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Genetic and morphological differences among relict marginal occurrences of *Stenobothrus eurasius* (Orthoptera)

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Abstract

Steppes form large zonal habitats in Asia but only consist of localised outposts in Europe. An ideal subject for the study of differences within species between the main steppe zone and the localized more western outposts is the Orthopteran *Stenobothrus eurasius*, widespread across the Siberian and Central Asian steppes but present only in isolated relic populations at the western edge of its area. We used genetic and morphological analyses to detect possible differences among these relic populations.

We carried out a study on morphological parameters of wings in parallel with the comparison of four DNA fragments (cytochrome c oxidase subunit I, 12S rRNA and the mtDNA control region, cytochrome B, nuclear internal transcribed spacers plus the 5.8S rRNA region) involving 15 extrazonal populations of the species. *St. nigromaculatus* was used as an outgroup taxon in the genetic analyses.

Variability of the morphological characters of *St. eurasius* individuals was higher within the regions than amongst the regions. The two *Stenobothrus* species were not separated based on the CR gene. Samples of both *Stenobothrus* species were separated on the COI, cytB and ITS1-5.8S-ITS2 phylogenetic trees with high support (PP = 1) in Bayesian analyses but clear genetic lineages were not revealed, and populations of the focal species were not grouped according to their geographic locations. The similarity of this species in different steppe outposts supports the hypothesis that *St. eurasius* was widespread in the more extensive steppe areas that were once present, but the extension of agricultural landuse reduced the steppe habitats resulting in the current patchy distribution of *St. eurasius* limited to the remaining habitats.

Key words

COI, 12S-CR, cytB, ITS1-5.8S-ITS2, Natura 2000, phylogeography, steppe outposts

1. Introduction

The steppe zone stretches continuously from eastern Asia to eastern Europe but there are extrazonal outposts in the Mediterranean-, in Western- and in Central-Europe. In recent decades, our knowledge of phylogeographic patterns of steppe species in the extrazonal steppes has considerably increased (Ribera and Blasco-Zumeta 1998; Cremene et al. 2005; González-Sampériz et al. 2010; Kajtoch et al. 2016; Kirschner et al. 2020). Most work focuses on the importance of refugia during Pleistocene glaciations and interglacials (Taberlet et al. 1998; Petit et al. 2003; Bhagwat and Willis 2008; Holderegger and Thiel-Egenter 2008; Kryštufek et al. 2009; Stewart et al. 2010; Schmitt and Varga 2012). Postglacial climate variations have also played a major role in shaping the current distribution patterns of species (Divíšek et al. 2022). The consequences of the isolation of European steppe outposts are also at the forefront of the recent research (Kirschner et al. 2020). Examination of the steppe species that have fragmented populations in Western- and Central Europe is also relevant from the point of view of nature conservation (Dengler et al. 2014). European steppe outposts usually are characterised by assemblages very rich in species that are among the most threatened in Europe due to natural (mainly climatic) and anthropogenic factors (afforestation, intensification, trampling, fragmentation) (Cremene et al. 2005; Habel et al. 2013; Fekete et al. 2014; Kajtoch et al. 2016).

Currently isolated extrazonal populations of steppe species often differ from zonal populations and it is far from clear whether these differences are relics from the past or have developed more recently (Hensen et al. 2010). A study by Kirschner et al. (2020) on the orthopterans *Stenobothrus nigromaculatus* (Herrich-Schäffer, 1840) and *Omocestus petraeus* (Brisout de Barneville, 1856), revealed that extrazonal lineages are restricted to parts of the European Alps, the Italian Peninsula and western and southern Europe and meet zonal lineages at the periphery of the Pannonian basin. There have been two main hypotheses on the origin of the biodiversity of steppe grasslands in Central Europe: (i) long-term species persistence in situ since the early Pleistocene versus (ii) immigration from the south-east, either after the last glacial maximum (LGM) or after the Neolithic landscape deforestation (Divíšek et al. 2022).

In phylogeographic studies, the importance of previously described minor morphological differences can be confirmed or rejected by considering genetic distances detected by phylogenetic analyses (Borissov et al. 2021). An ideal subject for the above topic could be *Stenobothrus eurasius* Zubovski, 1898 which is a common species in the Siberian and Central Asian steppes (Sergeev 1998) but is present only in isolated relict populations at the western edge of its distribution area, including Central Europe and the Balkan Peninsula (Mařan 1958; Willemse 1974; Sergeev 1986; Ingrisch and Köhler 1998; Rácz 1998; Holuša and Holuša 2002; Gavlas 2005; Nagy and Puskás 2007; Willemse and Willemse 2008; Lemonnier-Darcemont et al. 2014; Nagy and Rácz 2014; Şirin et al. 2017; Iorgu and Iorgu 2018; Kenyeres et al. 2020). Based on its local rarity, the species is included in Annex II and Annex IV of the EU Habitats Directive (Council Directive 92/43/EEC 1992), is protected in several countries, and included in regional Red Lists (Rakonczay 1990; Krištín 2001; Kočárek et al. 2013). Six subspecies of *St. eurasius* have been described from the western edge of its area (Cigliano et al. 2023; Chládek 2018). *St. eurasius slovacus* Mařan, 1958 is found in the northern part of the Carpathian basin (Holuša et al. 1999; Macháčková and Fikáček 2014), while in the Czech Basin, Hungary, and Austria *St. eurasius bohemicus* Maran, 1958 (Vidlička in Ambróz et al. 2011; Zuna-Kratky et al. 2017) occurs. In addition, *St. eurasius moravicus* Chládek, 2018 was described from Slovakia (Chládek 2018). Because all of the related taxonomic analyses of *St. eurasius* are based on morphological studies and genetic analyses of the species are missing, we carried out an analysis of the published morphological characteristics in parallel with the comparison of four DNA fragments (cytochrome c oxidase subunits I – COI, 12S rRNA and control region mtDNA – 12S-CR, cytochrome B – cytB, nuclear internal transcribed spacers plus 5.8S rRNA region – ITS1-5.8S-ITS2) involving 15 extrazonal populations of the species.

