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A first look at mitochondrial genetic diversity in *Miniopterus schreibersii* in Serbia

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Abstract: Schreiber's bent-winged bat *Miniopterus schreibersii* (Chiroptera) is a widespread, cave-dwelling, regionally migrating species whose genetic diversity was studied throughout its distribution area using mitochondrial and nuclear markers. Previous studies revealed little to no structuring of populations and established Anatolia to be a single refugium during the last glacial maximum. The Balkans were well covered in these studies but usually lacked samples from Serbia. We sequenced the mitochondrial hypervariable region 1 (*HV1*) gene in *M. schreibersii* collected at seven sites in Serbia to assess their genetic relatedness to other European and Asia Minor populations and check whether the Balkans exhibit a higher genetic diversity than Western Europe due to its closeness to Anatolia. We recorded nine haplotypes from Serbia, six of which had not been previously reported, with a haplotype diversity of 0.585. The remaining three were shared with individuals from Portugal, Greece, and Turkey. A single most common haplotype was present throughout the species distribution range, pointing to a well-connected population and as indicated by the shape of the haplotype network, a common origin, and a sudden population expansion. Results complement existing data on *M. schreibersii* having a non-structured population, adding valuable new data from the Balkans supporting the previous hypothesis about its Anatolian origin.

Keywords: Schreiber's bat; Chiroptera; hypervariable region 1 (HV1); control region; haplotypes

INTRODUCTION

Schreiber's bent-winged bat, *Miniopterus schreibersii* (Kuhl, 1817), is among Serbia's most commonly found cave-dwelling bat species [1]. The distribution of the species is Mediterranean-wide, from southwest Europe across the Balkans and Caucasus to Asia Minor [2]. Previously, the distribution of this species was much wider, and in addition to Europe, it included Asia and north Africa. After multiple genetic and morphologic studies, it was concluded that the complex of species in question was *M. pallidus* in the Near East [2,3], *M. maghrebensis* in North Africa [4], and *M. schreibersii*, living in sympatry with other two species in certain parts of the distribution area.

Miniopterus schreibersii uses underground roosts exclusively and forms very large and dense colonies [5]. It is capable of flying long distances (up to 833 km) due to its wing morphology [5], but usually migrates within a 40-100 km range between summer and winter roosts [6]. It was previously thought that gene flow in this species is exclusively male-mediated since females exhibit a very strict philopatry and stay loyal to their natal groups [7]. However, based on the ringing-recapture data from Portugal, it was subsequently shown that males also exhibit pronounced philopatry, though they tend to disperse more than females [8,9]. The importance of appropriate mediatory roosts for the contact of individuals from different colonies over larger distances and the general rule that dispersion proceeds from larger colonies towards the smaller ones was shown [8]. This species has been thoroughly studied over the years, and its genetic diversity has been analyzed with various markers across its entire distribution range [9-15]. Conclusions of the studies evolved, depending on the method and the scale of the sampling. For example, in Portugal, populations of *M. schreibersii* exhibited a significant isolation-by-distance based on both mtDNA and microsatellites [7]. Similar findings were observed in southern and central Europe [9]. Conversely, other studies indicated a less pronounced genetic differentiation in *M. schreibersii* as seen from

mtDNA [14], and no differentiation was observed in nuclear markers [13]. However, a more recent study based on restriction site-associated DNA sequencing (RAD-seq) and single nucleotide polymorphisms (SNPs) reinforced the initial findings that there is, after all, a strong isolation by distance and a significant genetic structuring in *M. schreibersii* across its European range [11]. Regarding the sampling efforts described in previously published studies, the whole distribution area of *M. schreibersii* was well covered, including the Balkan Peninsula. However, some countries, such as Serbia, are largely under-represented, with only a single sequence of the mtDNA *HV1* used in one study [15].

