Splitting the Roman snail *Helix pomatia* Linnaeus, 1758 (Stylommatophora: Helicidae) into two: redescription of the forgotten *Helix thessalica* Boettger, 1886

Ondřej Korábek[1], Lucie Juřičková[2] and Adam Petrusek[1]

[1]*Faculty of Science, Department of Ecology, Charles University in Prague, Viničná 7, Prague 2 CZ-128 44, Czech Republic; and*
[2]*Faculty of Science, Department of Zoology, Charles University in Prague, Viničná 7, Prague 2 CZ-128 44, Czech Republic*

Correspondence: O. Korábek; e-mail: ondrej.korabek@gmail.com

(Received 10 May 2015; accepted 1 July 2015)

ABSTRACT

The Roman snail *Helix pomatia* Linnaeus, 1758 is a large terrestrial gastropod very common in central and southeastern Europe, and is the type species of the genus. A recent molecular phylogenetic study has uncovered two divergent lineages within the species as currently conceived. One represents the true *H. pomatia*, the second corresponds to a previously recognized subspecies *H. pomatia thessalica* Boettger, 1886. Using more samples, new molecular data and thorough morphological investigations, we redescribe the latter as a separate species. Morphological differences, especially colouration and surface sculpture of the shell and dark colouration of distal genitalia, distinguish this taxon reliably from *H. pomatia*. The species is widespread in the southern Balkans, but its range extends also to central Europe along the Carpathian Arc. Thus, its distribution broadly overlaps with that of *H. pomatia* and we have found evidence for occasional hybridization between the two species. Some lineages of *H. thessalica* seem to be restricted to the Carpathians and their vicinity; these might have survived locally during the last glacial. By clarifying the taxonomy of *H. pomatia*, we make the crucial first step towards elucidating the phylogeographic history of this iconic member of the European land snail fauna.

INTRODUCTION

The Roman snail *Helix pomatia* Linnaeus, 1758 is a large, broadly distributed and ubiquitous land snail and a prominent member of the European gastropod fauna. It is the type species of the genus *Helix* Linnaeus, 1758 (ICZN, 1926), which comprises the largest Palaearctic land snails. Its latitudinal range spans from Greece to Scandinavia, and its longitudinal extent from westernmost Russia to England (Neubert, 2014). It lives in a broad variety of habitats including forests, shrubs, gardens and roadsides. As a valued delicacy, the Roman snail is also of some economic importance (e.g. Gheoca, 2013).

The species exhibits a broad variability in shell shape and colouration (Hazay, 1880; Buchner, 1899; Urbański, 1963). This has led to the description of *c.* 50 nominal taxa that are now considered synonyms of *H. pomatia*. Despite this considerable number of described varieties, no subspecies of *H. pomatia* were recognized in the latest revision of the genus *Helix* (Neubert, 2014); the use of subspecific taxa within this species was largely abandoned during the first half of the 20th century (but see, e.g. Grossu, 1983). Much of the conchological variation observed in *H. pomatia* is deemed to be environmentally induced (Clessin, 1897; Buchner, 1899; Urbański, 1963). Still, the conchological variability is unevenly distributed geographically. In particular, high diversity in shell shapes and colouration can be observed in Romania (Kobelt, 1906; Knipper, 1939; Urbański, 1963).

Recently, we have analysed most of the recognized *Helix* species in the first comprehensive study of the genus that has used molecular data (Korábek *et al.*, 2015). We provided the first sequences for *H. pomatia* from the Balkans and revealed the presence of two distinct lineages within the species as currently (Neubert, 2014) delimited. Based on conchological characters, we suggested that these lineages correspond to the division of *H. pomatia* into two subspecies as proposed by Urbański (1963): *H. pomatia pomatia* and *H. pomatia thessalica* O. Boettger, 1886, of which the taxonomic status needs to be reevaluated. A division into only two subspecies, one of them inhabiting the Balkans, was previously suggested by Knipper (1939), but these were vaguely defined. Neubert (2014) did not distinguish any subspecific taxa, but noted two conchological forms in the Balkans different from the typical *H. pomatia* and speculated that one of these could be affiliated with the divergent lineage of Korábek *et al.* (2015). Urbański (1963), however, described in detail the conchological differences between the two above-mentioned subspecies as recognized by him, providing a comprehensive taxonomic hypothesis. He also assessed the distribution of both presumed subspecies. According to his account, populations from the greater part of the range of *H. pomatia* should belong to the nominotypical subspecies, whose type locality was described vaguely by Linnaeus (1758: p. 771) as "in Angliae, Galiae nemoribus" (in forests of Anglia and France). *Helix pomatia thessalica* appears to inhabit Greece, the former Yugoslavia,

Bulgaria and Romania; the type locality is in Thessaly, Greece (Boettger, 1886).

We propose that *H. thessalica* is a valid species, separate from *H. pomatia*. We redescribe this taxon here and provide a detailed account of the diagnostic characters allowing for a reliable distinction between the two species, a revised overview of their distribution and new data on their phylogenetic relationships.

MATERIAL AND METHODS

Sampling

Since *Helix thessalica* appeared to be conchologically distinguishable (Urbański, 1963), we searched our own collections and collections of *H. pomatia* in the National Museum, Prague (NMP), Museum für Naturkunde der Humboldt Universität, Berlin (ZMB), Forschungsinstitut und Naturmusem Senckenberg, Frankfurt am Main (SMF) and Naturhistorisches Museum, Wien (NHMW) for corresponding morphotypes, which allowed us roughly to establish the geographic range of the putative *H. thessalica* and the range of the typical *H. pomatia* across the whole of Europe. The examined specimens totalled thousands of shells, which are not referred to here individually; however, most of them originated from Central Europe (Czech Republic, Germany, Austria). These preliminary investigations showed a surprisingly broad distribution of the morphotype corresponding to *H. thessalica*, broader than that described by Urbański (1963) and extending as far as the southeastern Czech Republic. In order to investigate the status of *H. thessalica* and its distribution, we genetically analysed samples of *H. pomatia* in its current sense, including those corresponding to *H. thessalica*, from an area where the latter morphotype has been found, spanning from the Czech Republic and Slovakia to Romania, Bulgaria and Greece (Fig. 1). Samples from France have been used as representatives of *H. pomatia* in the strict sense and a few samples from other parts of Europe have been also included for comparison. Where available, multiple specimens were analysed from sites where the two morphotypes were syntopic or where unusually high genetic or conchological variability were observed.

To resolve the relationships between *H. thessalica* and *H. pomatia* and those among the members of the European clade of the genus as defined by Korábek *et al.* (2015), we have analysed all currently recognized species in this clade, including also the divergent mitochondrial lineages uncovered by Korábek *et al.* (2015). As outgroup taxa, *H. melanostoma* Draparnaud, 1801, *H. cincta* Müller, 1774 and *H. philibinensis* Rossmässler, 1839 were used.