During the study, we tested the following hypotheses: Hyp-1: *St. eurasius* colonized the studied area in several different waves during the Pleistocene and Holocene, so the age of the populations is different, which should be reflected in higher genetic and morphological differences among the populations (justifying the formerly described subspecies-level differences). Hyp-2: *St. eurasius* colonized the study area in roughly the same time, as a result of one or more waves during the Holocene that covered the dry parts of Central Europe belonging to western margin of the steppe area. If the latter is true, we should not be able to find any substantial genetic and morphological differences among the recent populations.

2. Methods

2.1. Sample collection

Between 2017 and 2018, we examined *St. eurasius* at 15 sites throughout the western margin of its area (Fig. 1 and Table S1). Our study covered all regions which are characterised by the presence of *St. eurasius* : (1) three localities in Dobrogea (*DOB*) (Cerni: CE, Greci: GR, Izovoarele: IZ), (2) four localities in the Eastern part of the Hungarian Middle Mts (*EHM*) (Bélapátfalva: BE, Bódvarákó: BO, Füzér: FU, Tokaj: TO), (3) three localities in the Western part of the Hungarian Middle Mts (*WHM*) (Budaörs: BU, Szárliget: SZ, Tés: TE), (4) one locality in the Southern periphery of the Fatra (*SPF*) (Malé Kršteňan: MKV), (5) one locality in the Vienna Basin (*VIB*) (Hainburg: HA), (6) one locality in Moravia (*MOR*) (Miroslav: MI), (7) two localities in the Czech Basin (*CZB*) (Mnichov: MN, Raná: RA). *St. nigromaculatus* samples, as an outgroup taxon, were used from the following sites: (1) two localities in the Czech Basin (*CZB*) (Horenec: HO, Raná: RA), (2) two localities in Moravia (*MOR*) (Miroslav: MI, Vevcice: VE), (3) one locality in the Southern periphery of the Fatra (*SPF*) (Nitra: NI), (4) four localities in the Western part of the Hungarian Middle Mts (*WHM*) (Csór: CS, Lábatlan: LA, Perbál: PE, Tés: TE). Geographical units were used for the boundaries of each region.

For the analyses, we used 15 male and 24 female specimens of *St. eurasius*, and 7 male and 20 female speci-

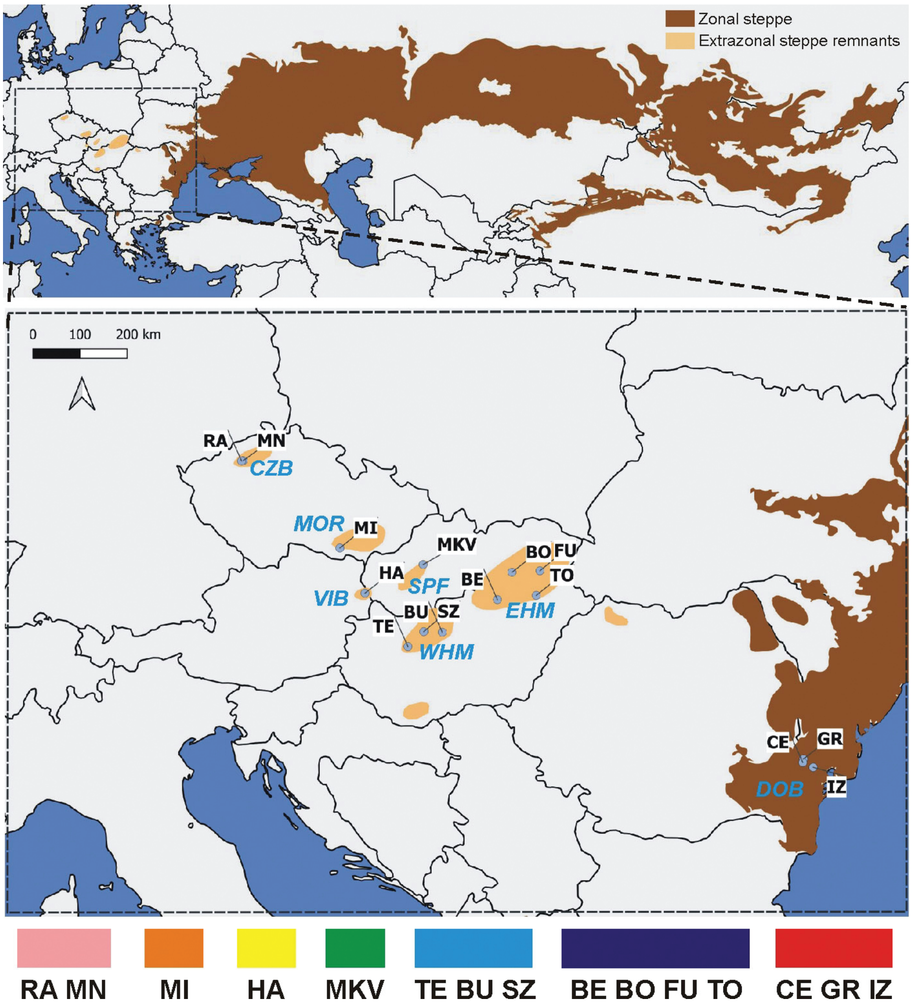

Figure 1. Study sites in the area of *Stenobothrus eurasius*. (Details see in Suppl. Table 1.) — Abbreviations of the settlement names: **BE** – Bélapátfalva; **BO** – Bódvarákó; **BU** – Budaörs; **CE** – Cerni; **FU** – Füzér; **GR** – Greci; **HA** – Hainburg; **IZ** – Izovoarele; **MI** – Miroslav; **MKV** – Malé Kršteňan; **MN** – Mnichov; **RA** – Raná; **SZ** – Szárliget; **TE** – Tés; **TO** – Tokaj. Abbreviations of the regions: ***CZB*** – Czech Basin; ***MOR*** – Moravia; ***VIB*** – Vienna Basin; ***SPF*** – Southern periphery of the Fatra; ***WHM*** – Western part of the Hungarian Middle Mts; ***EHM*** – Eastern part of the Hungarian Middle Mts; ***DOB*** – Dobrogea)

mens of *St. nigromaculatus*. Insects were released after collecting the test material (a right leg and a digital photograph of their wings); we wanted to sample several robust populations from each of the large outposts – but with minimal intervention in the threatened populations. The study sites were selected based on aerial photographs in Quantum GIS 3.16.1 (QGIS Development Team 2016) software, located and recorded by a Trimble Juno 3 GPS-PDA.