During the Last Glacial Maximum (LGM), European Peninsulas – Iberian, Italian, and Balkan, served as refugia for many species [16]. The Balkans were an important refugium for numerous bat species [17,18], harboring a significant genetic diversity for *M. schreibersii* populations [13,19]. However, results [15] suggest that Anatolia served as a single refugium during the LGM for *M. schreibersii*, from where it spread towards the Balkan Peninsula and further on to the west of Europe. This can be seen through the level of genetic diversity that decreases from east to west of the species distribution area [15,20]. According to the newest hypothesis [11], Europe's three largest peninsulas might have saved remnants of historical populations and served as microrefugia for *M. schreibersii*. In our endeavor to understand the role of the Balkan Peninsula in the phylogeography of this species, we aimed to assess Serbia's sample placement relative to other European and Anatolian samples through the analysis of the mtDNA *HV1* gene and thereby contribute novel insights to the already substantial *M. schreibersii HV1* database. We hypothesized that Serbia (and the Balkans) should exhibit higher genetic diversity compared to Western European samples, given its proximity to the presumed place of the species' origin. Alternatively, if samples from Serbia demonstrated a high level of uniqueness and diversity, this would align with the microrefugia hypothesis.

MATERIALS AND METHODS

Ethics statement

Capture and marking of bats was done under permits issued by the relevant state authorities and ethical and safety protocols: Licenses for bat catching, marking, and tissue sampling in Serbia: Permit no. 353-02-502/2017-17, issued by the Ministry of Environmental Protection of Serbia; Permit nos. 353-01-1432/2017-04 and 353-01-675/2018-04, issued by the Ministry of Environmental Protection of Serbia.

Sampling

Bats were caught with standard chiropterological nets at roost entries at dusk during emergence at seven sites shown on the map (Fig. 1A) as follows: (i) Mali Kamenolom (3 females); (ii) Petrovaradin (5 males); (iii) Drenajićka (3 males, 2 females); (iv) Petnička (5 females); (v) Bela Sala (2 females); (vi) Toplik (1 male, 1 female); (vii) Temska (1 female). A small piece of skin (3 mm in diameter) was taken from each wing for tissue preservation in absolute ethanol and further genetic analyses. As part of long-term monitoring, the processing of animals also included taking basic body measurements (forearm length, mass), determining sex and age, and banding bats with wing markers (rings) with unique numbers. All bats were released at trapping sites immediately after processing.

DNA isolation, amplification, and sequencing

Tissues of up to 5 individuals per site were incubated overnight in digestion buffer with proteinase K [21], after which whole genomic DNA was extracted with a Quick-DNA Miniprep commercial extraction kit (Zymo Research, USA), following the manufacturer's protocol. For the analyses of mitochondrial DNA, we amplified the fragment of the gene for cytochrome b, entire proline tRNA and threonine tRNA, and a fragment of the hypervariable region 1 (*HV1*) control region. For more simple terminology, these sets of gene fragments will be referred to as *HV1* according to the longest gene fragment of this group. Amplification was performed using the primer set C and E [22]. The PCR reaction was prepared in a 25-μL total volume, containing 1 × Dream Taq buffer, 0.6 mM dinucleotides, 0.8 μM of each primer, 1 mM $MgCl_2$, 0.625 U of Dream Taq polymerase, 2 μL DNA and ddH_2O to a final volume. The reaction had 3 min of initial denaturation at 94°C, followed by 40 cycles, 30 s at 94°C, 60 s at 56°C, and 60 s at 72°C, followed by 5 min of final extension at 72°C. The PCR products were visualized using 1% agarose gel