All the assembled material has been conchologically identified based on comparison with types in the genus *Helix* and the most recent taxonomic revision (Neubert, 2014). Conchological characters distinguishing *H. thessalica* from *H. pomatia* and geographical distribution of the species were mostly assessed using our own collections, because the differences between these two lineages appeared partly to fade in old museum specimens. Our samples were complemented by some well-identifiable museum samples and specimens with clearly observable diagnostic characters in order to establish the range of the species. A list of the samples used is provided in Supplementary Material Table S1. Where preserved animals were available, the genital system was checked for the colouration of the penis and vagina, because an unusual pigmentation of these body parts has been observed by Wohlberedt (1909) and Knipper (1939) in specimens that may have corresponded to *H. thessalica*. For taxa unavailable for dissection, information on colouration of the genital system has been derived from Neubert (2014); no data were available for *H. dormitoris dormitoris* (Kobelt, 1898). In addition to the reported specimens, we have examined the genital systems of numerous individuals of *H. pomatia* mainly from the Czech Republic prior to this study.
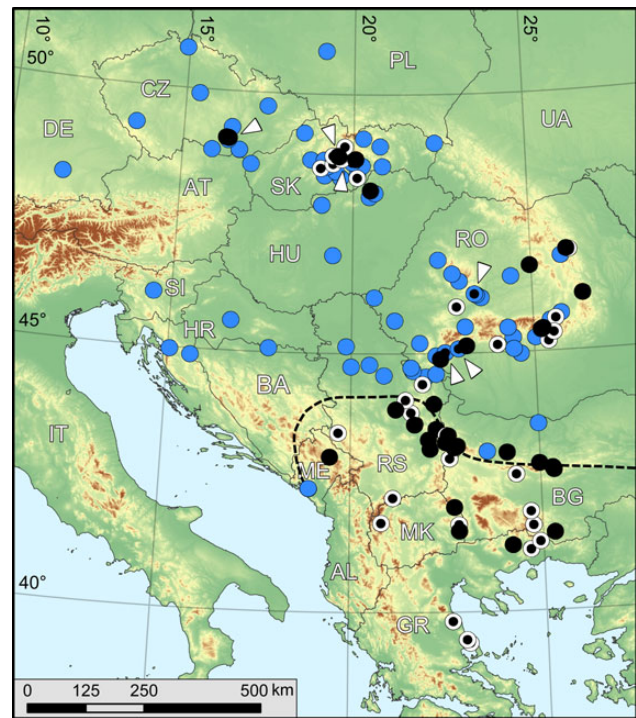
Figure 1. Origin of examined specimens of *Helix thessalica* and sampling sites of *H. pomatia* used for molecular analyses. Black circles indicate localities of *H. thessalica*; white rim indicates that only shells were available. Blue (grey in print) rim stands for localities where only *H. pomatia* haplotype was obtained in a population identified as *H. thessalica*. Blue (grey) circles indicate localities of *H. pomatia* sampled for molecular analyses, further sampling localities of this species north and west of the map limits are reported in Supplementary Material Table S1. Arrowheads indicate localities where specimens carrying haplotype different from that suggested by morphology were recorded. The black dashed line shows approximate southern limit of *H. pomatia* distribution derived from our own observations, museum collections and literature. Countries are identified by ISO two-letter codes; details of each sample are provided in Supplementary Material Table S1.

Molecular analyses

For the initial molecular phylogenetic assessment of the status of *H. thessalica*, we used the mitochondrial markers employed in previous work on *Helix* (Korábek, Juřičková & Petrusek, 2014; Psonis *et al.*, 2015; Korábek *et al.*, 2015). Two datasets were used for phylogenetic analyses. The first one consisted only of samples of *H. pomatia* and putative *H. thessalica*; the objective was to establish distribution of the two lineages recognized by Korábek *et al.* (2015) and to evaluate whether the mitochondrial data corresponded with the prior identification based on morphology. For this purpose, a fragment of the 16S rRNA gene was amplified. Initially, a *c.* 390 bp (excluding primers) fragment of the gene was targeted, as by Korábek *et al.* (2015). For more recently collected samples, a longer fragment of almost 820 bp, overlapping the shorter one, was sequenced.

A second dataset consisting of sequences from 22 specimens representing all known major lineages within the European clade of *Helix*, major lineages within the *H. thessalica* and *H. pomatia* lineages revealed by preliminary analyses (outlined above) and the three outgroup taxa, was used to resolve the relationships between *H. pomatia* and *H. thessalica*. Fragments of three mitochondrial genes were used here: 16S rRNA and the cytochrome *c* oxidase subunit I (COI), as in previous studies, and additionally the 12S rRNA gene. The COI fragment was also available for a few additional specimens of *H. thessalica* and *H. pomatia*, for which it was sequenced earlier.

DNA extraction, amplification of COI and the shorter 16S fragment, and sequencing followed the methods described by Korábek *et al.* (2015). The primer pair and PCR conditions described by Cadahía *et al.* (2014) were used for 12S. The longer fragment of 16S was amplified using the primer pair Scs1 and Scs2 designed by Chiba (1999) under the conditions described therein. For some previously sequenced specimens, the available shorter 16S sequence was extended at the 3′ end to the length of *c.* 800 bp by amplifying an additional, partially overlapping *c.* 450 bp fragment using the forward primer Scs1 in combination with the reverse primer 16S_MN3: 5′-GCTACCTTTG CACAGTCAGWG-3′ (M.T. Neiber, personal communication). The 16S sequence was also assembled from these two fragments when the amplification of the whole target fragment failed. All resulting chromatograms were visually checked for ambiguities and sequencing errors. The sequences were deposited in GenBank (Supplementary Material Table S1).

Phylogenetic analyses

Each of the three analysed loci was aligned using MAFFT v. 7.214 (Katoh & Standley, 2013) with default settings as implemented at http://mafft.cbrc.jp/alignment/server/. For all the three resulting alignments as well as for the concatenated one, a substitution model was selected by PartitionFinder v. 1.1.1 (Lanfear *et al.*, 2012) and the datasets were analysed under the respective models in MrBayes v. 3.2.2 (Ronquist *et al.*, 2012) to check for potential incongruences among the markers and to obtain a preliminary phylogeny.