2.2. Morphological analysis

The subspecies of *St. eurasius*, which were only partly confirmed by Harz (1975), have been reported in recent publications (Holuša et al. 1999; Vidlička in Ambróz et al. 2011; Macháčková and Fikáček 2014; Zuna-Kratky et al. 2017; Chládek 2018). The basic morphological differences between the subspecies were as follows: in *St. e. bohemicus*, the cubital vein (CuA) of the tegmina (forewings) are fused either completely or in the middle, and the cubital field is only indicated in the basal part of the elytra. In *St. e. slovacus*, the cubital veins of the tegmina are completely separated or fused only in the apical third of the elytra, and the cubital field is always clearly formed in its middle. Finally, in *St. e. moravicus*, the media area of the hindwings is not wider than the radial area, and in the apical part, it is narrowed, and the apical part is clearly smoky (Mařan 1958; Harz 1975; Chládek 2018).

Based on the above-mentioned, the following parameters were measured on the forewings and hindwings. For males: (a) length and (b) maximum width of the forewing, (c) maximum width of the costal area on the forewing, (d) maximum width and (e) apical width of the hindwing median area. For females: (f) length and (g) maximum width of the forewing, (h) maximum width and (i) apical width of the hindwing median area (Table S3).

For the data collection of the above morphological parameters, we took photographs in the field of the wings (both fore- and hindwing) on a white paper base marked with a 5 × 5 mm scale. Because we did not find the species on the sites studied at Miroslav (MI), we used the morphological analysis of the published photograph of Chládek (2018) (*St. e. moravicus*, holotype, Miroslav). Further, we involved some other type specimens after figures from Willemse (1974) [*St. e. slovacus* (topotype, Domico)(Sts), *St. e. bohemicus* (paratype, Mily)(Stb), *St. e. eurasius* (topotype, Russia)(Ste)].

Using the taken photographs, we digitised the wing morphology of the examined *St. eurasius* specimens using CorelDRAW12 software (Fig. S1). On the vector graphics we measured the above mentioned parameters (see from (a) to (i)) for morphological analyses (Table S3). We measured the parameters in thousandths of millimeters (see Table S3), and produced the following variables: var1 = b/a, var2 = c/b, var3 = e/d, var4 = g/f, var5 = i/h. Var1, 2, and 3 were used for cluster analysis of males, and var4 and 5 of females. Cluster analyses were carried out by Ward's method (Euclidean similarity, data were transformed by isometric Burnaby). Statistical analyses were performed using the Past 3.14. software package (Hammer et al. 2001). For the clear interpretation, samples from each region (*DOB*, *EHM*, *WHM*, *SPF*, *VIB*, *MOR*, *CZB*) are marked in the same color on the figures.

2.3. Genetic analysis

For the genetic analyses, we used sharp scissors to collect one right hind leg from 14 males and 24 females (see Table S1) from six regions (*DOB*, *EHM*, *WHM*, *SPF*, *VIB*, *CZB*); as mentioned above, *MOR* region was involved only in morphological analysis using the published picture of Chládek (2018). The insects were released alive in good condition. Legs were kept in ethanol (96%) and deposited in a regular fridge (at 6°C degrees) until the start of genetic studies. As a control for the genetic analyses, we collected the hind legs of 7 males and 20 females of *St. nigromaculatus* (see Table S2).

DNA was extracted from leg-muscle tissue using the NucleoSpin tissue kit (Macherey–Nagel, Germany) according to the manufacturer's protocol. However, the DNA extraction method failed to isolate DNA from the *St. eurasius* collected in the *SPV* region (MKV). The Polymerase chain reaction (PCR) and sequencing of four markers were carried out using the following primers: LCO and HCO for COI (Folmer et al. 1994), SR-J14610 and T1-N18 for 12S-CR (Simon et al. 2006), CBJ and CBN for cytB (Simon et al. 1994), 18S–28S and 28S–18S for ITS1-5.8S-ITS2 (Weekers et al. 2001).

The PCR was performed in 20 μl reaction volume containing 0.1 μl Taq DNA Polymerase (EURx, Poland), 2.0 μl 10x PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP mixture, 10 μM of each primer, DNA template and ddH_20. For COI and cytB the PCR procedure consisted of 36 cycles at 94°C for 1 min, 48°C for 1 min and 72°C for 2 min with the final extension at 72°C for 7 min. The PCR conditions for the 12S-CR fragment were as follows: 35 cycles at 92°C for 20 s, 48°C for 30 s and 60°C for 3 min, with the final extension at 72°C for 7 min. To amplify the ITS1-5.8S-ITS2 fragment, the following PCR protocol was used: 30 cycles at 95°C for 1 min, 48°C for 1 min 50 s, 72°C for 2 min, with the final extension at 72°C for 10 min. All PCR products were purified using EPPiC Fast (A&A Biotechnology, Poland), following the standard protocol. The sequencing reaction was carried out in 10 μl reactions containing: 1.5 μl of sequencing buffer, 1.0 μl of BrilliantDye^{TM} v3.1 Terminator Cycle Sequencing Kit (NimaGen, The Netherlands), 1.0 μl of primer (forward or reverse), 3.0 μl of the purified DNA and 3.5 μl of sterile water. The sequencing protocol was as follows: the initial melting step of 3 min at 94°C was followed by 25 cycles of 10 s at 96°C, 5 s at 55°C and a final step of 90 s at 60°C. The sequences generated for this study were deposited in the GenBank database under the accession numbers given in Table S4.