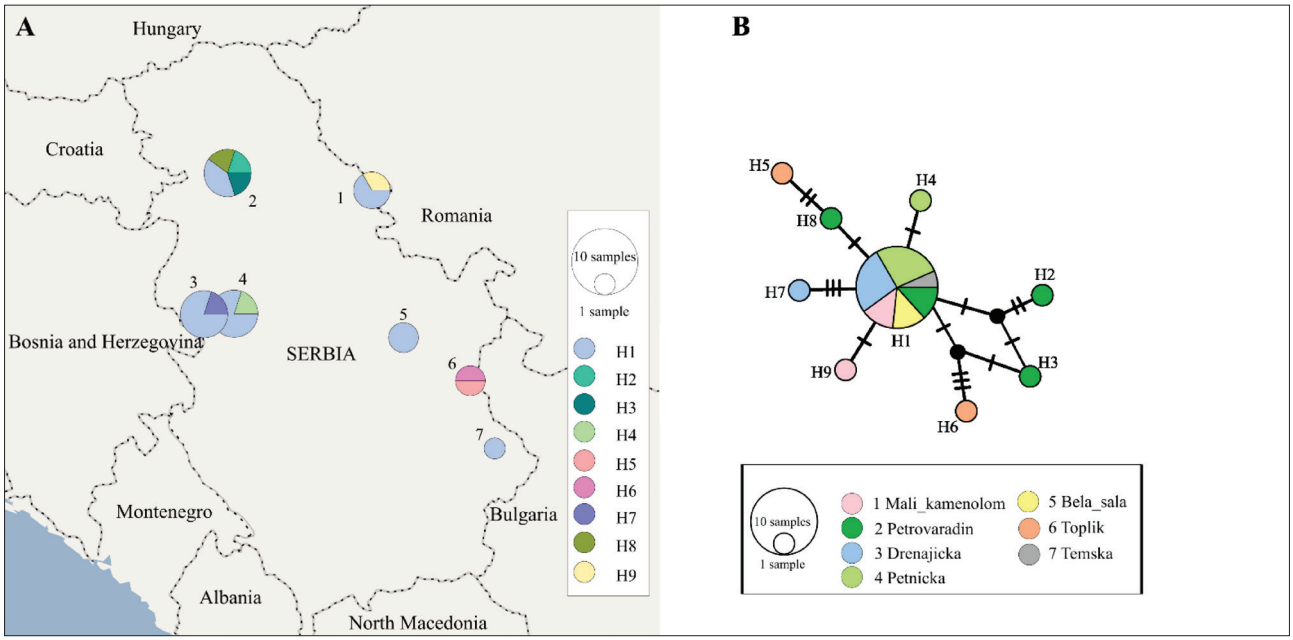

Fig. 1. A – Map of sampling sites of *Miniopterus schreibersii* bat in Serbia with mtDNA *HV1* gene haplotype distribution. **B** – Median-joining haplotype network of mtDNA gene *HV1* detected in Serbian samples. Circle sizes correspond to the number of individuals having each haplotype; dashes on the branches represent mutational steps between haplotypes. The black circle represents the putative intermediary haplotype that was not detected here.

electrophoresis with Midori Green (Nippon Genetics) and sent for commercial sequencing. The samples from the Petnička site were sequenced in Hungary as a part of a project and collaboration with the University in Pécs (referred to in the Acknowledgements).

After the sequencing and rejection of poor-quality or shorter sequences, the final dataset contained 23 sequences of *M. schreibersii* of 553 bp length. The sequences were aligned and edited using CodoneCodeAligner 4.2.7 software (CodoneCode Corporation, Centerville, USA). In DnaSP6 [23] and Arlequin [24] software, parameters of molecular diversity of sequences (number of haplotypes, haplotype diversity (Hd), number of polymorphic sites, number of nucleotide differences (k), nucleotide diversity (π)) were calculated and collapsed into haplotypes. Haplotype networks were analyzed and visualized in PopART 1.7 software [25] using the median-joining algorithm and default settings (ε=0). Analysis of demographic history was performed by comparing the distribution of pairs of nucleotide differences by mismatch distribution analysis. Mismatch analysis was performed in DnaSP6 [23] to determine the existence of potential historical population fluctuations in the studied bat species under the null hypothesis that the observed data correspond to the model of sudden expansion. The significance of the overlap of the observed mismatch distribution with the expected one was estimated based on the sum of the squared deviations (SSD) and Harpending's index (r – raggedness index). Mismatch graphs were created in DnaSP6 software to determine if populations show signs of spatial distribution expansion or a stationary population history [26]. Two tests of neutrality, which are based on the distribution of allele frequencies, Fu's Fs and Tajima's D tests were used to investigate demographic changes in the past and were also performed in DnaSP v.6 [23].

To assess the placement of Serbia's samples compared to other European and Anatolian samples through the analysis of the mtDNA *HV1* gene, we added 98 sequences of sufficient length from GenBank [7,19,27,28]. The expanded dataset had 121 sequences, which were again collapsed into haplotypes in DnaSP v.6 [23] and visualized in PopART 1.7 software [25] using the median-joining algorithm and default settings (ε=0). The analysis of evolutionary divergence between the sequences of the expanded dataset was performed in MEGA v.6 software [29] using the maximum composite likelihood model [30].