Following the results of the preliminary analyses, the 16S sequences belonging either to the *H. pomatia* or *H. thessalica* lineage were selected and analysed separately. To analyse the relationships between the *H. pomatia* and *H. thessalica* lineages, a concatenated alignment of the three markers as described above was prepared for selected specimens. The dataset (labelled as 'MAFFT' hereafter, final length 2,114 bp) was aligned using MAFFT; about 20 bp from the 3′ end of 16S and 5′ end of 12S was removed due to low coverage. Because both 16S and 12S rRNA genes contained variable regions where the alignment may be ambiguous and contain nonhomologous sites, thus impeding the phylogenetic analyses, we also explored the effects of alignment filtering as well as of an alternative approach to alignment on the results. We analysed the MAFFT alignment with the TCS algorithm of the T-Coffee package (Chang, Di Tommaso & Notredame, 2014, at http://tcoffee.crg.cat/apps/ tcoffee/do:core under default settings) and discarded all positions with a score lower than the highest possible score of 9 ('TCS9' alignment, 1,837 bp). Alternatively, the full dataset was realigned using SATé v. 2.2.7 (Liu *et al.*, 2012) under default settings ('SATé' alignment, 2,113 bp). SATé simultaneously estimates the alignment and phylogeny, and potentially increases the accuracy of the subsequent phylogenetic analyses (Liu *et al.*, 2012). Also, because of low support values of nodes between the *H. pomatia* and *H. thessalica* lineages, and the apparently unstable position of the Greek sample of *H. schlaeflii* Mousson, 1859 and the sample of *H. dormitoris dormitoris* (isolates SC2 and DT1) as indicated also by leaf stability index calculations from the boostrap analysis, the two taxa were removed from the comparatively best-performing SATé alignment ('stable' alignment) and the analyses of that alignment were repeated.

All the concatenated alignments were split into five partitions (16S, 12S and three COI codon positions); this partitioning scheme was evaluated and appropriate substitution models selected by PartitionFinder: 16S + 12S: TVM + I + G (HKY + I + G for 'TCS9'); COI 1st position: TIM + G; COI 2nd position: F81; COI 3rd position: K81uf + G (K81uf + I + G for 'TCS9'). Bayesian phylogenies were reconstructed and posterior probabilities (PP) calculated using MrBayes; the analyses were run in two parallels of four chains each for 5,000,000 generations sampling every 1,000th generation, with the first half of the sampled trees discarded as burn-in. Maximum likelihood analyses were performed using Garli v. 2.0 (Zwickl, 2006) with ten replicates and bootstrap (BS) searches were done with 500 pseudoreplicates.

RESULTS

Mitochondrial phylogeny

The results of the phylogenetic analyses are summarized in Figure 2 and Supplementary Material Figure S1. Within the traditionally conceived *Helix pomatia* (Neubert, 2014), the data confirm the presence of two divergent mitochondrial lineages as already shown by Korábek *et al.* (2015), here identified as *H. pomatia* and *H. thessalica*, respectively. However, despite the twofold increase in length of the sequences used to disentangle the relationships within the European clade of *Helix* as compared with our previous work (Korábek *et al.*, 2015), the relationships between the species of this clade were not resolved unambiguously. While the topologies remained largely stable in different analyses and also matched the results from Korábek *et al.* (2015), the support values for the interspecific nodes (in particular, the BS proportions) were low.

In all analyses, *H. vladika* (Kobelt, 1898), *H. straminea* Briganti, 1825, *H. dormitoris arnautorum* Knipper, 1939 and *H. schlaeflii* (from Macedonia and Albania) were resolved as close relatives of *H. thessalica*. However, the branch uniting these received substantial support only in analyses of the 'SATé' (PP) and 'stable' (PP and BS) alignments. Excluding the two unstable taxa (*H. dormitoris dormitoris* and the Greek sample of *H. schlaeflii*, both from the type areas of these taxa) from the alignment also increased support for the sister relationship between this clade and *H. pomatia* (Fig. 2A). Filtering the alignment somewhat increased the support for the basal position of *H. lutescens* Rossmässler, 1837 within the European clade, but had no effect on support for the relationship between *H. pomatia* and *H. thessalica*. However, it is noteworthy that none of the analyses suggested a close relationship of the *H. thessalica* lineage to *H. pomatia* in the strict sense. Of the preliminary single gene analyses, the COI tree matched closely the final topology of the concatenated analysis.

Within the *H. thessalica* clade, a total of six divergent mitochondrial lineages were uncovered (Fig. 2A, B). The most basal (cross in Fig. 2A, B) originated from western Macedonia and Serbia, but the relationships between the remaining lineages are unresolved. Two lineages (triangles) from the Rilo-Rhodope massif (Bulgaria, northern Greece) and one from central Bulgaria (near Shipka, diamond) were represented by single samples. The remaining populations belong either to a group broadly distributed in Bulgaria, Serbia, Macedonia and Montenegro, which extends also to Slovakia and the Czech Republic (square) or to a clade limited to the Carpathians (Romania and Hungary; circle). *Helix pomatia* s. s. is genetically diverse; however, no split deeper than those observed in earlier studies (Korábek *et al.*, 2014, 2015) was found. The haplotypes fall into four groups, with well-resolved mutual relationships (Fig. 2A, C). The clade that includes the samples from western and northern Europe (circle) is the most widely distributed and is considered the typical *H. pomatia*. Its sister clade (square) comprises two lineages that appear to be limited to the Balkans and the Carpathian Arc. Surprisingly, the most basal mitochondrial lineage within *H. pomatia* (triangle), previously known only from Serbia and Croatia, was also found in central Europe. In some populations, it occurred together with the two remaining *H. pomatia* clades.
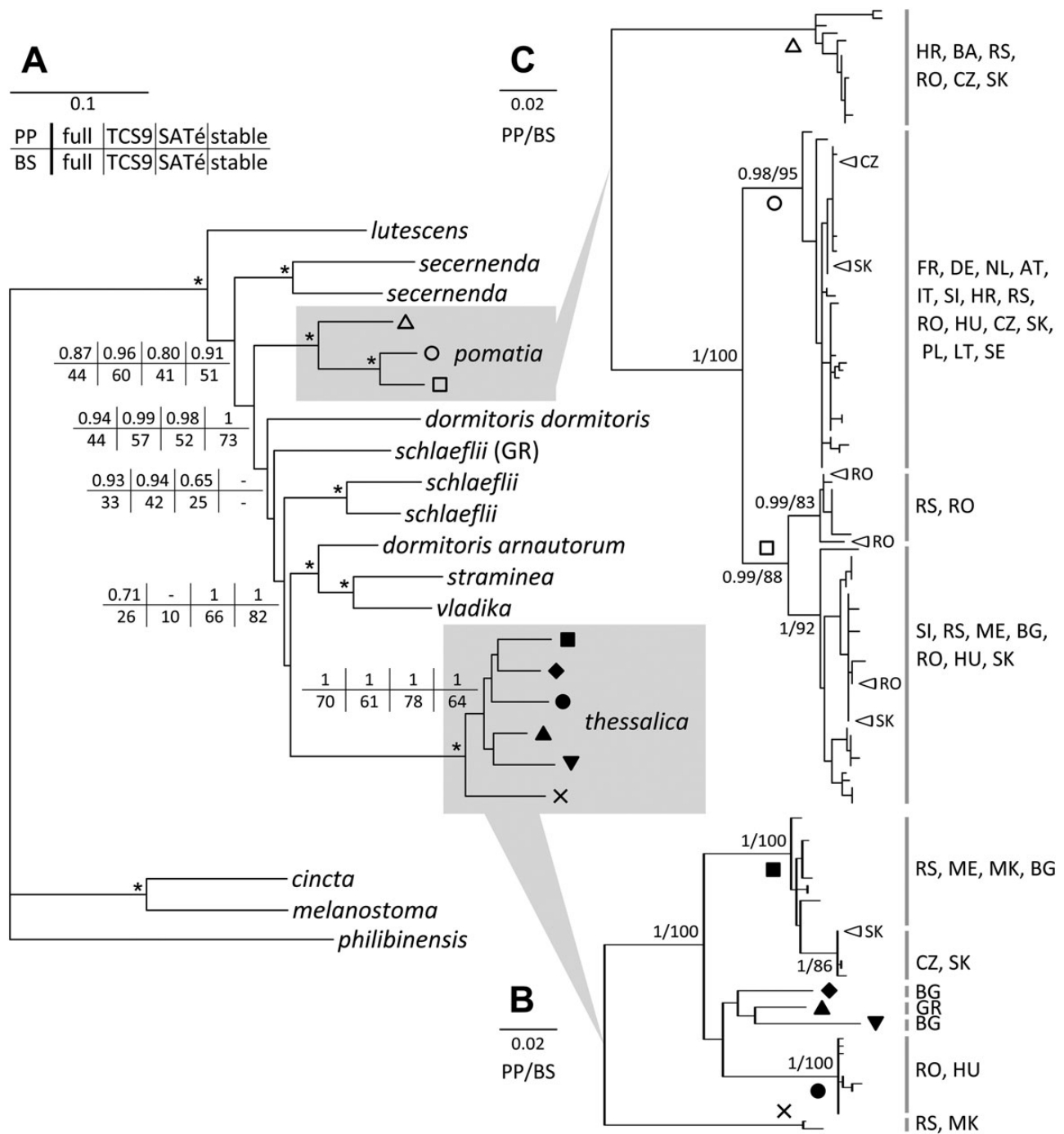
Figure 2. Phylogenetic relationships between and within *Helix thessalica* and *H. pomatia* based on mitochondrial data. **A.** Relationships within the European *Helix* clade *sensu* Korábek *et al.* (2015). Best ML tree from analysis of concatenated alignment of 16S, COI and 12S ('MAFFT', see Material and Methods). Support values obtained from analyses of alternative alignments are indicated; nodes without substantial support in any of the analyses are omitted. Asterisks indicate PP ≥0.99 and BS >95 in all analyses. **B, C.** Relationships among *H. thessalica* (**B**) and *H. pomatia* (**C**) 16S haplotypes. Symbols indicating main branches correspond to those in **A**. ISO two-letter country codes are used to indicate sample origin. Arrowheads indicate specimens identified morphologically as the other species. Detailed version of the figure with further sample information is available as Supplementary Material Figure S1.

