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Genetic differentiation of *Liparus glabrirostris* (Curculionidae: Molytinae) populations from the fragmented habitats of the Alps and Carpathian Mountains

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Abstract

Populations of *Liparus glabrirostris* (Curculionidae: Molytinae), a weevil inhabiting higher altitudes of Central Europe, were sampled from 24 localities in the Alps and Carpathian Mountains, and the geographical structuring of genetic variation was analyzed. Comparison of the concatenated mitochondrial cytochrome oxidase subunit I and subunit II sequences revealed consistent genetic divergence between the populations of *L. glabrirostris* from different mountain ranges. In phylogenetic analysis using maximum parsimony and median-joining networks, concatenated mitochondrial haplotypes from the Alps and Carpathians clustered as separate lineages, with high bootstrap support. Substantial genetic distances determined between the separated groups ranged from 2.6 to 3.0%, with divergence estimated to have initiated approximately 0.85–0.98 million years ago. The nuclear elongation factor 1α gene was additionally amplified and haplotype analysis showed very low evolutionary divergence (0.2%), with separate clustering as well. The observed divergence suggests that the populations have been isolated for a long time, as a consequence of environmental changes resulting in varying fragmentation of habitats in the Alps and Carpathians, interrupting genetic exchange events and altering the genetic structure of *L. glabrirostris* populations. On the other hand, comparison of morphological characteristics showed no differences to confirm genetically well differentiated groups of populations. A polymerase chain reaction and restriction fragment length polymorphism-based method was therefore developed to discriminate between the Alpine and Carpathian lineages.

Keywords: *Liparus glabrirostris*, mitochondrial DNA, nuclear elongation factor 1α, allopatric speciation

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Introduction

Great distances or geographical barriers are major factors preventing gene flow between populations, triggering genetic divergence (Campbell *et al.*, 1999). Intense geological processes such as glaciation, emergence of mountain ranges, formation or disruption of land bridges, formation of lakes, land

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erosions, etc. can fragment habitats and facilitate further range shift, adaptation and diversification of different species (Riddle, 1996). The range shifting of populations caused by habitat fragmentation is one of the mechanisms driving the large changes in species distributions and population differentiation.

Analysis of DNA sequences is often a method of choice in biogeographic studies to infer divergence, diversification and changes in population distributions produced by range shifts. Mitochondrial genes are powerful markers in terms of high mutation rates, small effective population size and maternal inheritance, successfully used as evolutionary tools to investigate the taxonomic status of populations, genetic variation, divergence patterns and allopatric or sympatric speciation, including cryptic endemism as well (Hernańdez-Vera *et al.*, 2010; Toševski *et al.*, 2011, 2013; Stepanović *et al.*, 2015). Nuclear genes on the other hand are more conservative, biparentally inherited with larger effective population size but still can contribute to analysis of phylogenetic relatedness and record gene flow events.

The Earth has experienced the Quaternary glaciation, which refers to a series of glacial and interglacial events from approximately 2.6 million years ago (mya) to the present day (Elias, 2007). Molecular biogeographic studies of different plant and animal species showed variable effects of historical events during the Quaternary on distribution of genetic diversity, evolutionary diversification and speciation (Riddle, 1996; Schmitt, 2007). Many different taxa survived the glacial periods within geographically separate refugia of mountainous areas, which resulted in genetic differentiation between the refugial areas and spatial structuring of distinct genetic lineages (Provan & Bennet, 2008).

The genus *Liparus* Olivier, 1807 (Curculionidae: Molytinae) contains some of the largest weevils of the European fauna with body length ranging from 9 to 20 mm (Reitter, 1924). It includes about 15 Palearctic species present in the mountainous regions of Central and Southern Europe (Hoffmann, 1954). *Liparus* species prefer xerothermic and shady habitats, namely humid mountain forests, near rivers where they live on plants from the families Asteraceae and Apiaceae.

Liparus glabrirostris Küster 1849 is a weevil inhabiting mainly mountainous and upland areas in the central part of Europe, from the Pyrenees to the Carpathians (Hoffmann, 1954). It has been reported from the Spanish, Italian and French mainlands; and from Germany, Austria, Poland, Slovakia, the Czech Republic, Ukraine, Denmark, Switzerland, Lithuania and Latvia (Hoffmann, 1954; Wanat & Mokrzycki, 2005; Germann & Luscher, 2007; Benedikt *et al.*, 2010; Balalaikins & Bukejs, 2012; https://www.faunaeur.org). This weevil chooses slopes with altitudes up to 2000 m, dwelling in moist areas close to the shores of lakes and banks of rivers and streams. The adults of *L. glabrirostris* reach up to 19 mm in length and can be found feeding on the host plants from April until August (Hoffmann, 1954). Larvae consume the roots of plants from the family Asteraceae, most often from the genera *Heracleum* (e.g., *H. pyrenaicum*) and *Petasites* (e.g., *P. officinalis, P. hybridus, P. albus*) (Reitter, 1924; Hoffmann, 1954).

Liparus glabrirostris has been reported in the literature as inhabiting both the Alps and Carpathian Mountains. The Alps are the highest and most extensive range of Europe, covering approximately 1200 km and stretching from Italy through France, Switzerland and Austria up to Germany (Williams & Ferregno, 2005). The mountains emerged around 65 mya during the Mesozoic Era, with the landscape further modeled throughout the Quaternary glaciation (Elias, 2007). The Alps are divided into Western, Central and Eastern segments, each further fragmented into several distinct ranges (Williams & Ferregno, 2005). Great variations in the elevations, shapes and exposure of the Alps result in distinct differences in climate within and between the ranges and consequently in diversity of plants and animal species present at different altitudes. The Carpathian Mountains are a chain of mountains forming an arc about 1500 km long across Central and Eastern Europe (Elias, 2007). They are divided into regions of the Western (Czech Republic, Poland, Slovakia), Eastern (Southeastern Poland, Eastern Slovakia, Ukraine, Romania) and Southern (Romania, Serbia) Carpathians. The Carpathians do not actually form a continuous chain, but consist of several geologically distinct groups of mountains, with a structural variety as great as that of the Alps.

In view of the specificity of both mountain ranges and the flightless behavior of the weevil, the aim of this study was to evaluate the impact of habitat fragmentation on genetic divergence of the insect populations and selection pressure imposed by their geographical isolation. The geographical structuring of genetic variation was investigated using mitochondrial cytochrome oxidase subunit (mtCOI) I and subunit II (mtCOII) sequence data. An additional nuclear marker was employed to elucidate the phylogenetic relationship between the separated taxa from the Alps and Carpathians. Distinctive morphological differences were looked for to support the molecular evidence of geographical structuring of *L. glabrirostris* populations from different mountain ranges. A polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP)-based diagnostic method was developed to discriminate between the Alpine and Carpathian populations.