RESULTS

Genetic diversity of mtDNA of *M. schreibersii* from Serbia

Until now, there has been a lack of genetic data for *M. schreibersii* from Serbia, with only a single sequence published [15]. To address this knowledge gap, we sampled and analyzed genetic diversity at seven sites in Serbia. We recorded 9 haplotypes, 6 of which had not been previously reported. Indicators of DNA molecular polymorphism for our dataset of 23 sequences of the *HV1* gene (553 bp) in *M. schreibersii* showed haplotype diversity Hd=0.585±SD 0.122, nucleotide diversity π=0.00277±SD 0.00082, and average values of nucleotide differences k=1.53. The number of polymorphic positions was 13, of which 4 were parsimony informative.

The nine haplotypes recorded from Serbia (Fig. 1B) were named H1-H9, and the unique sequences of each are stored in GenBank with accession numbers OR948566-OR948574. Haplotype H1 was the most common, accounting for 65% of animals in our sample, and was present in 6 out of 7 sampling sites (Fig. 1B). The other haplotypes (H2-H9) were represented with a single specimen sequence. The largest haplotype diversity was observed in the Petrovaradin site (Fig. 1A, site no. 2). The median-joining haplotype network was star-like in shape, with H1 in the middle, surrounded by the rest of the haplotypes, differing in 1 to 5 mutational steps (Fig. 1B). Haplotypes H2, H3, and H6 differed from H1 more than the others.

Mismatch distribution analysis showed a multimodal distribution, indicating a stable population size. Observed values showed a slightly better fit to the values expected for the constant population size scenario (Supplementary Fig. S1A). The value of Harpending's index was r=0.096 (n.s.), and its positive and non-significant values point to population expansion in the past. Values of neutrality tests, Fu's (Fs=-4.044, P<0.05) and Tajima's (D=-2.111, P<0.05), both being negative and significant, point to the more recent population growth scenario.

M. schreibersii from Serbia in the expanded dataset

The expanded dataset had 121 sequences trimmed to a length of 434 bp. The number of haplotypes identified herein was 66, of which 45 were represented with a single sequence (Fig. 2). Detailed information about GenBank sequences used is given in Supplementary Table S1. Due to the large number of haplotypes, the labels in Fig. 2 only show those found in Serbia. The most common haplotype in Serbia (H1) was also retrieved from Portugal, Greece and Turkey, and it has a central position in the haplotype network. Six haplotypes (H2-3, H5-7, H9) were unique to Serbia and had not been recorded elsewhere. Haplotype H4 was also found in Turkey [28], and haplotype H8 was found in Greece and Turkey [7,19]. Divergence between the sequences of the expanded dataset was 0.2-2.2% (Supplementary File S2).

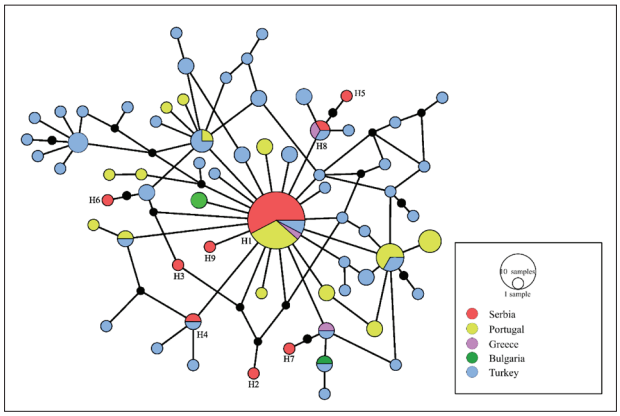

Fig. 2. Median-joining haplotype network of *HV1* mtDNA gene of *Miniopterus schreibersii* expanded dataset, with combined original sequences and GenBank available sequences. Black dots represent 2 base pair changes between the haplotypes, and lines connecting different haplotypes represent 1 base pair change.