The nucleotide sequences were edited and aligned in CodonCode Aligner 9.0 (CodonCode Corporation; https://www.codoncode.com/aligner) with default parameters. All sequences were checked for stop-codons in MEGA v. 11 (Tamura et al. 2021) and verified using BLAST of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Due to the different sizes of the datasets, the alignments of four molecular markers (COI, cytB, 12S-CR and ITS1-5.8S-ITS2) were analysed separately without concatenation of the sequences. For COI *Chorthippus binotatus* (GenBank Accession Number: KY709671) was used as the outgroup species, for cytB – *Chorthippus binotatus* (JN187524), 12S-CR – *Chorthippus parallelus* (X93574) and ITS1-5.8S-ITS2 – *Stenobothrus festivus* (KM385195).

Phylogenetic relationships were estimated by neighbor-joining (NJ) and Bayesian Inference (BI) using MEGA v. 11 (Tamura et al. 2021) and MrBayes v. 3.2 (Ronquist et al. 2012), respectively. Relative support for the NJ tree was obtained by bootstrapping using 1000 replicates. A sequence evolution model was selected using MrModeltest v. 2 software (Nylander 2004). Posterior probabilities were based on two independent Markov chain Monte Carlo (MCMC) runs, each composed of four chains (three heated and one cold). Markov chains were run for 4 million generations with sampling every 100 generations. The first 25% of trees were discarded as burn-in and a majority-rule consensus tree was generated from the remaining trees. The convergence of analyses was validated by evaluating likelihood values using Tracer v. 1.7 (Rambaut et al. 2018). The haplotype network was created using the median-joining algorithm in the PopART software (Leigh and Bryant 2015).

The genetic diversity indices were calculated for each group (*DOB*, *EHM*, *WHM*, *VIB*, *CZB*) using DnaSP v. 6 (Rozas et al. 2017), including the number of haplotypes (h), haplotype diversity (Hd) and the nucleotide diversity (Pi) of mitochondrial genes. Nucleotide pairwise distances among *St. eurasius* specimens were calculated only for mitochondrial sequences using the Kimura-2-parameter distance model in MEGA v. 11.

3. Results

Statistical analysis of the morphometric parameters of digitised wings of the collected *St. eurasius* specimens sorted the specimens in the male and female groups (Fig. 2). However, all groups contain specimens from different regions (e.g. CE and MN) or placed individuals from the same sampling site into distinct groups (e.g. BU). Further, on the resulting graph of males, we can see the topotype of *St. e. slovacus* and the topotype of *St. e. eurasius* are close to each other. Cluster analysis of morphological variables (Ward's method, Euclidean similari-

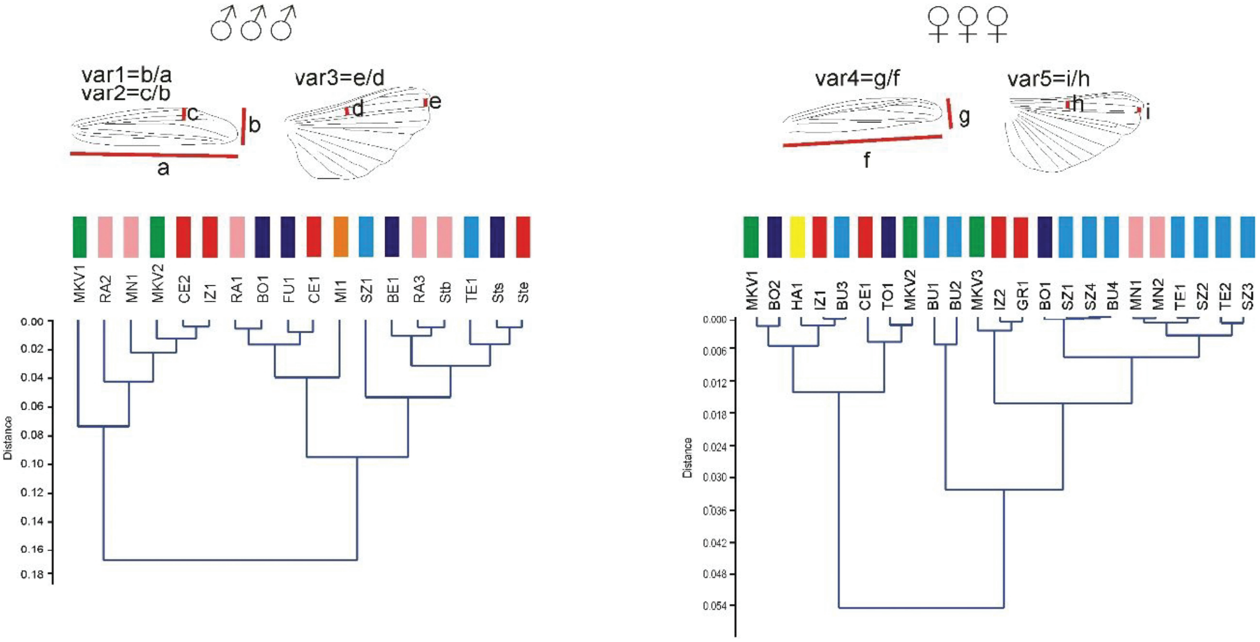

Figure 2. The result of cluster analysis of morphological variables (Ward's method, Euclidean similarity, data were transformed by isometric Burnaby). — Abbreviations: **BE** – Bélapátfalva; **BO** – Bódvarákó; **BU** – Budaörs; **CE** – Cerni; **FU** – Füzér; **GR** – Greci; **HA** – Hainburg; **IZ** – Izovoarele; **MI** – Miroslav; **MKV** – Malé Kršteňan; **MN** – Mnichov; **RA** – Raná; **SZ** – Szárliget; **TE** – Tés; **TO** – Tokaj; **Stb** – *St. e. bohemicus* (paratype, Mily); **Ste** – *St. e. eurasius* (topotype, Russia); **Sts** – *St. e. slovacus* (topotype, Domico) after Willemse (1974))