Material and methods

Sampling area of insect material

In 2006, 2014 and 2015, *L. glabrirostris* adults were sampled from 19 localities in the Carpathian Mountains (Ukraine, Slovakia, Czech Republic) and five in the Alps (Austria, Switzerland) (table 1). ArcGIS® software (Esri, California) was used to map the distribution of *L. glabrirostris* samples collected from 24 localities in five countries and quantify the values of environmental variables in the distribution area. The sampled adults were stored in 96% ethanol at −20°C until subjected to molecular and morphological analyses. Adults of a congener *Liparus germanus* were sampled at one locality in Switzerland and one in the Czech Republic and used as an outgroup in phylogenetic analyses (table 1).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from individuals using the QIAGEN Dneasy® Tissue Kit. Two holes were punched in the abdomen of adults prior to incubation overnight at 56°C. After the weevils were removed from the buffer the following day, the remaining solution was treated following the manufacturer's instructions. All specimens have been deposited in the collection of the Department of Plant Pests in Belgrade.

We used the two mitochondrial genes COI and COII to analyze geographic structure of population differences. Additionally, the nuclear elongation factor 1α gene (EF-1α) was employed to elucidate phylogenetic relationships

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Table 1. The list of sampling localities for *Liparus glabrirostris* populations with concatenated mtCOI and COII haplotypes and EF-1α haplotypes.

Country	Sampling locality	GPS coordinates	Altitude (m)	Samples ID	Haplotypes detected[1]
Czech Republic	Chvalčov, Hostýnské vrchy	N 49,39222222 E 17,70527778	386	S1, S2, S3, S4, S5, S6, S14, S15, S30, S31	H1T3, H6T2, H5T7, H6T6, H4T2, H6T7, Ec1
Czech Republic	Strážné. lom	N 50,66370400 E 15,61505500	781	S32, S33, S51	H2T6
Czech Republic	Obří Důl, Krkonoše	N 50,71055556 E 15,72611111	924	S35, S36, S37	H2T6
Czech Republic	Horni Maršov, Krkonoše	N 50,66666667 E 15,81944444	657	S55, S58, S59	H2T6
Czech Republic	Slámova chata, Hostýnské vrchy	N 49,38916667 E 17,71138889	381	S71	H6T5
Czech Republic	Rajnochovice, Hostýnské vrchy	N 49,41298700 E 17,81318200	402	S72, S73, S75	H2T6, H6T7
Czech Republic	Tesák	N 49,37264700 E 17,78910700	630	S76, S77, S79, S80, S83	H6T3, H6T5, H6T7
Czech Republic	Kozlůvky, Chvalčov	N 49,40672800 E 17,78177700	624	S82	H6T7, Ec3
Czech Republic	Troják	N 49,35398200 E 17,81535700	582	S84, S85, S86	H6T7, H2T7
Czech Republic	Májová-Držková	N 49,32398200 E 17,78689300	465	S87, S88, S89	H6T7
Czech Republic	Rusava	N 49,34465700 E 17,70867800	570	S90, S91, S93, S94	H1T1, H5T7
Czech Republic	Kozlůvky right river bank	N 49,30117000 E 18,06461200	408	S95, S98	H6T2, H6T7
Czech Republic	Kozlůvky left river bank	N 49,32489500 E 18,03920600	633	S23, S99, S100	H6T7
Czech Republic	Mala Moravka, M. Jeseník	N 50.02111111 E 17.31194444	1350	S143, S144, S145	H4T2, Ec1
Slovakia	Slanske vrchy	N 48,78423800 E 21,25126600	235	S27	H6T7
Slovakia	Belianske Tatry Mts., dolina Siedmi pramenov	N 49,24213100 E 20,21579000	1727	S28, S29	H6T7
Slovakia	Vysoke Tatry, Bielovodska dolina	N 49,19163500 E 20,11676800	1310	S25, S26	H2T6, H6T7, Ec1
Slovakia	Gaderská dolina, K.n. Blatnica, Velika Fatra	N 48,91666667 E 18,94694444	592	S65, S66, S68, S69, S70	H6T4, H6T7, Ec2
Ukraine	Carpathian Biosphere Reserve Kuzii Rachiv	N 47,59431430 E 24,11698700	1177	S19, S20, S107, S108, S109	H3T6, H4T6, Ec1
Austria	Puchenstuben, Treffling valley	N 47,93333333 E 15,28333333	958	S7, S8, S9, S10, S11, S12, S13, S16, S47, S48, S49, S50, S113, S114, S115, S116, S117, S118, S119, S120, S121	H8T9, H7T9, H7T8, H8T8, Eg1
Switzerland	Märenschlag nr. Alpnach canton Obwalden	N 46,96046000 E 8,20412400	1310	S38, S39[2], S40[2]	H8T8, Eg2
Switzerland	Dtto, site 2, Alpnach canton Obwalden	N 46,96223220 E 8,20894200	1433	S41, S42, S60	H8T8, Eg1, Eg2
Switzerland	Dtto, site 3, Alpnach canton Obwalden	N 46,96154900 E 8,20566400	1364	S61, S62	H7T8, H8T8

Continued

Table 1. (*Cont.*)

Country	Sampling locality	GPS coordinates	Altitude (m)	Samples ID	Haplotypes detected[1]
Switzerland	Dtto, site 4, Alpnach canton Obwalden	N 46,95672900 E 8,21394400	1198	S64	H8T8, Eg2
Czech Republic	Těchobuz, nr. Pacov	N 49,52193700 E 14,94485400	563	S22[2]	*Liparus germanus*

[1]T1–T9 mtCOII haplotypes and H1–H8 mtCOI haplotypes are concatenated for 87 samples forming 18 haplotypes of 1394 bp long sequences; Ec1–Ec4 – Carpathian elongation factor EF-1α haplotypes, Eg– Eg2 – Alpine elongation factor EF-1α haplotypes.
[2]Collected *Liparus germanus* used as outgroup to root phylogenetic trees.

between the taxa separated on the basis of mitochondrial DNA (mtDNA) sequences.