Mismatch between morphology and molecular data: evidence for hybridization

In total, we found five localities of syntopic occurrence of *H. thessalica* and *H. pomatia*: two in the Czech Republic (Ivančice and Mohelno), one in Slovakia (Podbrezová) and two in Romania (Podu Dâmboviței and Oituz). At Ivančice, *H. thessalica* inhabits

small patches on conglomerate rock slopes surrounded by a *H. pomatia* population and uninhabitable fields; at Mohelno we found a spatially restricted, but dense, *H. thessalica* population with sparse *H. pomatia* within less than 100 m. At the Slovak locality in Podbrezová, patches of one or the other species were found. At Podu Dâmboviței, *H. pomatia* lives within the village while *H. thessalica* was found in a limestone gorge just behind it.

Near Oituz the two species apparently live syntopically (based on shell evidence); however, only one live specimen of each species was collected at the site. In all five cases, direct contact between both species offers the possibility for hybridization.

Most of the 38 genetically analysed samples identified morphologically as *H. thessalica* belonged to the mitochondrial clade assumed to represent *H. thessalica* by Korábek *et al*. (2015), but there were three exceptions in which such individuals carried a *H. pomatia* haplotype. All of these were found in the region where the distributions of *H. thessalica* and *H. pomatia* morphotypes overlap, two of them at sites of their syntopic occurrence (Ivančice and Podbrezová). A *H. thessalica* haplotype was, however, also found at these two localities. In one case in Romania (Munţii Mehedinţi), a *H. pomatia* haplotype was obtained from an unambiguously identified *H. thessalica* specimen, but no other *Helix* individual was found at that site. The discrepancy between phenotype and the respective haplotype was not limited to *H. thessalica* morphotypes; a *H. thessalica* haplotype was obtained from one specimen with typical *H. pomatia* shell at the Slovak site of cooccurrence in Podbrezová.

Apart from the cases of mismatch between mitochondrial haplotype and phenotype reported above, we observed further signs of likely hybridization between the two taxa at the locality of Ivančice. Although almost all of the shells collected there were unambiguously attributable to either *H. thessalica* or *H. pomatia*, there were some near Ivančice that did not fully correspond to the morphology of either species. Moreover, *H. pomatia* (identified by conchology and genetics) with a coloured genital system was found there.

In addition, *H. pomatia* haplotypes were obtained from juvenile *Helix* from three sites where all the collected empty adult shells belonged to the *H. thessalica* morphotype (Slovakia: Muráň; Romania: Tismana and Cheile Ampoiţei; Supplementary Material Table S1). Unfortunately, only one living juvenile specimen was found at each of these three localities and thus no other individuals could be analysed genetically from the respective localities.

SYSTEMATIC DESCRIPTION

HELICIDAE Rafinesque, 1814

Helix Linnaeus, 1758

Helix thessalica O. Boettger, 1886
(Figs 3A–H, 4, 5, Supplementary Material Fig. S2)

Helix (*Helicogena*) *pomatia* var. *thessalica* Boettger, 1886: 56 (village Makrinitsa near Volos, Pelion, Greece; lectotype SMF 9568, designated by Zilch, 1952: 165).

Helix (*Helicogena*) *pomatia* var. *serbica* Kobelt, 1906: 252, pl. 363, figs 1, 2 (Maidanek [Majdanpek], Serbia; holotype SMF 9540; not *H. serbica* Kobelt, 1872).

Helix (*Helicogena*) *pomatia* var. *christinae* Kobelt, 1906: 255; pl. 364, figs 1, 2 (Runku oberh. Bukarest [Dâmboviţa County, Runcu, *c*. 30 km east of Câmpulung], Romania; lectotype SMF 9542, designated by Zilch, 1952: 164; not *H.* (*Plectotropis*) *christinae* Adams, 1870).

Helix (*Helicogena*) *pomatia* var. *rhodopensis* Kobelt, 1906: 257; pl. 364, figs 5, 6 (slopes of the Rhodope Mountains near Plovdiv, Bulgaria; lectotype, here designated, SMF 9599/2a).

Helix pomatia burmesteri Blume, 1920: 90 (Südhängen des Brena (2100 m)…Höhe von 15–1700m…Brena befindet sich zirka 30 km westlich von Gjewgjelii im Wardartal, Macedonia; types unknown).