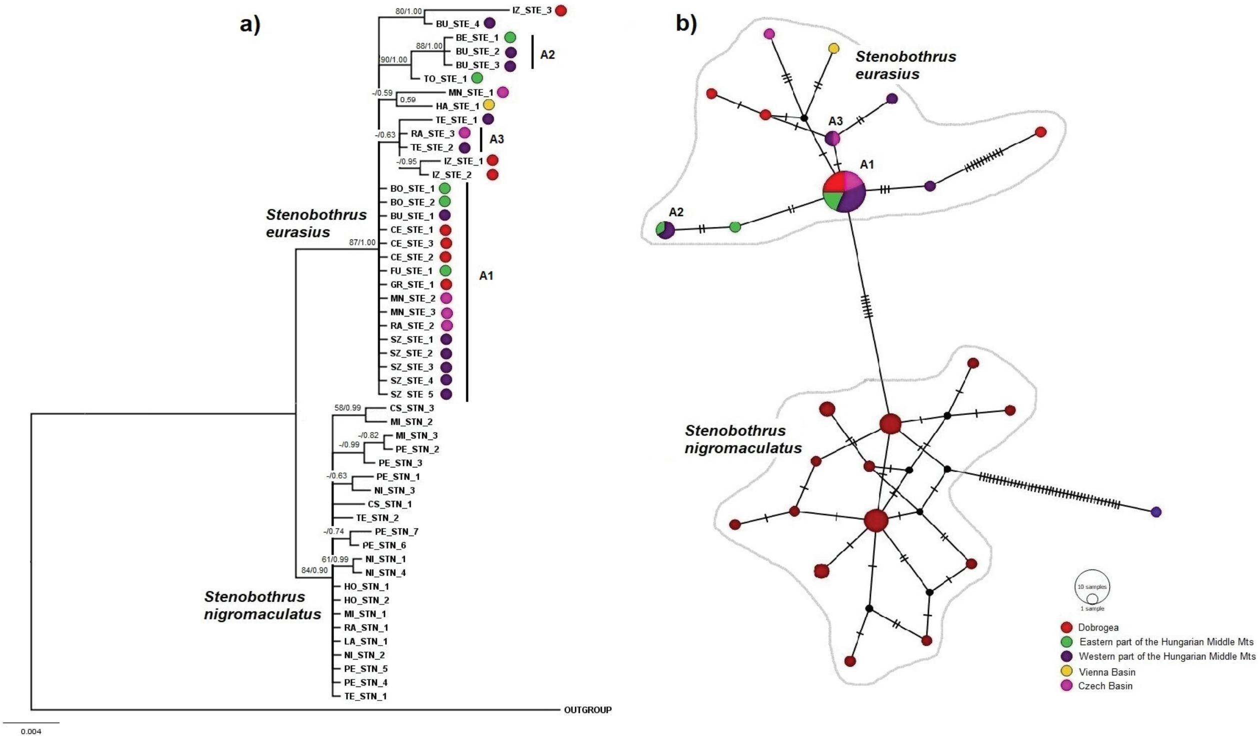

Figure 3. Phylogenetic tree reconstructed based on the neighbor-joining (NJ) and Bayesian Inference (BI) methods using COI gene (**a**), and the haplotype network was constructed based on haplotypes of COI (**b**). (The numbers on branches indicate the bootstrap values (NJ) and posterior probabilities (BI) being separated by a slash. Haplotype frequencies are related to the size of the circle. Different colors within the nodes of *St. eurasius* refer to different sampling sites.)

ty, data were transformed by isometric Burnaby) showed that variability of the morphological characters of the individuals is higher within the regions than amongst the regions (Fig. 2).

DNA was successfully extracted from 60 samples of *Stenobothrus* specimens (33 *St. eurasius* and 27 *St. nigromaculatus*) from 21 localities in five regions: *DOB*, *EHM*, *WHM*, *VB*, *CZB*. The number of sequences and the