A partial sequence of the mtCOI gene was amplified using the primer pair HCOf/TL2-N-3014 (Simon *et al.*, 1994) (table 2). Each PCR was carried out in a volume of 20 µl, containing 1 µl of extracted DNA, 11.8 µl of H_20, 2 µl of High Yield Reaction Buffer A (with 1 × mg), 1.8 µl of $MgCl_2$ (2.25 mM), 1.2 µl of dNTP (0.6 mM), 1 µl of each primer (0.5 µM) and 0.2 µl of DNA polymerase (0.05 U μl^{-1}). The amplification protocol included initial denaturation at 95°C for 5 min, 40 cycles consisting of 1 min at 95°C, 1 min at 45°C, 2 min at 72°C and final extension at 72°C for 10 min.

The mtCOII gene was amplified using the primers TL2-J-3038 (Emerson *et al.*, 2000) and TK-N 3782 (Harrison Laboratory, Cornell University, Ithaca, NY, USA) (table 2). The amplification reaction ran in a volume of 20 µl, containing 1 µl of extracted DNA, 9.45 µl of H_20, 2 µl of High Yield Reaction Buffer A (with 1 × mg), 2.8 µl of $MgCl_2$ (3.5 mM), 1.6 µl of dNTP (0.8 mM), 1.5 µl of each primer (0.75 µM) and 0.15 µl of KAPA*Taq* DNA polymerase (0.0375 U μl^{-1}) (Kapabiosystems). The PCR protocol included initial denaturation at 95°C for 5 min, 40 cycles consisting of 1 min at 95°C, 1 min at 54°C and 2 min at 72°C; and final extension at 72°C for 10 min.

The nuclear EF-1α gene was amplified with the primer pair EF1-Bf/EF1-Br (Hernández-Vera *et al.*, 2013) (table 2). The PCR reactions ran in volumes of 20 µl containing 1 µl of extracted DNA, 11.8 µl of H_20, 2 µl of High Yield Reaction Buffer A (with 1 × mg), 1.8 µl of $MgCl_2$ (2.25 mM), 1.2 µl of dNTP (0.6 mM), 1 µl of each primer (0.5 µM) and 0.2 µl of DNA polymerase (0.05 U μl^{-1}). The amplification protocol consisted of initial denaturation at 94°C for 2 min, 40 cycles consisting of 45 s at 94°C, 60 s at 50°C and 90 s at 72°C; and a final extension at 72°C for 7 min.

Amplified products of the three genes were run on 1% agarose gel, stained with ethidium bromide and visualized under a UV transiluminator. DNA sequencing was performed using automated equipment (Macrogen Inc., Korea), and the COI, COII and EF-1α sequences were deposited in the GenBank database (www.ncbi.nlm.nih.gov).

Phylogenetic analyses

Sequences of COI, COII and EF-1α were manually edited in FinchTV ver.1.4.0 (www.geospiza.com) and aligned using the Clustal*W* program integrated in MEGA5 (Tamura *et al.*, 2011). Concatenated COI and COII sequences were submitted to maximum-likelihood best fit model analysis using the MEGA5 program. According to the obtained Akaike Information Criterion scores, out of 24 different nucleotide substitution models, the Tamura–Nei model was the best fit model for estimation of evolutionary divergence between the concatenated mitochondrial sequences (Tamura & Nei, 1993). A maximum parsimony (MP) tree was constructed using the MEGA5 software, with 500 bootstrap replicates performed to assess the branch support. *Liparus germanus* was used as an outgroup to root the tree. A median-joining network using a MP calculation (Bandelt *et al.*, 1999) was constructed for concatenated COI and COII haplotypes and EF1-α with the NETWORK ver. 4.6.1.2 program (http://www.fluxus-engineering.com).

Published data suggest that mitochondrial divergence rates for arthropods range from 1.2 to 4.9% per million years (Brower, 1994; Wares, 2001; DeSalle *et al.*, 2005). We apply a

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Table 2. The list of primers used to amplify mitochondrial and nuclear gene fragments.

Gene	Primer abbreviation	Primer sequence
COI	HCOf	5′ TGATTTTTTGGTCAGCCTGAAGTTTA 3′
	TL2-N-3014	5′ TCCAATGCACTAATCTGCCATATTA 3′
COII	TL2-J-3038	5′ TAATATGGCAGATTAGTGCATTGGA 3′
	TK-N 3782	5′ GAGACCATTACTTGCTTTCAGTCATCT 3′
EF-1α	EF1-Bf	5′ AGAACGTGAACGTGGTATCA 3′
	EF1-Br	5′ CTTGGAGTCACCAGCTACATAACC 3′

mean estimate rate of 3.05% divergence per million years, on the basis of the previously reported weevil research of Hernańdez-Vera *et al*. (2010), Toševski *et al*. (2011) and Toševski *et al*. (2013).

Morphological analysis

Collected specimens were identified using the descriptions given by Reitter (1924) and Hoffmann (1954). Body size was compared by measuring the length with the rostrum excluded. Specimens were analyzed for morphological peculiarities. All collected males were analyzed for genitalia differences, with length and width of the aedeagus measured for each specimen. All measured parameters were analyzed using the Statistica 8.0 software package (Weiss, 2007).

RFLP analysis for discrimination of taxa

In the absence of clear morphological characters to support the molecular divergence, we have developed a PCR–RFLP method to differentiate between the Alpine and Carpathian populations. Sequences of all identified haplotypes of the COI and COII genes were first virtually digested using the pDRAW32 software (AcaClone Software, http://www.acaclone.com) to identify specific discriminating sites and determine the suitable restriction enzyme(s). Thereafter, digestion reactions were performed with COI and COII amplicons using the restriction enzymes selected according to the obtained virtual RFLP patterns. Restriction analyses were performed at 37 or 65°C for 16 h according to the manufacturer's instructions (Fermentas, Lithuania).

Restriction products were separated by the QIAxcel advanced system (Qiagen) for automated capillary electrophoresis, using a high resolution (Qiagen). The QX alignment marker for 15 bp/5 kb (Qiagen) was used to align the resulting restriction fragments and the QX DNA size marker phiX174/HaeIII (Qiagen) for fragment size comparisons. Finally, virtual restriction patterns were compared with the RFLP profiles obtained for COI and COII haplotypes to confirm trueness of the diagnostic PCR/RFLP protocol.