Helix pomatia brenaensis Blume, 1920: 91 (Südhängen des Brena (2100 m)…Höhe von 15–1700m…Brena befindet sich zirka 30 km westlich von Gjewgjelii im Wardartal, Macedonia; types unknown).

Diagnosis: Large *Helix* species with rounded shell, low spire, voluminous last whorl, spacious rounded aperture, thick ochre to olive-green periostracum, shell surface with dense radial striation and usually also spiral sculpture forming fine granulation, 4 reddish-brown bands; atrium, penis and vagina pigmented dark grey.

Description: Shell large (height: 34–49 mm, width: 35–50 mm), globose, relatively thin-walled, with high and voluminous last whorl only slightly descending below periphery; suture shallow; aperture large, rounded, with white to violet lip, labial callus sometimes also violet; umbilicus slit-like, open; upper shell surface usually densely radially striated, frequently with conspicuous fine granulation; periostracum thick; basic colour ochre to greenish-brown, usually with 4 prominent reddish-brown spiral bands, the middle 2 broad; bands sometimes reduced or completely absent, especially when aperture is white. Body light greyish-yellow to brownish-grey.

Genital anatomy: Penis, atrium, vagina and lower part of bursa copulatrix stalk grey to almost black with pigment dispersed in inner tissues; mucous glands richly branched; diverticulum reduced to short projection or lacking.

Comparison with similar taxa: Though similar in shell shape and size, *H. schlaeflii* Mousson, 1859 lacks the thick periostracum and fine sculpture; its colour is paler, dominantly greyish-brown and typically with a pattern of whitish or grey axial streaks (Neubert, 2014). The aperture of *H. schlaeflii* is typically orange-brown, without violet tones. *Helix vladika* has a conical rather than globular shell with a small aperture. It has a relatively smooth surface and the colour is brown. Shells of *H. straminea* are similar in banding pattern and colouration of the aperture to some populations of *H. thessalica*, but have a broader overall appearance with a smaller and differently shaped aperture; there is no granulation on the shell surface. All these three species usually lack an umbilicus. *Helix buchii* Dubois de Montpéreux, 1839 from northeastern Turkey and the Caucasus has a straight, not rounded columella; the shell surface lacks granulation. From species of the genus *Maltzanella* Hesse, 1917, which has a greenish periostracum and has been found in the European part of Turkey (Korábek *et al*., 2015), *H. thessalica* is easily distinguishable by a smaller protoconch.

The differences between *H. thessalica* (Fig. 3A–H) and *H. pomatia* (Fig. 3I–P) described by Urbański (1963) are largely valid, even though not all of them are applicable in all cases due to overlapping variability of both taxa. Shells of *H. thessalica* are on average more robust, more regularly globose with a relatively lower spire and higher last whorl, and consequently with a larger aperture; the suture is shallower. It usually has a better-developed palatal callus, especially conspicuous on corroded empty shells (Fig. 4D). Shell walls are thinner in *H. thessalica* and the surface is covered by a well-developed periostracum. However, *H. pomatia* also develops globular-shelled forest forms with a thicker periostracum that flakes off (Clessin, 1897; Falkner, 1984). The surface sculpture is in general finer in *H. thessalica*. In most individuals it is densely striated rather than ribbed, usually also with well-developed fine spiral sculpture, making the upper surface of the shell appear granulated. This granulated sculpture is most apparent on the penultimate whorl just above the aperture (Fig. 4B), while the radial striation is best developed on the upper surface of the second and third whorl (Fig. 4A). The granulation gives fresh shells a distinct
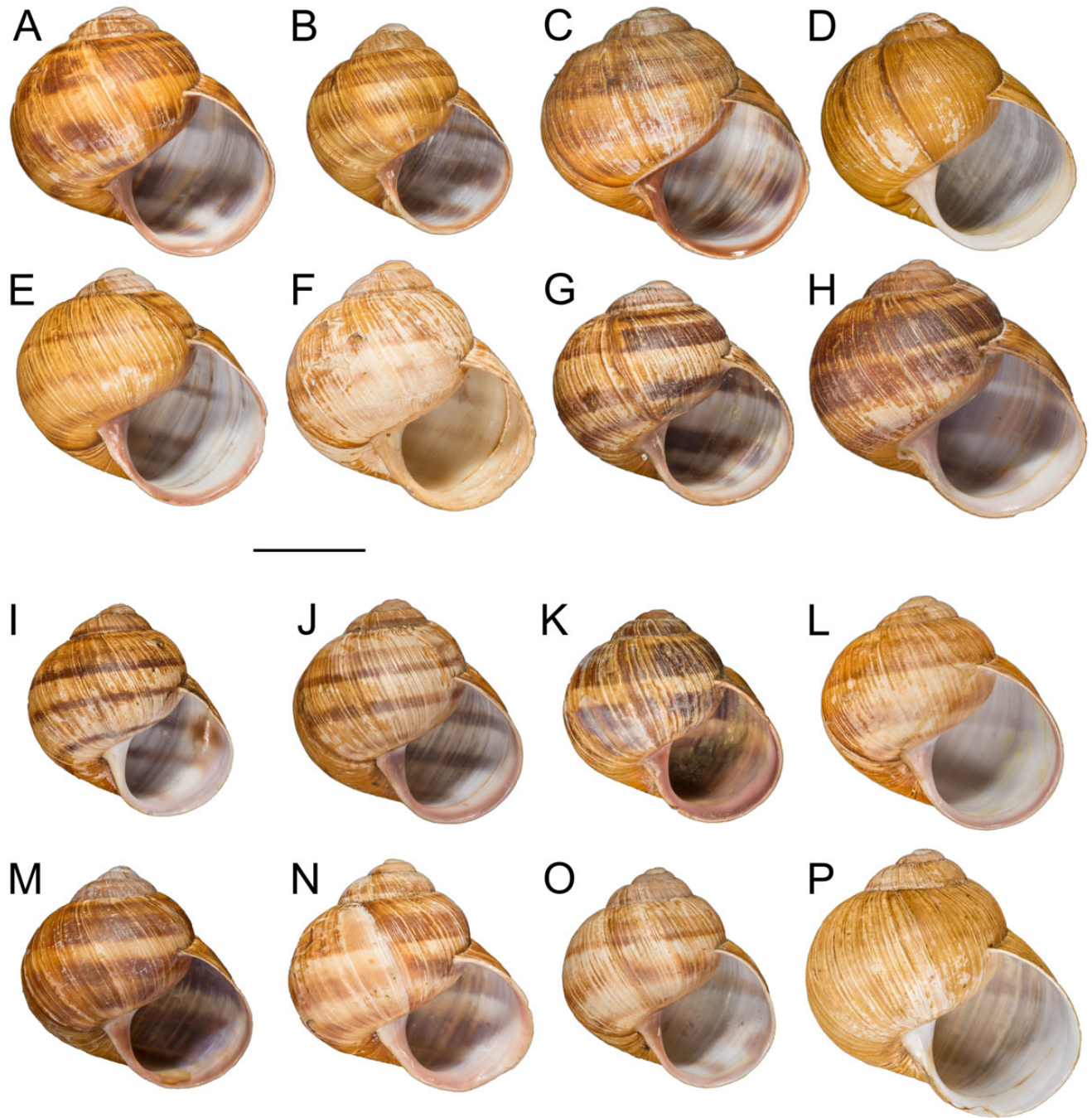
Figure 3. Conchological variability of *Helix thessalica* (**A–H**) and *H. pomatia* (**I–P**) in the study area. **A.** Montenegro, between Polja and Bistrica; SMF 346099. **B.** Bulgaria, Stara Zagora province, near Shipka; SMF 346093. **C.** Bulgaria, Gabrovo/Stara Zagora province, Stoletov peak near Shipka pass; SMF 346090. **D.** Greece, East Macedonia and Thrace, Sidirónero; NMBE 522265. **E.** Romania, Argeş County, Podu Dâmboviţei, limestone canyon; SMF 346102. **F.** Hungary, Bükk Mts, Mályinka; SMF 346095. **G.** Slovakia, Podbrezová; SMF 346128. **H.** Czech Republic, Ivančice env.; SMF 346094. **I.** Montenegro, Cetinje; SMF 346045. **J.** Serbia, Podunavlje District, Kolari; SMF 346069. **K.** Bulgaria, Pleven province, Lozitsa; SMF 346024. **L.** Romania, Alba County, Vârtop; SMF 346051. **M.** Romania, Argeş country, Podu Dâmboviţei village; SMF 346054. **N.** Hungary, Bükk Mts, Lillafüred; SMF 346041. **O.** Slovakia, Podbrezová; SMF 346078. **P.** Czech Republic, Pálava hills, Děvín; SMF 346034. Scale bar = 2 cm.