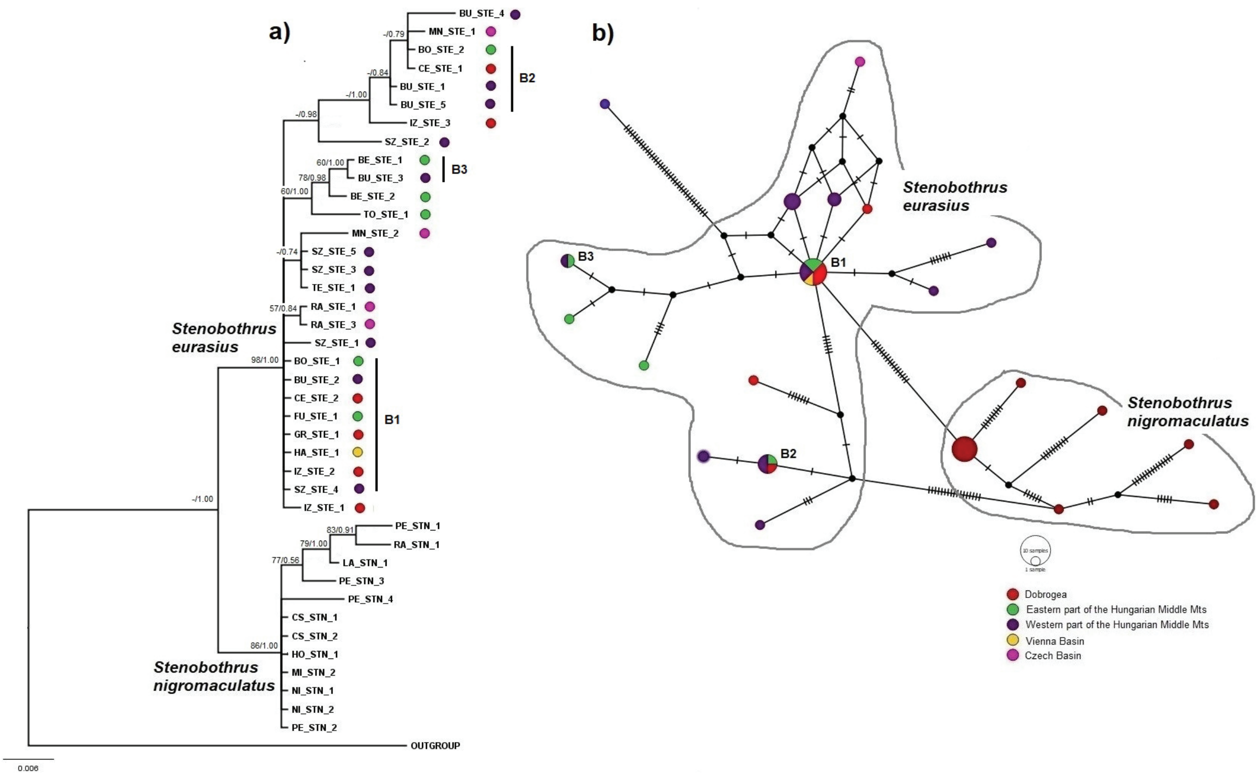

Figure 4. Phylogenetic tree reconstructed based on the neighbor-joining (NJ) and Bayesian Inference (BI) methods using cytB gene (**a**). The haplotype network was constructed based on haplotypes of cytB (**b**). (The numbers on branches indicate the bootstrap values (NJ) and posterior probabilities (BI) being separated by a slash. Haplotype frequencies are related to the size of the circle. Different colors within the nodes of *St. eurasius* refer to different sampling sites.)

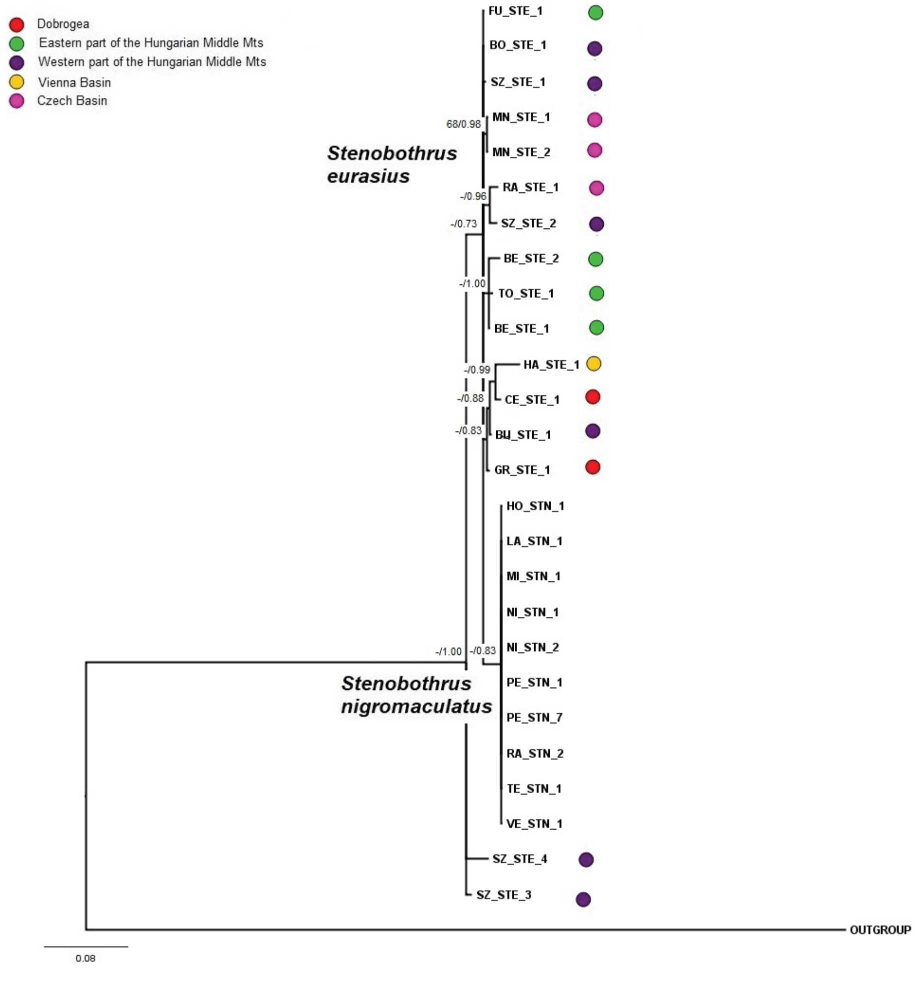

Figure 5. Phylogenetic tree reconstructed based on the neighbor-joining (NJ) and Bayesian Inference (BI) methods using 12S-CR marker. (The numbers on branches indicate the bootstrap values (NJ) and posterior probabilities (BI) being separated by a slash. Different colors within the nodes of *St. eurasius* refer to different sampling sites.)