Results

Distribution area of L. glabrirostris

The weevil's presence was recorded at 24 sites, from slopes to higher altitudes in the Alps and Carpathians, throughout the Czech Republic, Slovakia, Ukraine, Switzerland and Austria (table 1, fig. 1). In total 90 adults of *L. glabrirostris* were collected between May and September, from *Petasites* sp., mostly at semi-shaded sites and in humid forests. In the Western Carpathians, material was collected from 14 sites in the Czech Republic at altitudes of from 381 to 924 m with average annual temperatures ranging from 3.5 to 7.8°C and mean annual precipitation of 739.4 ± 118.7 mm. The sampling sites in Slovakia were at altitudes ranging from 235 to 1727 m in places with annual temperatures from 0 to 8.2°C and average annual precipitation of 1131 ± 389.8 mm. Those in the Eastern Carpathians were located in the Carpathian Biosphere Reserve in Ukraine, at an altitude of 1177 m with average annual temperature of 5.1°C and precipitation of 831 mm. In the Alpine region, weevil specimens were collected from one site in Austria (958 m; t_a = 5.6°C; annual precipitation of 963 mm); and four localities in Switzerland (1198–1433 m; annual temperatures of 4–5.2°C; annual precipitation of 1545 ± 62.7 mm).

Analysis of concatenated mtCOI and mtCOII fragments

Fragments of the mtCOI gene were amplified and sequenced for 90 specimens of *L. glabrirostris*, covering all sampled localities across the Carpathians and Alps (table 1). A total of six COI haplotypes were identified among specimens collected from the Carpathian Mountains, designated as H1, H2, H3, H4, H5 and H6 (table 3). A comparison of the 756 bp long mtCOI sequences showed eight mutations, three of which produced amino acid substitutions. In the case of the Alpine group, only two COI haplotype (H7 and H8) differing in a single non-synonymous nucleotide mutation were detected. Comparison of H7 and H8 with the six haplotypes from the Carpathian group detected 32 mutations, 28 being parsimony informative and seven causing amino acid substitutions. Sequences of the mtCOII gene were successfully amplified for 87 specimens from all of the investigated localities (table 1). In comparison of COII sequences, seven haplotypes (T1–T7) were determined in the Carpathian group and two (T8, T9) in specimens from the Alps (table 1). Analysis of 638 bp long sequences of T1–T7 haplotypes detected eight mutations, four of which induced amino acid substitutions. A total of two haplotypes were collected in the Alps, i.e. T8 and T9, differing in a single nucleotide. On the other hand, these haplotypes diverged substantially from the Carpathian group with 18 mutations detected, 12 of which parsimony informative, while eight amino acid substitutions were produced.

Sequences of COI and COII fragments were concatenated for 87 specimens, producing 1394 bp long sequences submitted to evolutionary divergence estimates and used for construction of the phylogeny tree and median-joining network. The names assigned to concatenated haplotypes consist of the name of a certain COI (H1–H8) and COII haplotypes (T1–T9). In total 18 concatenated mitochondrial haplotypes were identified, 14 from the Carpathian Mountains and four from the Alpine region (tables 1 and 4). The most dominant Carpathian haplotype was H6T7, determined within 21

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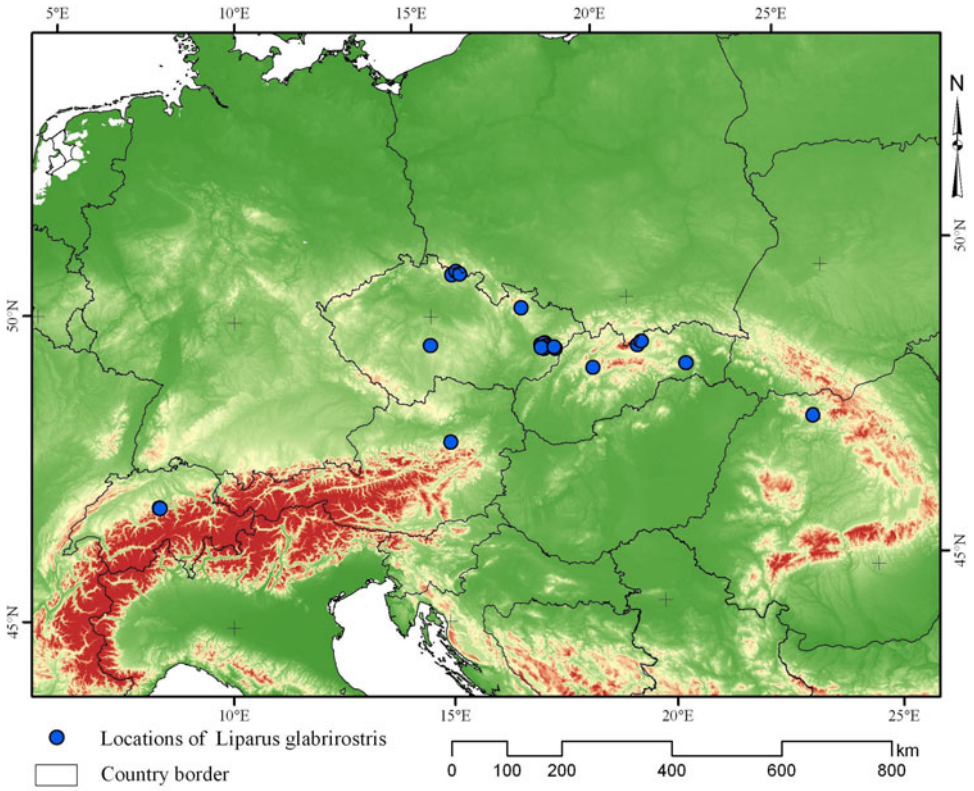

Fig. 1. Sampling localities of *Liparus glabrirostris* populations in the Alps and Carpathians.

Table 3. The list of mtDNA COI and COII haplotypes identified within the *Liparus glabrirostris* populations sampled from the Carpathians and Alps, including the outgroup species *Liparus germanus* and accession numbers assigned after submission to the GeneBank.

Mitochondrial region	Haplotype	Accession number in GeneBank
COI	H1	KU199741
	H2	KU199742
	H3	KU199743
	H4	KU199744
	H5	KU199745
	H6	KU199746
	H7	KU199747
	H8	KU199748
	Liparus germanus	KU199740
COII	T1	KU199750
	T2	KU199751
	T3	KU199752
	T4	KU199753
	T5	KU199754
	T6	KU199755
	T7	KU199756
	T8	KU199757
	T9	KU199758
	Liparus germanus	KU199749

specimens sampled from eight localities in the Czech Republic and four in Slovakia. Haplotype H2T6 was reported for 12 weevils collected from both, Czech Republic and Slovakia. The remaining 12 concatenated mitochondrial haplotypes of Carpathian origin were detected in one to four specimens collected either in the Czech Republic (H1T1, H1T3, H2T7, H4T2, H5T7, H6T6, H6T2, H6T5 and H6T3), Slovakia (H6T4) or Ukraine (H3T6 and H4T6). The prevalent mitochondrial haplotype in the Alps was H8T8, which was registered in 19 specimens collected from all inspected sites in Switzerland and Austria (table 4). The other three haplotypes (H7T9, H7T8 and H8T9) were detected in one to six weevils.