DISCUSSION

Indicators of DNA molecular polymorphism of *M. schreibersii* for samples collected in Serbia showed relatively high haplotype diversity (Hd=0.585), considering the sample size of only 23 sequences. A similar but slightly lower Hd was shown in a larger sample from Portugal (Hd=0.531, n=307 sequences) [14], potentially confirming that genetic diversity in *M. schreibersii* diminishes from east to west of its distribution. According to mismatch distribution analysis, we assume that *M. schreibersii* in Serbia has a stable population size and that in recent history, it went through population growth, as shown by Harpending's r and neutrality tests. The star-like shape of the haplotype network also supports the scenario of rapid population expansion. A dominant presence of 1 haplotype in the center of the haplotype network points to a possible single-glacial refugium

origin. Samples from Serbia were characterized by a high number of exclusive haplotypes, haplotypes present only in one sampling site. Thus, each roost, apart from Bela Sala, had its' own unique haplotype(s): H9 was recovered at Mali Kamenolom, H2, H3, and H8 at Petrovaradin, H7 at Drenajićka Pećina, H4 at Petnička Pećina, H5, and H6 at Toplik. However, the exclusivity of these haplotypes is more likely due to the small sample size and a high probability that other individuals carrying these specific haplotypes were missed rather than being a sign of a structured species population. The sampling sites in Serbia were chosen to cover different regions of the country. Samples were collected in late summer and autumn and likely included individuals from transitory roosts and the remnants of the nursery roosts. *M. schreibersii* is a regional migrant, and its migrations provide opportunities for gene flow. The importance of intermediary roosts between summer and winter colonies that would serve as transitory stepping-stones in migrations was previously identified [8]. Thus, another possible explanation of the exclusive haplotypes in each sampling site might be the lack of intermediary roosts between different regions of Serbia.

Since 1994, there has been comprehensive data on bat movement and connections of different roosts in Serbia based on ringing and recapture programs [31]. Monitoring underground roosts and finding marked bats allows us to infer that bats regularly migrate between some of the studied roosts. For example, the site with the highest number of haplotypes was Petrovaradin Fortress, which is consistent with the fact that this site is interconnected with many other sites in Serbia, based on long-term ringing and monitoring schemes [32-33]. It would be interesting to comprehensively explore and compare *M. schreibersii* genetic diversity in sites in Serbia that have confirmed long-lasting connectivity. In addition, *M. schreibersii* individuals ringed in Serbia have also been found in neighboring countries, which proves constant migratory activities between Balkan localities [33].

When comparing with the expanded dataset available from GenBank, the most common haplotype in Serbia was H1, and it was the most common in the entire sample, including Portugal, Greece, and Turkey. The fact that this haplotype was so widely distributed speaks in favor of a unique metapopulation of *M. schreibersii* in its entire range [13]. It is evident that the expanded dataset contains the most sequences originating from Turkey and is comprised of the most frequently identified haplotypes, making a single-refugium origin from Anatolia probable. The haplotype H4 from this study was also retrieved from Turkey, and H8 from Greece and Turkey. The other haplotypes detected in our samples (H2, H3, H5, H6, H7, and H9) were not previously reported from other countries and represent the unique contribution of this study to the knowledge of the genetic diversity of Schreiber's bent-winged bat. Sequence divergence calculated between this study's sequences and those of the expanded dataset is consistent with the average divergence rate within a vertebrate species [34].

The contribution of this study is six new, previously undescribed haplotypes originating from Serbia and three haplotypes that occur in other parts of Europe (Portugal and Greece) and Asia Minor. Although many of the haplotypes from Serbia were site-exclusive and have not been previously reported, we do not have sufficient information to refute or endorse the microrefugia hypothesis. Nevertheless, the fact that we obtained relatively high genetic diversity in a modest sequence sample with a representative number of sites provides a promising basis for future comprehensive studies in Serbia.