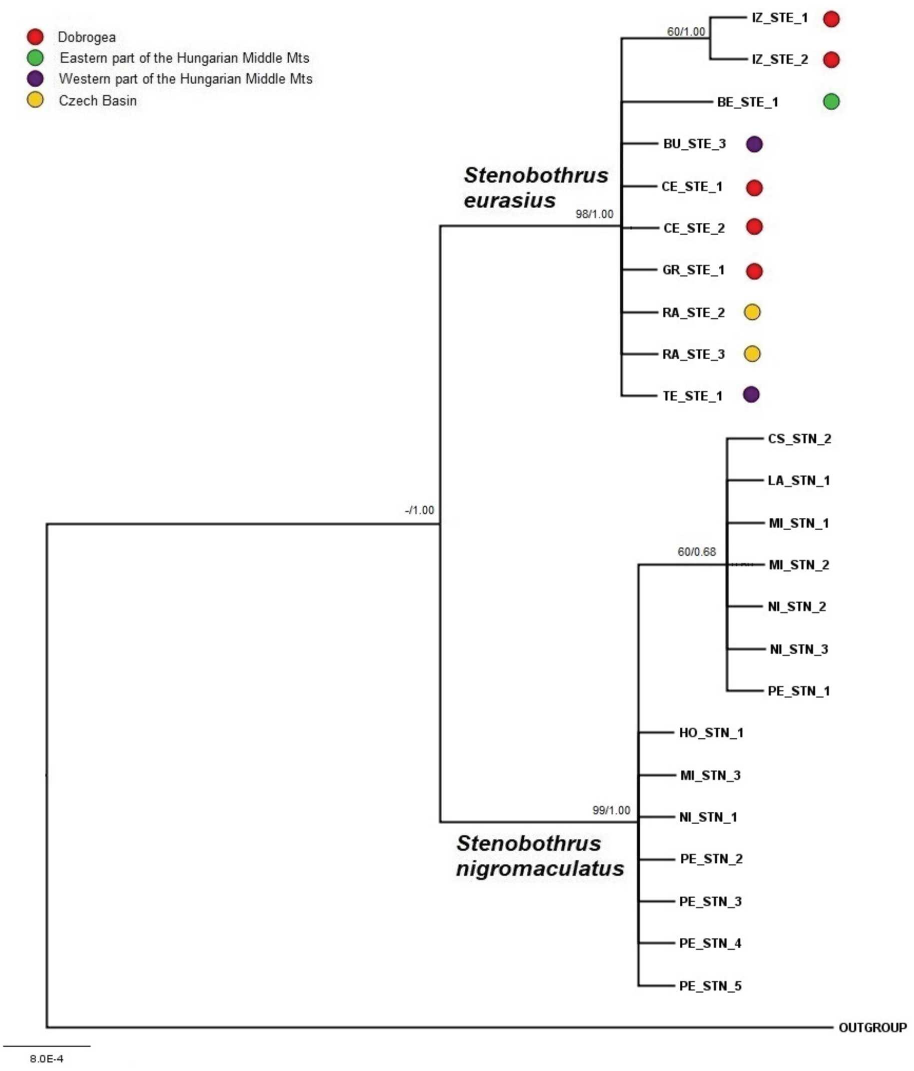

Figure 6. Phylogenetic tree reconstructed based on the neighbor-joining (NJ) and Bayesian Inference (BI) methods using ITS1-5.8S-ITS2. (The numbers on branches indicate the bootstrap values (NJ) and posterior probabilities (BI) being separated by a slash. Different colors within the nodes of *St. eurasius* refer to different sampling sites.)

Table 1. Mitochondrial standard genetic indices calculated for *St. eurasius* species.

	COI				
	EHM	***DOB***	***CZB***	***WHM***	***VIB***
S	5	7	5	11	1
H	3	4	3	5	—
Hd	0.700	0.714	0.700	0.709	—
Pi	0.00351	0.01088	0.00351	0.00447	—
	cytB				
S	6	6	4	11	1
H	5	4	3	7	—
Hd	0.933	0.800	0.833	0.909	—
Pi	0.01279	0.01331	0.01260	0.01283	—

Abbreviations: ***EHM*** – Eastern part of the Hungarian Middle Mts; ***DOB*** – Dobrogea; ***CZB*** – Czech Basin; ***WHM*** – Western part of the Hungarian Middle Mts; ***VIB*** – Vienna Basin; **S** – number of sequences; **H** – number of haplotypes; **Hd** – haplotype diversity; **Pi** – nucleotide diversity.

Table 2. Pairwise DNA sequence differences within *St. eurasius* species.

	COI			
	EHM	**WHM**	**DOB**	**VIB**
WHM	0.004	—	—	—
DOB	0.008	0.008	—	—
VIB	0.008	0.007	0.010	—
CZB	0.004	0.004	0.007	0.006
	cytB			
WHM	0.012	—	—	—
DOB	0.013	0.013	—	—
VIB	0.007	0.008	0.007	—
CZB	0.014	0.013	0.013	0.008

Abbreviations: ***EHM*** – Eastern part of the Hungarian Middle Mts; ***WHM*** – Western part of the Hungarian Middle Mts; ***DOB*** – Dobrogea; ***CZB*** – Czech Basin; ***VIB*** – Vienna Basin

length of DNA fragments (bp) for COI, cytB, 12S-CR, and ITS1-5.8S-ITS2 were shown in Table S4. Sequences of 12S-CR and ITS1-5.8S-ITS2 were used only for the reconstruction of phylogenetic relationships because they were obtained for a limited number of individuals. The neighbor-joining (NJ) and Bayesian analyses (BI) resulted in congruent topologies for all molecular markers. Samples of the two *Stenobothrus* species (*St. eurasius* and *St. nigromaculatus*) were separated on the COI, cytB and ITS1-5.8S-ITS2 trees with high support (PP = 1) in BI (Figs. 3A, 4A, 5) but clear genetic lineages were not revealed, and populations were not grouped according to their geographic locations. Additionally, the two *Stenobothrus* species were not separated based on the 12S-CR marker (Fig. 6).

The complete network of haplotypes of *St. eurasius* and *St. nigromaculatus* was constructed only for COI and cytB genes (Figs 3B, 4B). Three *St. eurasius* haplotypes were shared by samples from different regions in COI (A1, A2, A3, on Fig. 3B) and cytB (B1, B2, B3, on Fig. 4B) genes.

Genetic diversity measures for *St. eurasius* collected in *DOB*, *EHM*, *WHM*, *VIB*, and *CZB* are shown in Table 1. Haplotype diversity (Hd) observed for both mitochondrial markers (COI, cytB) ranged between 0.700 and 0.933. Haplotype and nucleotide diversity were zero in samples collected from *VIB*, as only a single sequence was obtained. Low levels of nucleotide diversity (Pi) were observed across all samples from *EHM* and *CZB* followed by COI. Sequences of the COI and cytB revealed little genetic variation between specimens of *St. eurasius* from different regions (Table 2).

4. Discussion

Taking into account that four loci do not provide enough information for answering detailed population genetic questions, but, given the results obtained using the control taxon (*St. nigromaculatus*), we have to state that our examinations did not find any substantial genetic or morphological differences among the studied occurrences of *St. eurasius*. We expected that mitochondrial and nuclear DNA sequences may resolve relationships within the lineages. Despite the geographic distances of the sampled populations (coordinates see in Table S1), the molecular analysis did not reveal obvious differences. The morphological and molecular resemblance detected between the investigated *St. eurasius* populations suggests that they have not been separated for long.