The topology of the MP tree revealed the existence of two separate mitochondrial lineages (fig. 2). The first group of 14 haplotypes included specimens of *L. glabrirostris* collected in the Western and Eastern Carpathians which clustered with bootstrap support of 98%. On the other hand, the four concatenated mitochondrial haplotypes originating from the Alps, i.e. specimens from Switzerland and Austria grouped with 99% support (fig. 2). The average evolutionary divergence between the 14 haplotypes in the Carpathian group as estimated with the Tamura–Nei model was 0.3% (range of 0.2–0.6%), while the four Alpine haplotypes differ by only 0.1%. On the contrary, the average divergence rate between the two separate mitochondrial lineages ranged from 2.6 to 3.0%. When compared with *L. germanus* used as an outgroup, both the Alpine and the Carpathian concatenated mitochondrial haplotypes of *L. glabrirostris* diverged significantly from 19.5 to 20.0%.

The median-joining network recognized the same two groups of concatenated mitochondrial haplotypes, Alpine and Carpathian connected with a confidence limit of 95% (fig. 3). In total, 33 mutational steps were recognized

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Table 4. The list of concatenated mitochondrial haplotypes identified within the *Liparus glabrirostris* populations sampled from the Carpathians and Alps.

Haplotypes of concatenated COI and COII sequences[1]	Samples	Country of origin (no. of localities with sampled haplotype)
H1T1	S90, S91	Czech Republic (1)
H1T3	S31	Czech Republic (1)
H6T3	S80	Czech Republic (1)
H6T4	S66, S68, S69, S70	Slovakia (1)
H6T5	S71, S76, S79	Czech Republic (2)
H6T2	S5, S95, S15	Czech Republic (2)
H5T7	S93, S14	Czech Republic (2)
H6T7	S1, S3, S4, S6, S23, S26, S27, S28, S29, S65, S73, S77, S82, S83, S85, S86, S87, S88, S89, S98, S99	Czech Republic (8), Slovakia (4)
H6T6	S2	Czech Republic (1)
H3T6	S107, S108, S109	Ukraine (1)
H2T6	S25, S32, S33, S35, S36, S37, S51, S55, S58, S59, S72, S75	Czech Republic (4), Slovakia (1)
H2T7	S84	Czech Republic (1)
H4T6	S20	Ukraine (1)
H4T2	S30, S143, S144, S145	Czech Republic (2)
H8T8	S7, S8, S9, S10, S11, S12, S13, S16, S38, S41, S42, S47, S48, S49, S60, S61, S64, S119, S120	Switzerland (4), Austria (1)
H8T9	S50	Austria (1)
H7T9	S114, S117	Austria (1)
H7T8	S62, S113, S115, S116, S118, S121	Switzerland (1), Austria (1)

[1]The name of concatenated mitochondrial haplotypes consists of the particular COI sequence (H1–H8) and COII sequences (T1–T9).

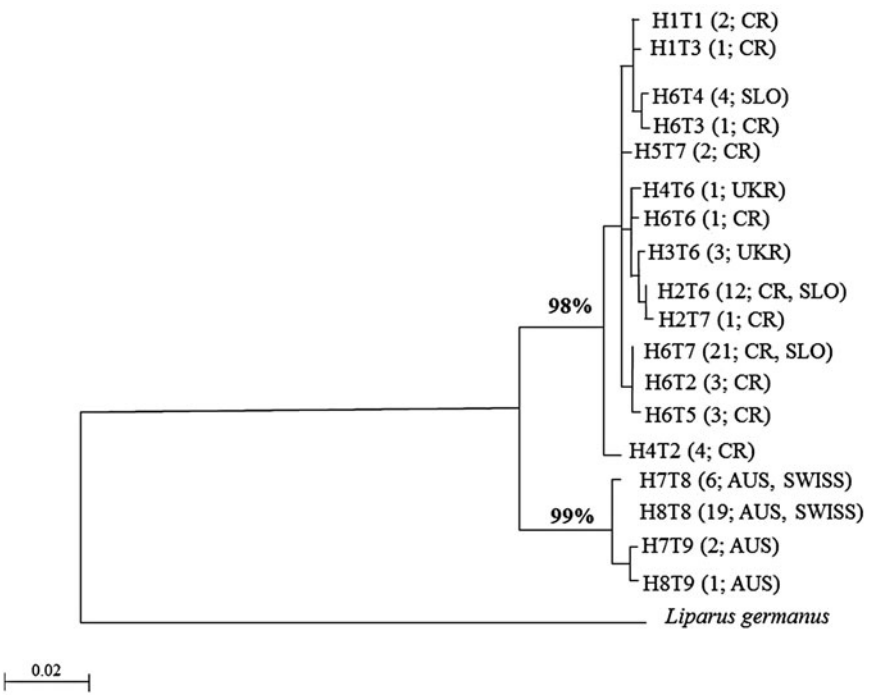

Fig. 2. Phylogenetic tree based on the concatenated COI and COII fragments obtained using the MP method. There were a total of 1394 positions in the final dataset. Bootstrap values >90% are indicated above the branches. The scale bar indicates the number of substitutions per site. Branch lengths are in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the Tamura–Nei parameter method. Carpathian haplotypes: H1T1, H1T3, H2T6, H2T7, H3T6, H4T6, H4T2, H5T7, H6T7, H6T6, H6T2, H6T4, H6T5, H6T3; Alpine haplotypes – H7T8, H7T9, H8T8, H8T9. The numbers and letters in parentheses refer to the number of sequences for each haplotype and geographical origin of sequences, respectively. Abbreviations for countries of origin: CR – Czech Republic, SLO – Slovakia, UKR – Ukraine; SWISS – Switzerland, AUS – Austria.