matt lustre. In *H. pomatia*, the granulation is often absent and the size of granules more variable. The radial sculpture of *H. pomatia* is on average more robust, radial structures are usually thicker and more widely spaced. However, the surface sculpture may also be coarse in some individuals of *H. thessalica*.

The periostracum of *H. thessalica* peels off more easily and is often more contrasting with the underlying shell than in *H. pomatia*. In many specimens of *H. thessalica*, the periostracum

is first lost on the top of the granules (Fig. 4B), which gives the shell a characteristic worn look. Also, the periostracum in many cases remains in between the radial striation, making the striation more conspicuous. The basic colour of *H. thessalica* is in shades of ochre, in many cases with olive-green tones, which are never observed in the largely brown *H. pomatia*. Usually, four reddish-brown longitudinal bands are developed in *H. thessalica*, two thicker ones in the middle and two thinner ones on either
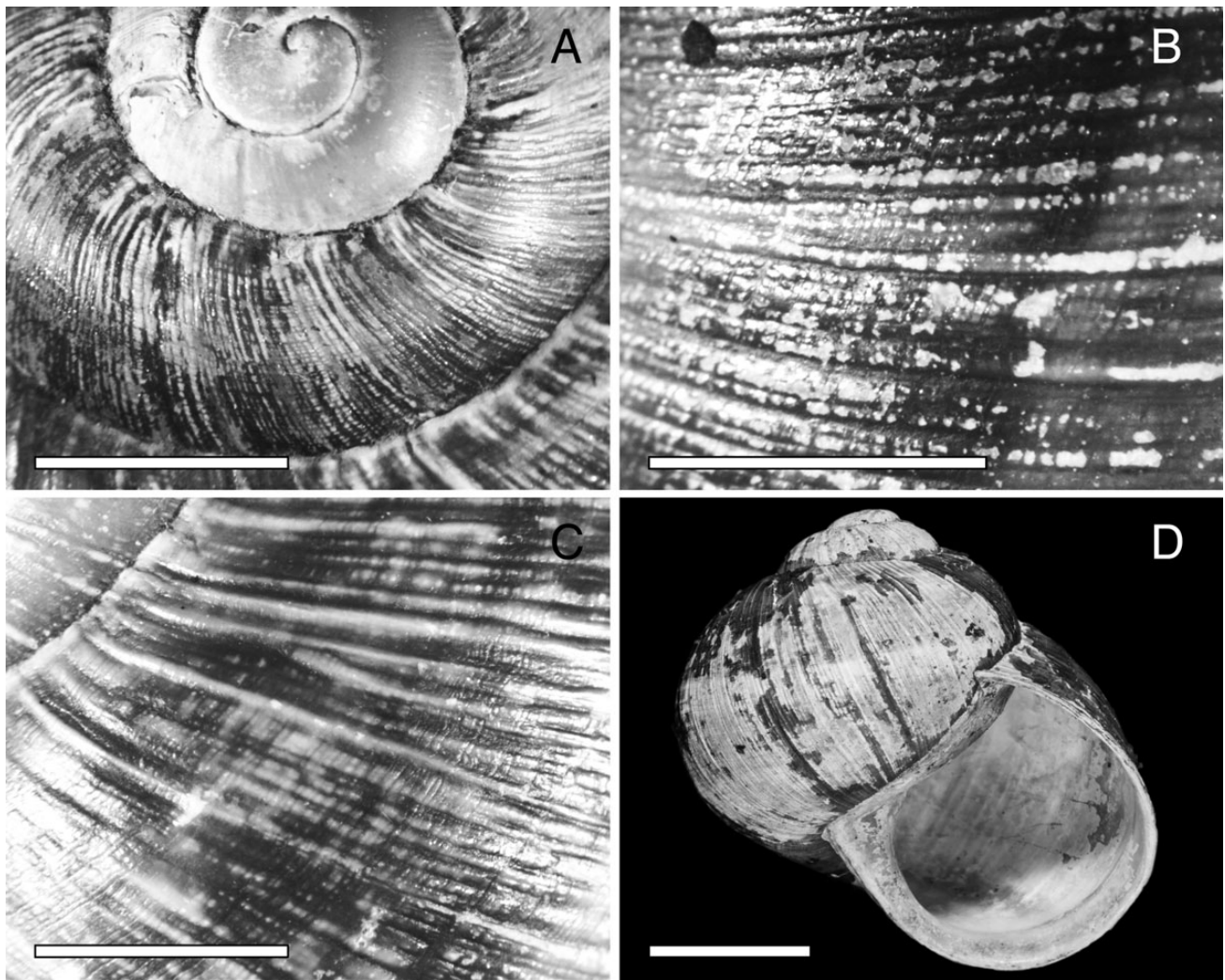
Figure 4. Shell surface structures of *Helix thessalica*. Note that well-developed examples are shown and there is a considerable overlap with *H. pomatia* in these characters. **A.** Apex and upper surface towards the aperture showing dense fine striation. Serbia, Nišava District, Sićevo; SMF 346122. **B.** Frontal surface of last whorl above aperture showing granulate sculpture and characteristic pattern of periostracum loss on top of the granules. Bulgaria, Lovech/Gabrovo Province, Kravenik; SMF 346092. **C.** Upper surface above suture close to aperture. Macedonia, Maleševska Planina, Suvi Laki; SMF 346098. **D.** Corroded shell showing a darkened, peeling periostracum and a conspicuous palatal callus. Czech Republic, Ivančice; SMF 346094. Scale bars: **A−C** = 5 mm; **D** = 20 mm.

side, but there is a large variability in the banding pattern. *Helix pomatia* may exhibit the same banding pattern (apparently plesiomorphic, being found also in most other species of the European *Helix* clade). In many cases, the bands are not uniformly coloured, but rather marbled (Fig. 4C). The dark colouration of the apertural margins may also extend to the palatal callus, which is not the case in *H. pomatia*. Bands are largely absent in populations from Greece and the Rhodope Mountains and these have a white aperture.