Our results could be more robust if samples from the Asian zonal distribution of the focal species had been available, but even without the above, data supported our second hypothesis. In Central Europe, in all probability, the recent populations of *St. eurasius* colonized the region in one wave during periods suitable for steppe expansion, covering similar habitats from east to west up to the Czech Basin (Divíšek et al. 2022; Kirschner et al. 2022). Further, in the postglacial period, there were also several waves, which potentially covered, due to the macrogeomorphology of the region, approximately the same areas. In the latter case *St. eurasius* could have recolonized again and again its suitable habitats in the region.

Our results do not support the taxonomic differences outlined in recent publications. Our results reaffirm the position of Hartz (1975) of the doubtful validity of differences at the subspecies level amongst the relic *St. eurasius* populations, known not only in Central Europe but also in other parts of the species range (Willemse 1974). Tarasova et al. (2021) also found only very slight morphological differences (venation of hind wing) between *St. eurasius eurasius* Zubowsky, 1898, and *St. eurasius hyalosuperficies* Vorontsovskii, 1927, but they recently changed the status of the subspecies to the species level. Time requirements of the marked acoustic and morphological differences of a taxon within its area are insufficiently known (Fjeldsaå and Lovett 1997; Orr 2001; Lee and Palci 2015).

According to the basic theory, *St. eurasius* immigrated from the Angarian refuge into Central Europe during a warm period of a postglacial epoch, probably in the Boreal Age (Mařan 1964; Rácz 1998). After that, in the opinion of Ložek (2000), populations in the outposts survived the most humid epoch helped by stone age agriculture on nutrient rich soils, which stopped the expansion of forests on southern foothills. Furthermore, it is known that *St. eurasius* had massive outbreaks in arable land (Bey-Bienko and Mistshenko 1963; Antonova 1965; Sergeev 1998) in the Siberian and Central Asian steppes, suggesting that this habitat is favorable for the species. In contrast, in the Central European outposts, the species has been restricted to relic habitats (Berman et al. 2011) not only because of the Holocene climate fluctuations (cold, rainy periods; warmer in comparison to the Last Glacial Maximum; rainy in the Atlantic age), but also because of the human influence on agrarian habitats by transforming their vegetation and using more and more productive and profitable landuse methods (Wesche et al. 2016; Divíšek et al. 2022).

Recent genetic analyses (Kajtoch et al. 2016; Kirschner et al. 2020; Willner et al. 2021; Chytrý et al. 2022) showed that the presence of distinct clusters of genetically similar individuals of steppe species in Central European relict habitats suggests an ancient (Pleistocene) origin of these populations (Divíšek et al. 2022). Contrary to extrazonal populations having lineages with extrazonal occurrences absent from the zonal steppes, our findings belong to the recent colonisation scenario of steppe species, under which extrazonal populations of steppe species are young descendants of zonal steppe lineages (Kirschner et al. 2020). Our results would support the hypothesis that *St. eurasius* was widespread in the more extensive steppe areas that were once present in the Pannonian and Czech Basin. The extension of agricultural landuse reduced the steppe habitats to remnants concentrated in hilly and middle mountanous regions unsuitable for agriculture: the result is the current patchy distribution of *St. eurasius* in the remaining steppe habitats.

Our findings are the first steps towards understanding the evolutionary history and isolation level of the occurrences of the steppe flagship species. However, certain limitations of our study should be considered when interpreting the results. The genetic analyses were based on four DNA fragments while the ideal molecular diversity analysis should include alternative methods with higher throughput, including next-generation sequencing (Hawlitschek et al. 2023). Genome-wide sequencing data can also yield much more information and increase the resolution of genetic data, which may be limited when analyses are based on few mitochondrial and nuclear genes as it was demonstrated in Orthoptera, especially in Gomphocerinae (Hawlitschek et al. 2017). Despite these limitations, taxonomic and genetic analyses based on selected genes are still widely used to understand the genetic diversity of insects and also allow direct comparison of results with older studies. Further complex genetic, acoustic, and morphological studies are needed to clarify the taxonomic questions and population structure throughout the whole area of the species.

5. Conclusion

Our results suggest that populations of *St. eurasius* colonized Central European steppes at the same time during periods suitable for steppe expansion, covering similar habitats from east to west up to the Czech Basin. The current marginal populations of the species are related to isolated remnant steppe patches occurring within an anthropogenic landscape being fragmented mainly due to agricultural land expansion and other human impacts. The current distribution pattern of the species minimizes the number of potential connections among the recent populations, but they show just a slight genetic and morphological differences. The formerly described taxa (subspecies) were not confirmed by our study. Our results draw attention to the fact that taxonomic and biogeographical questions should be addressed by a combined analysis of distribution pattern, genetic and morphological differences.

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Supplementary Material 1

Tables S1–S4

Authors: Kenyeres Z, Bauer N, Kociński M, Grzywacz B (2024)
Data type: .pdf
Explanation notes: Table S1. Habitats of the studied *Stenobothrus eurasius* specimens (fm = female, m = male). — **Table S2**. Habitats of the involved *Stenobothrus nigromaculatus* specimens (fm = female, m = male). — **Table S3**. Used data for morphological analysis (the meaning of the codes see in Fig. 2. and Table S1)(var1 = b/a, var2 = c/b, var3 = e/d, var4 = g/f, var5 = i/h). — **Table S4**. GenBank accession numbers of *S. eurasius* and *S. nigromaculatus*. In the brackets the length of DNA fragments was presented.

Link: https://doi.org/10.3897/asp.82.e116541.suppl1

Supplementary Material 2

Figure S1

Authors: Kenyeres Z, Bauer N, Kociński M, Grzywacz B (2024)
Data type: .pdf
Explanation notes: Figure S1. Digitised wing morphology of the examined *Stenobothrus eurasius* specimens.

Link: https://doi.org/asp.82.e116541.suppl2

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