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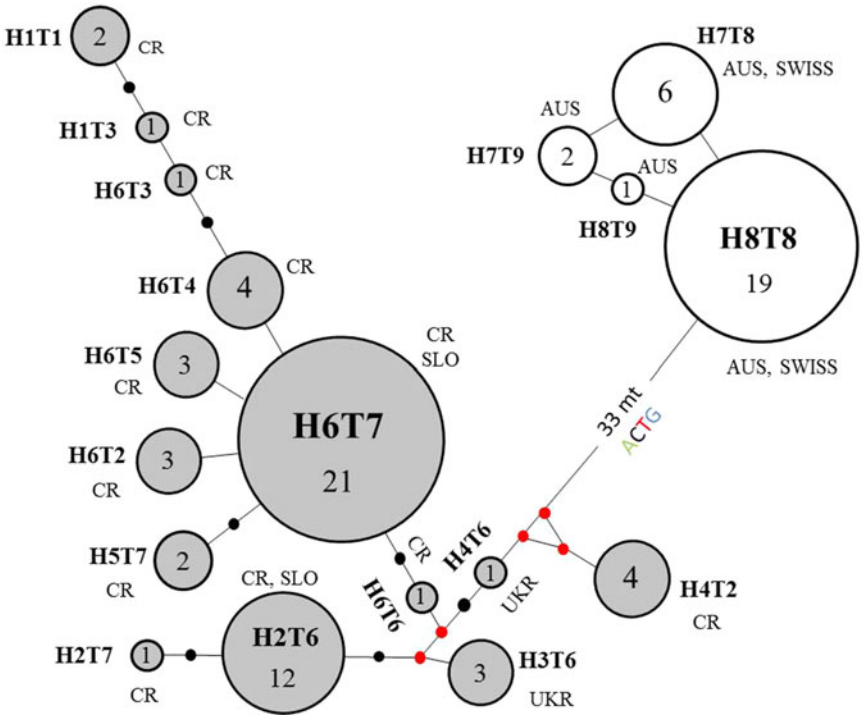

Fig. 3. Median-joining network of concatenated mtCOI and COII haplotypes obtained for 87 *Liparus glabrirostris* specimens. Gray circles represent specific haplotypes from the Carpathian Mountains, white circles ones from the Alps. Circle size reflects the number of individuals with that haplotype (not to scale). Numbers in circles refer to the number of specimens with a particular haplotype. Smaller black circles represent missing haplotypes; red circles are median vectors. Carpathian haplotypes: H1T1, H1T3, H2T6, H2T7, H3T6, H4T6, H4T2, H5T7, H6T7, H6T6, H6T2, H6T4, H6T5, H6T3; Alpine haplotypes – H7T8, H7T9, H8T8, H8T9. Lines between the circles are mutational steps. Due to the large number of mutational steps between the two groups, nucleotide substitutions were designated as 33 mt ACTG. Geographical distribution of the sequenced specimens is abbreviated next to the haplotype circles (SLO – Slovakia, CR – Czech Republic, UKR – Ukraine, SWISS – Switzerland, AUS – Austria).

Table 5. The list of nuclear EF1-α haplotypes identified within the *Liparus glabrirostris* populations sampled from the Carpathians and Alps.

Haplotype	Sequences codes	Country of origin (number of localities with sampled haplotype)	Accession number in GeneBank
Ec1	S1, S2, S4, S6, S26, S143, S144, S145, S109	Czech Republic (2), Slovakia (1), Ukraine (1)	KU199759
Ec2	S66	Slovakia (1)	KU199760
Ec3	S82	Czech Republic (1)	KU199761
Eg1	S7, S12, S47, S48, S49	Austria (1)	KU199762
Eg2	S38, S41, S42, S60, S64	Switzerland (3)	KU199763

connecting the two separated groups of sequences with no ambiguities (fig. 3).

Analysis of the nuclear EF-1α

For amplification of the nuclear elongation factor, 19 specimens were selected, covering both groups separated according to mitochondrial genes analysis (table 5). All nuclear sequences were successfully amplified. The coding region of EF-1α was unambiguously aligned due to the absence of insertion or deletion mutations. The final data set of indel-free 572 bp long sequences was compared and five haplotypes were detected, three within the specimens from the Carpathians (Ec1, Ec2 and Ec3) and two in ones from the Alps (Eg1 and Eg2) (table 5). The average evolutionary distance between the EF-1α haplotypes was 0.2% (range from 0.2 to 0.5%). Overall, five mutations were detected in nuclear sequences, with only one causing the amino acid substitution.

The median-joining network shows the connection between EF-1α haplotypes from the Carpathian Mountains and the Alps (fig. 4).

PCR–RFLP-based method for discrimination of L. glabrirostris *mitochondrial lineages*

Virtual restriction analysis identified *Hpy188*III (New England) and *Bfa*I (Fermentas, Lithuania) as restriction enzymes suitable to distinguish mtCOII haplotypes of the two separated *L. glabrirostris* lineages. Digestion of 731 bp long COII sequences with the *Bfa*I (C'TA_G) endonuclease

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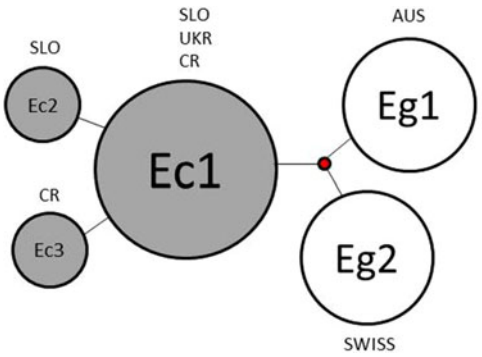

Fig. 4. Median-joining network of EF-1α haplotypes obtained for 19 *Liparus glabrirostris* specimens. Gray circles represent specific haplotypes from the Carpathian Mountains (Ec1, Ec2, Ec3), white from the Alps (Eg1, Eg2). Circle size reflects the number of individuals with that haplotype (not to scale). Lines between the circles are mutational steps and the red circle is a median vector. Geographical distribution of the sequenced specimens is abbreviated next to the haplotype circles (SLO-Slovakia, CR – Czech Republic; UKR – Ukraine; SWISS – Switzerland; AUS – Austria).

recognized two restriction sites at the 207- and 543-bp positions for the Carpathian haplotypes, while sequences from the Alpine group have only one recognition site at the 207-bp position (fig. 5). In the case of the *Hpy188*III enzyme (TC'nn_GA), both groups of COII haplotypes have two recognition sites, sharing the one at the 111-bp position, but differing with respect to the other which was determined at the 207- bp and 526-bp positions in the Carpathian and the Alpine groups, respectively (fig. 5). The actual digestion performed with the *Bfa*I and *Hpy188*III restriction enzymes produced the same profiles as the virtual RFLP, clearly discriminating the Carpathian from the Alpine haplotypes (fig. 5).