In summary, *H. thessalica* is conchologically best distinguished from *H. pomatia* by a combination of a more rounded shell with larger aperture, thick ochre-coloured periostracum, the manner of periostracum loss and denser surface sculpture. We were unable reliably to distinguish juvenile individuals of the two species from each other. Often, identification of old museum material also proved difficult, because differences in colouration disappear over time in the collection and surface sculpture becomes obscured by dust.

Anatomically, *H. thessalica* differs by its dark grey atrium, penis, epiphallus, vagina and the distalmost stalk of the bursa copulatrix (reported before from Pljevlja, Montenegro and Vlajnica near Gostivar, Macedonia; Wohlberedt, 1909; Knipper, 1939; Fig. 5). The colour may be pale in some specimens or populations of *H. thessalica*, but is always present on both male and female parts of the genitalia. In all the examined specimens from the Central European sites (Hungary, Slovakia and Czech Republic) the colouration was well developed and dark (as in Fig. 5B, E, F), thus being a reliable character for differentiating *H. thessalica* from *H. pomatia*. *Helix pomatia* has unpigmented genitalia; only sometimes are light grey transverse bands present on the penis, or the penis (including epiphallus) is light grey (Fig. 5H–J). However, this is inconspicuous and the penis is never dark grey; the atrium and female genital organs are always whitish. The colouration in *H. thessalica* tends to become obscured in fixed specimens with opaque tissues. This appears to be the case in the specimen figured by Neubert (2014: fig. 22), in which there are traces of dark colour visible especially on the atrium and vagina. Dark colouration of the distal genitalia is unusual within the genus *Helix*. It is known in some populations of *H. buchii* (Mumladze, Tarkhnishvili & Pokryszko, 2008); *H. lutescens* sometimes has a coloured penis, and a light-grey colouration of the penis has also been observed in *H. dormitoris arnautorum* (Neubert, 2014; personal observation).

Figure 5. Genitalia of *Helix thessalica* (**A–G**) from various parts of its range, and of *H. pomatia* (**H–J**) from area of syntopic occurrence of both species in the Czech Republic showing the varying extent of dark colouration. The specimens differ by how long they were stored in alcohol; arrows point to traces of the dark colouration in the specimens most affected by storage. **A.** Romania, Mehedinţi county, Munţii Mehedinţi, Crovuri; 1 month in alc. **B.** Slovakia, Podbrezová; fresh specimen. **C.** Bulgaria, Dobrich province, Brestnitsa; 13 months in alc. **D.** Romania, Argeş county, Podu Dâmboviţei, limestone canyon; 1 month in alc., aphallic. **E.** Hungary, Bükk Mts, Mályinka; fresh specimen, flagellum damaged. **F.** Czech Republic, Ivančice; 2 months in alc. **G.** Serbia, Pomoravlje district, Suljkovac; 13 months in alc. **H.** Czech Republic, Ivančice env., Řeznovice; fresh specimen, individual with lightly coloured penis. **I.** Czech Republic, Ivančice; 11 months in alc. **J.** Czech Republic, Mohelno; fresh specimen, proximal parts missing. Not to scale.

Distribution: *Helix thessalica* has been found in Greece (see also Westerlund & Blanc, 1879; Boettger, 1886; Neubert, 2014), Macedonia (Sturany & Wagner, 1915), northeastern Montenegro (Wohlberedt, 1909), Serbia south of the rivers Sava and Danube (Pavlović, 1912), Bulgaria and Romania (Urbański, 1963), northern Hungary, central Slovakia and the southeastern Czech

Republic (this study). The Greek occurrences are concentrated near the Bulgarian border in the foothills of the Rhodope Mountains and in the Pelion Mountains in Thessaly (Neubert, 2014). The record of '*H. pomatia*' from Spileo in the foothills of the Pindos (Neubert, 2014; NMBE 522903) was based on an atypical population of *H. schlaeflii*, while that from Euboea was an error made during collection cataloguing (Neubert, 2014 and personal communication). *Helix thessalica* was relatively easy to find and common in hilly landscapes in Serbia south of Beograd, and in Bulgaria south of the Balkan mountain range Stara Planina. The distribution in Serbia is similar to that of *H. vladika*, with which it sometimes lives syntopically, though apparently broader (Korábek *et al.*, 2014). The localities of *H. thessalica* in Romania and Hungary were discovered along and within the Carpathian Arc. In Slovakia, the species has a considerable range in the central parts of the country, where it is locally common. Patchy populations have been uncovered in the southeast of the Czech Republic in the valley of the Jihlava River near Ivančice and Mohelno (Fig. 1); these represent the westernmost known localities of the species. After acceptance of this paper, we have recorded *H. thessalica* from two sites in Russia (near Valuyki, Belgorod Oblast and Ulyanovsk, Ulyanovsk Oblast; not shown in Fig. 1) (Velichkovskiy, 1910; Artem'eva & Semenov, 2008). The haplotypes obtained from both sites belonged to the clade distributed in Romania (full circle in Fig. 2). Thus, although it is unclear if any of the populations of *H. pomatia s. l.* in Russia are native (e.g. Likharev & Rammel'meyer, 1952; Snegin & Artemchuk, 2014), this finding indicates that *H. thessalica* may live also in Ukraine. Despite substantial efforts, we have not found *H. thessalica* in the Ukrainian Carpathians. However, Urbański (1963) tentatively attributed a sample from near Zalishchyky, Ternopil Oblast, to this species.

Ecology: Most known localities of *H. thessalica* are in hilly or mountainous areas. At the type locality in the Pelion Mountains, it lives in beech forests (Stussiner & Boettger, 1885–1886); it has also been reported from the same type of habitat at Šar Planina, Macedonia (Sturany & Wagner, 1915). We likewise found it in beech forests (frequently with hornbeam and maple), among various shrubs (e.g. *Corylus*, *Euonymus*, *Rubus*, *Ligustrum* and *Sambucus*), along streams (under willow and alder, and in herbs such as *Urtica*, *Petasites* and *Carex*) and on limestone rocks. Population densities, as judged from empty shells, were low in the beech forests, but higher along streams and at forest margins. At many localities in Romania, we were unable to find live adults in the first half of August, despite substantial effort, and only first and second year juveniles could be collected; searches in Slovakia in early September gave similar results. This was in sharp contrast to most localities of *H. pomatia*, where live adults were usually easy to find during these months. The reproductive biology is poorly known; in northern Greece eggs are being laid from April to mid-June, depending on altitude, and hatch *c*. 20 d later (Hatziioannou *et al.*, 1989). In Mohelno, Czech Republic, we observed several specimens laying eggs on 23 June 2015.

