Zoolgia 58: 81–83. https://doi.org/10.1080/11250009109355732

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75(1): 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

Szarski H (1983) Cell size and the concept of wasteful and frugal evolutionary strategies. Journal of Theoretical Biology 105: 201–209. https://doi.org/10.1016/S0022-5193(83)80002-2

Tiersch TR, Wachtel SS (1991) On the evolution of genome size of birds. Journal of Heredity 82(5): 363–368. https://doi.org/10.1093/oxfordjournals.jhered.a111105

Teeling EC, Vernes SC, Dávalos LM, Ray DA, Gilbert MTP, Myers E (2018) Bat1K Consortium. Bat Biology, Genomes, and the Bat1K Project: To Generate Chromosome-Level Genomes for All Living Bat Species. Annual Review of Animal Biosciences 6: 23–46. https://doi.org/10.1146/annurev-animal-022516-022811

Torgasheva AA, Malinovskaya LP, Zadesenets KS, Karamysheva TV, Kizilova EA, Akberdina EA, Pristyazhnyuk IE, Shnaider EP, Volodkina VA, Saifitdinova AF, Galkina SA, Larkin DM, Rubtsov NB, Borodin PM (2019) Germline-restricted chromosome (GRC) is widespread among songbirds. Proceedings of the National Academy of Science USA 116(24): 11845–11850. https://doi.org/10.1073/pnas.1817373116

Trivers R, Burt A, Palestis BG (2004) B chromosomes and genome size in flowering plants. Genome 47: 1–8. https://doi.org/10.1139/g03-088

Valente GT, Nakajima RT, Fantinatti BEA, Marques DF, Almeida RO, Simões RP, Martins C (2016) B chromosomes: from cytogenetics to systems biology. Chromosoma 126: 73–81. https://doi.org/10.1007/s00412-016-0613-6

Volleth M (1987) Differences in the location of nucleolus organizer regions in European vespertilionid bats. Cytogenetics and Cell Genetics 44: 186–197. https://doi.org/10.1159/000132371

Volleth M (1992) Comparative analysis of the banded karyotypes of the European *Nyctalus* species (Vespertilionidae Chiroptera). In: Horáček I, Vohralík V (Eds) Prague Studies in Mammology. Charles University Press: Prag, Czech Republic, 221–226.

Volleth M, Müller S, Heller K-G, Trifonov V, Liehr T, Yong H-S, Baker RJ, Khan FAA, Sotero-Caio CG (2021) Cytogenetic analyses detect cryptic diversity in *Megaderma spasma* from Malaysia. Acta Chiropterologica 23(2): 271–284. https://doi.org/10.3161/15081109ACC2021.23.2.001

Vujošević M, Blagojević JB (2004) Chromosomes in populations of mammals. Cytogenetics and Genome Research 106: 247–256. https://doi.org/10.1159/000079295

Vujošević M, Rajičić M, Blagojević J (2018) B chromosomes in populations of mammals revisited. Genes 9(10): e487. https://doi.org/10.3390/genes9100487

Wohlgemuth R, Devrient I, Garcia A, Hutterer R (2004) Long-distance flight of a Lesser noctule (*Nyctalus leisleri*) after rehabilitation. Myotis (41–42): 69–73.

Worthington Wilmer J, Barratt E (1996) A non-lethal method of tissue sampling for genetic studies of chiropterans. Bat Research News 37(1): 1–3.

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