Virtual digestion of 821 bp long COI sequences with available restriction enzymes showed that *Tai*I and *Tfi*I (Fermentas, Lithuania) are suitable for diagnostics (fig. 6). The *Tai*I (_ACGT') endonuclease cuts the Carpathian COI sequences at the 401- and 723-bp positions, with fragment sizes of 401, 322 and 98 bp. The Alpine haplotypes are cut at the 723 bp position, with fragment sizes of 723 and 98 bp (fig. 6). The *Tfi*I (G'AwT_C) restriction enzyme recognized two restriction sites at the 32- and 470-bp positions in the Carpathian group, while in the Alpine group only one restriction site was recognized, at the 32 bp position. The RFLP analyses performed with two restriction enzymes showed no differences compared with the virtual gel, confirming the suitability of *Tai*I and *Tfi*I for discrimination between the two groups of haplotypes (fig. 6).

Morphological analysis

All examined *L. glabrirostris* specimens fit the descriptions of Reitter (1924) and Hoffmann (1954) (fig. 7). Body length was measured for all 62 *L. glabrirostris* adults originating from the Carpathians (group 1) and 28 specimens from the Alps (group 2). The average length of adults was 15.96 ± 0.45 cm in group 1 and 16.07 ± 0.79 cm in group 2. In analysis of variance (ANOVA), no statistically significant difference in body length was detected between the weevil populations sampled from the Carpathians and the Alps (ANOVA: $F = 0.82$; significance level $P < 0.05$).

All males in the collected material, i.e. 69 males from the Carpathian group and 13 from the Alps, were analyzed for genitalia differences. No deviation in morphology was detected. The aedeagus of all specimens is rectangular, narrowed along the apical margin, slightly depressed (fig. 7). Mean width of the aedeagus measured for males from the Carpathians was 1.93 ± 0.06 mm (range: 1.8–2.0 mm; median 1.9 mm), its mean length 3.89 ± 0.06 mm (range: 3.8–4.0 mm; median 3.9 mm). Males from the Alps have an aedeagus on the average 1.90 ± 0.06 mm wide (range: 1.8–2.0 mm; median 1.9 mm) and 3.93 ± 0.08 mm long (range: 3.85–4.05 mm; median 3.9 mm). ANOVA showed no statistically significant differences in width ($F = 2.69$; $P < 0.05$) and length of the aedeagus ($F = 3.81$; $P < 0.05$) between males of *L. glabrirostris* collected from the Alps and the Carpathians.

Discussion

At a broad-scale our results indicate the existence of two monophyletic mitochondrial haplogroups inhabiting the Alps and Carpathians, with no evidence of haplotypes overlapping. It appears that the Carpathians are colonized by one common haplotype (H6T7) and the Alps by another (H8T8), with novel haplotypes that arose within each of the mountain ranges. The Carpathians were richer in haplotypes than the Alps, which could also be attributed to unequal sample size. Anyhow, the diversity and dispersal of mitochondrial haplotypes within both mountains ranges show prevalence of one haplotype suggesting recent and rapid range expansion. Low genetic divergence within the separate haplogroups clearly shows important gene flow between populations from the same mountain region. On the other hand, consistent and substantial genetic differentiation between the mitochondrial lineages strongly suggests a long-term isolation and interrupted gene flow between *L. glabrirostris* populations from the Alps and Carpathians.

Estimates of divergence time should always be interpreted with caution in the absence of available calibration of the genes of interest. According to the mitochondrial divergence rate for other weevils reported by Hernańdez-Vera *et al*. (2010), a split between the Alpine and Carpathian lineages could have taken place somewhere between 0.85 and 0.98 mya. This estimated divergence time places their initial divergence within a period of intensive geological events in Europe during the Pleistocene (2.6 mya to 10,000 years ago) (Elias, 2007).

Environmental changes during the Pleistocene such as climatic oscillations, erosions, creation of lakes and changes in sea levels and streams had a huge impact on biodiversity by forcing longitudinal and altitudinal range shifts via diverse pathways, including extraction, expansion and dispersal of populations (Stewart & Adrian, 2001; Schmitt, 2007). Habitat fragmentation during the Pleistocene may have led to geographical isolation of insect populations resulting in genetic differentiation and consequently allopatric speciation, in the absence of gene flow (Campbell *et al*., 1999). The dispersal ability of insects is an important factor affecting migration routes, level of gene flow and population differentiation. Fragmentation of the investigated habitats suggests that long-distance migration of the flightless weevil has been a rare event, and populations have been restricted to separate refugia in the Alps and Carpathians, separated by the river Danube as

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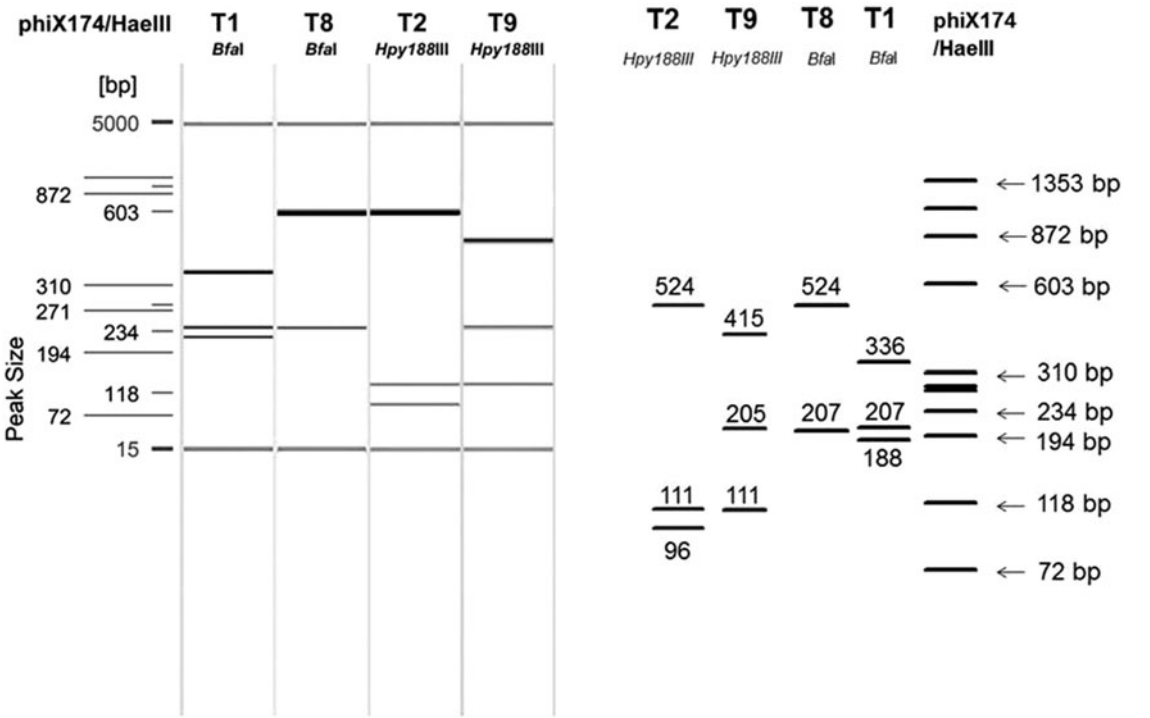