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Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/genbank/ under the accession numbers OR948566-OR948574 The expanded dataset of estimates of evolutionary divergence between sequences of HV1 gene in *Miniopterus schreibersii* is provided here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Bajic%20et%20al_Raw%20Dataset_S2.xls

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Sequences of *Miniopterus schreibersii HV1* mtDNA gene from GenBank, from previously published studies, used as an addition to this study's sequences

No.	GenBank accession no.	Authors of the study	Sampling site	Haplotype name
1	GQ149027.1	[7]	Portugal	H1 [a]
2	GQ149028.1	[7]	Portugal	H16
3	GQ149029.1	[7]	Portugal	H16
4	GQ149030.1	[7]	Portugal	H16
5	GQ149031.1	[7]	Portugal	H1 [a]
6	GQ149032.1	[7]	Portugal	H1 [a]
7	GQ149033.1	[7]	Portugal	H15
8	GQ149034.1	[7]	Portugal	H14
9	GQ149035.1	[7]	Portugal	H1 [a]
10	GQ149036.1	[7]	Portugal	H20
11	GQ149037.1	[7]	Portugal	H10
12	GQ149038.1	[7]	Portugal	H21
13	GQ149039.1	[7]	Portugal	H13
14	GQ149040.1	[7]	Portugal	H22
15	GQ149042.1	[7]	Portugal	H1 [a]
16	GQ149043.1	[7]	Portugal	H11
17	GQ149044.1	[7]	Portugal	H1 [a]
18	GQ149045.1	[7]	Portugal	H19
19	GQ149046.1	[7]	Portugal	H11
20	GQ149047.1	[7]	Portugal	H15
21	GQ149048.1	[7]	Portugal	H12
22	GQ149049.1	[7]	Portugal	H1 [a]
23	GQ149050.1	[7]	Portugal	H17
24	GQ149052.1	[7]	Portugal	H18
25	GQ149054.1	[7]	Portugal	H17
26	GQ149056.1	[7]	Portugal	H16
27	GQ149060.1	[7]	Portugal	H11
28	GQ149061.1	[7]	Portugal	H11
29	GQ149062.1	[7]	Portugal	H10
30	GQ149065.1	[7]	Portugal	H1 [a]
31	AY923064.1	[27]	Turkey	H1 [a]
32	AY923065.1	[27]	Bulgaria	H40
33	AY923067.1	[27]	Bulgaria	H39
34	AY923068.1	[27]	Greece	H23
35	AY923069.1	[27]	Greece	H8 [a]
36	AY923071.1	[27]	Turkey	H32
37	AY923072.1	[27]	Turkey	H44
38	AY923073.1	[27]	Turkey	H45
39	EU332355.1	[19]	Bulgaria	H39
40	EU332356.1	[19]	Turkey	H61
41	EU332357.1	[19]	Turkey	H12
42	EU332358.1	[19]	Turkey	H30
43	EU332359.1	[19]	Turkey	H57
44	EU332360.1	[19]	Turkey	H50

Table S1. continues.

No.	GenBank accession no.	Authors of the study	Sampling site	Haplotype name
45	EU332361.1	[19]	Turkey	H56
46	EU332363.1	[19]	Turkey	H27
47	EU332364.1	[19]	Turkey	H55
48	EU332365.1	[19]	Turkey	H60
49	EU332366.1	[19]	Turkey	H54
50	EU332367.1	[19]	Turkey	H54
51	EU332368.1	[19]	Turkey	H49
52	EU332369.1	[19]	Turkey	H1 [a]
53	EU332370.1	[19]	Turkey	H14
54	EU332371.1	[19]	Turkey	H11
55	EU332372.1	[19]	Turkey	H46
56	EU332373.1	[19]	Turkey	H23
57	EU332374.1	[19]	Turkey	H24
58	EU332375.1	[19]	Turkey	H48
59	EU332376.1	[19]	Turkey	H53
60	EU332377.1	[19]	Turkey	H59
61	EU332378.1	[19]	Turkey	H8 [a]
62	EU332379.1	[19]	Turkey	H28
63	EU332380.1	[19]	Turkey	H47
64	EU332381.1	[19]	Turkey	H58
65	EU332382.1	[19]	Turkey	H40
66	EU332383.1	[19]	Turkey	H52
67	EU332384.1	[19]	Turkey	H66
68	EU332385.1	[19]	Turkey	H32
69	EU332386.1	[19]	Turkey	H65
70	EU332388.1	[19]	Turkey	H64
71	EU332389.1	[19]	Turkey	H63
72	EU332390.1	[19]	Turkey	H62
73	EU332391.1	[19]	Turkey	H44
74	EU332392.1	[19]	Turkey	H51
75	FJ028608.1	[28]	Turkey	H38
76	FJ028609.1	[28]	Turkey	H31
77	FJ028610.1	[28]	Turkey	H43
78	FJ028611.1	[28]	Turkey	H36
79	FJ028612.1	[28]	Turkey	H4 [a]
80	FJ028613.1	[28]	Turkey	H26
81	FJ028614.1	[28]	Turkey	H30
82	FJ028615.1	[28]	Turkey	H12
83	FJ028616.1	[28]	Turkey	H12
84	FJ028617.1	[28]	Turkey	H25
85	FJ028618.1	[28]	Turkey	H42
86	FJ028619.1	[28]	Turkey	H35
87	FJ028620.1	[28]	Turkey	H41
88	FJ028621.1	[28]	Turkey	H34
89	FJ028622.1	[28]	Turkey	H33
90	FJ028623.1	[28]	Turkey	H37
91	FJ028624.1	[28]	Turkey	H32
92	FJ028625.1	[28]	Turkey	H29
93	FJ028626.1	[28]	Turkey	H11
94	FJ028627.1	[28]	Turkey	H28
95	FJ028628.1	[28]	Turkey	H27
96	FJ028629.1	[28]	Turkey	H24
97	FJ028630.1	[28]	Turkey	H31
98	FJ028631.1	[28]	Turkey	H1 [a]