Fig. 5. Actual (left) and virtual (right) restriction profiles of 731-bp COII genes. Haplotypes presented: T1,T2 – Carpathian group; T8,T9 – Alpine group. T1 and T8 were digested with *Bfa*I, T2 and T9 with Hpy188III. Molecular weight marker phiX174/HaeIII (digested) was used in virtual and actual digestion. Sequence fragment sizes and the marker are indicated.

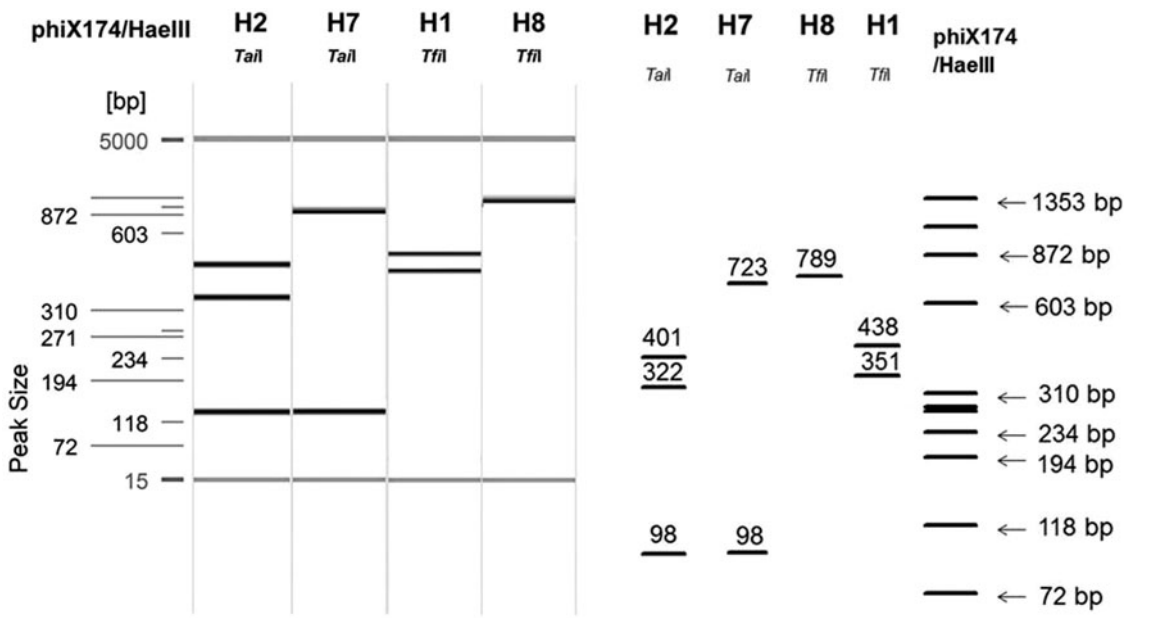

Fig. 6. Actual (left) and virtual (right) restriction profiles of 821-bp COI genes. Haplotypes presented: T1,T2 – Carpathian group; T8,T9 – Alpine group. T1 and T8 were digested with *Tfi*I, T2 and T9 with *Tai*I. Sequence fragment sizes and molecular weight marker phiX174/HaeIII (digested) are indicated.

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Fig. 7. *Liparus glabrirostris*: male adult (left); aedeagus (right).

a solid geographical barrier with no landscape bridges existing between the populations.

Adaptation and genetic divergence driven by geographical isolation of populations may not be accompanied by morphological differentiation, resulting in cryptic speciation (Campbell *et al*., 1999). The obtained molecular evidence indicating geographical structuring of *L. glabrirostris* populations was not supported by differences in phenotypes. All the species specific characters are common to all specimens collected from different localities and altitudes in the Alps and Carpathian Mountains. Cryptic biodiversity in terms of divergence and speciation has been observed as one of the genetic consequences of the ice age (Hewitt, 1996).

The diversity and distribution of *L. glabrirostris* mitochondrial haplotypes reflect a long history of responses to habitat changes produced by geological activities over the past million years. The Alps have been a major factor in shaping the genetic structure of European species, both as a barrier disrupting the populations' expansion and dispersal, as well as a refugium for species at lower latitudes. The present relief forms of the Alps resulted mostly from glaciations of the Pleistocene and are characterized by the presence of large glaciers, snowfields, high waterfalls, large lakes and other features which are lacking in the Carpathians (Gadek & Grabiec, 2008). While the Alps consist of a considerable number of ranges reaching 4000 m, the Carpathians are lower with an altitude of around 2500 m in only a few sites.

Due to their specific biogeographical features and diverse topography, the Carpathians represent one of the major refugia for diverse organisms during the glacial period, providing conditions for diversification through allopatric speciation (Steffen *et al*., 2009). In comparison with the Alps, structure of the Carpathians is less compact, being split into a number of individual mountain blocks separated by depressed areas. In the Carpathians, glaciation affected only the highest peaks, and the present relief forms have been shaped by the action of water.

Cold-tolerant species inhabiting mountains could expand their range and undergo re-colonization, probably from several refugia. An example of this has been reported by Karen *et al*. (2010), for the Alpine caddis fly *Allogamus uncatus* across the central European Alps and re-colonization from multiple refugia peripheral to the Alps. Krascsenitsova *et al*. (2013) described low genetic divergence, but with haplotype diversity and dispersal alterations between populations of the bark beetle *Ips typographus* inhabiting different parts of the Carpathian Mountains, suggesting several refugia. The genetic structure of its populations suggests that *L. glabrirostris* had multiple allopatric Pleistocene refugia in the Alps and Carpathians as survival points and source of postglacial expansion and re-colonization. Cold tolerance may have enabled *L. glabrirostris* to survive in isolated populations within cryptic refugia for a long time (Haranczyk *et al*., 2012), probably through several glacial and interglacial periods of Pleistocene, and consequently shaping the large genetic gap between the Alpine and Carpathian populations.

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