GenBank accession numbers of used sequences of fragment cytohrome b gene (*cytb*), tRNK proline gene, tRNK threonine gene, fragment of the hypervariabile region 1 (*HV1*) of the control region of *Miniopterus schreibersii*, published in previous studies. Haplotypes with the mark [a] correspond fully to the sequences obtained in this study.

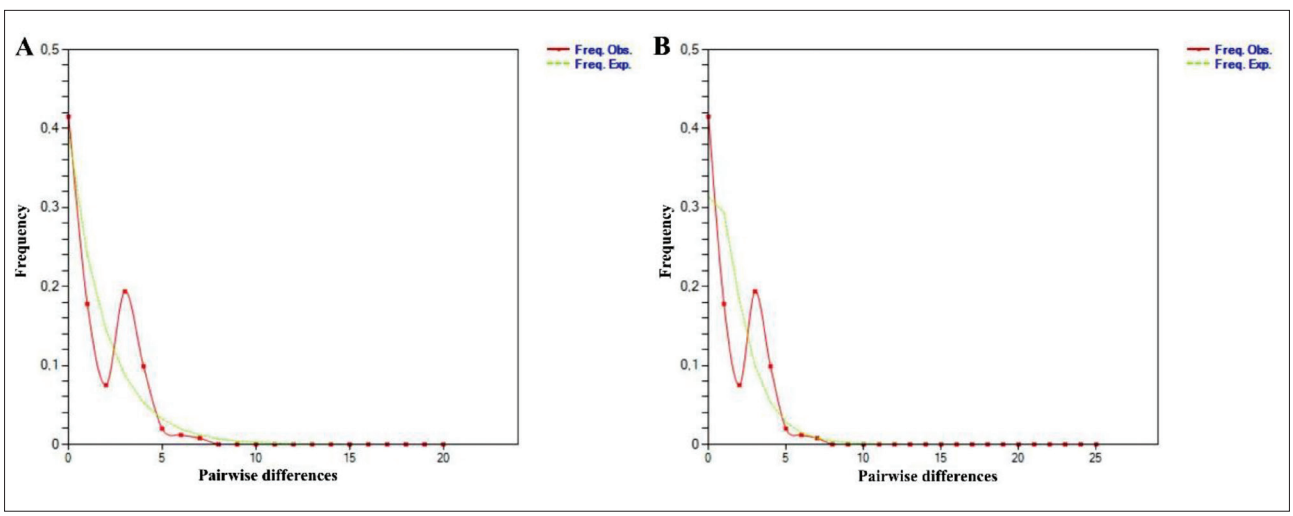

Fig. S1. Mismatch distribution of pairwise number of differences. **A** - Constant population size. **B** - Population growth-decline. Red line named Freq. Obs. in the legend represents frequencies observed, and green dashed line named Freq. Exp. represent frequencies expected of nucleotide pair differentiation.