Remarks: Synonyms of *H. pomatia* have been listed by Neubert (2014). Of these, the nominal taxa that are synonyms of *H. thessalica* are listed in the synonymy above; those remaining are here considered to be synonyms of *H. pomatia* s. s. Type specimens of the nominal taxa listed here as synonyms of *H. thessalica* were figured by Neubert (2014: figs 11, 15, 17–19). To the synonymy of *H. pomatia* given by Neubert (2014) the following information can be added. The type material of *H. pomatia* itself consists of syntypes preserved in the Linnean collections of the Linnean Society of London (Hanley, 1855) and the Museum of Evolution, Uppsala University (Wallin, 2001). Syntypes of *Helicogena pomatia* var. *Banatica* von Kimakowicz, 1890 (not *Helix banatica* Rossmässler, 1838) with the type locality Caransebeş, Caraş-Severin county, Romania, are held in

the Muzeul National Brukenthal, Sibiu, Romania (reg. nos 37102, 37105, 37106 and 37111).

DISCUSSION

Relationships between Helix thessalica *and* H. pomatia

The distinction between the true *H. pomatia* in the north and west and *H. thessalica* in the southeast of the range of *H. pomatia* in its traditional broad sense, suggested in a previous study (Korábek *et al.*, 2015), is here corroborated by both genetic and morphological data. There is a strong association between the haplotype lineage and the shell morphology corresponding to *H. thessalica*, which does not vanish even in areas of overlap and probable hybridization with *H. pomatia*.

Our data are inconclusive regarding the phylogenetic relationships within the European radiation of *Helix*, including the relationship between *H. thessalica* and *H. pomatia*. The topology obtained here (Fig. 1) is similar to the previous one based on shorter sequences (Korábek *et al.*, 2015) and remains largely stable irrespective of settings of the various analyses. Out of the three preliminary single-gene analyses only that based on COI yielded the same results, albeit with no substantial support. Since support for the reconstructed tree increased not with alignment filtering, but with SATé realignment, the phylogenetic signal for the short branches separating *H. thessalica* and its relatives appears to be only in the third codon positions of COI and the most variable parts of the rRNA genes. Thus, we cannot entirely refute the possibility that *H. thessalica* and *H. pomatia* are sister lineages. Moreover, given the observed nonmonophyly of several species within the European *Helix* clade (Korábek *et al.*, 2015), the results of our phylogenetic analyses may be severely hampered by shared ancestral polymorphisms and mitochondrial introgression, thus not reflecting the true species tree. The relationships within the European *Helix* clade may be resolved unequivocally only with an extensive use of nuclear markers. Variable markers, such as microsatellites or single-nucleotide polymorphisms, may also reveal the extent of hybridization and gene flow upon contact of presumed species.

Nevertheless, according to the mitochondrial data, the closest relative of *H. thessalica* does not seem to be *H. pomatia*, but rather one of the following species: *H. schlaeflii*, *H. dormitoris*, *H. straminea* or *H. vladika*. Of these, *H. schlaeflii* appears to be the most likely candidate. It is mostly similar to *H. thessalica* in general habitus and there has even been some confusion in the past over the separation of these taxa in Greece (Westerlund & Blanc, 1879; Knipper, 1939; Zilch, 1952; Urbański, 1963). However, the present results on the relationships between *H. thessalica*, *H. schlaeflii* and the clade comprising *H. straminea*, *H. vladika* and *H. dormitoris arnautorum* may change somewhat when some potentially important populations are sampled. First, the lineage diversity within *H. thessalica* may turn out to be higher than recorded here when the as yet unsampled populations from Thessaly become available. Second, the genetic diversity of *H. schlaeflii* is generally poorly known and in *H. dormitoris* there remains a geographically isolated montane population in Mali i Tomorrit, Albania.

The existence of a separate Balkan species within the traditionally conceived *H. pomatia* is not surprising, because populations of *H. thessalica* have been recognized as a distinctive form by some earlier authors. They have been assigned various subspecific names (although those taxa, as then understood, were not properly distinguished from true *H. pomatia*): *H. pomatia serbica* (Knipper, 1939), *H. pomatia dobrudschae* Kobelt, 1906 (not *H. (Campylaea) trizona* var. *dobrudschae* Clessin, 1886; Wohlberedt, 1909; Pavlović, 1912), *H. pomatia rhodopensis* (Wohlberedt, 1911; Dedov, 1998) and *H. pomatia thessalica* (Hesse, 1913). The coloured

distal genitalia noted by earlier authors (Wohlberedt, 1909; Knipper, 1939) also suggested the presence of an unrecognized species, because this character is unusual within the subfamily Helicinae, to our knowledge being previously described only in *H. buchii*, *Cepaea nemoralis* and *C. hortensis* (Lang, 1908; Hesse, 1920; Mumladze *et al.*, 2008). However, the occurrence of *H. thessalica* as far north as Hungary, Slovakia and the Czech Republic is an unexpected finding, since these countries have been malacologically well studied. The high conchological variability of true *H. pomatia* in the Carpathians and the Balkans (Fig. 3I–P) is associated with a relatively diverse mitochondrial clade (open square in Fig. 1), which appears to be limited to that area. The Balkan form figured by Neubert (2014: fig. 22) also belongs here. We have found no indication of further hidden species diversity within the traditional *H. pomatia* s. l.

The recognition of *H. thessalica* as a valid species shifts the southern limits of the distribution of *H. pomatia* s. s. towards the north. The revised range limit in the south thus runs from western Montenegro through Bosnia to Serbia, where it continues roughly along the Sava and the Danube rivers to northern Bulgaria. There it reaches the northern slopes of the Stara Planina Mountains, as also indicated by published illustrations of shells and genital systems (Wohlberedt, 1911; Urbański, 1963; Zapryanov, 2006; Neubert, 2014; Dedov & Antonova, 2015). The southern margin of the distribution of *H. pomatia* in eastern Bulgaria needs to be surveyed, but the species is apparently present south of Varna (Zapryanov, 2006).

Hybridization and mitochondrial introgression

The haplotypes of *H. pomatia* found in three snails identified morphologically as *H. thessalica* (and one case of the reverse) in their area of overlap indicate occasional hybridization and mitochondrial introgression between the two species. This is further supported by observations of morphologically intermediate specimens from one site of their cooccurrence (see Results). Signs of introgression are often found in land snails (well documented by Sauer & Hausdorf, 2010; Harl *et al.*, 2014; Morii *et al.*, 2015) and mitochondrial introgression has also been suggested for some *Helix* species by Korábek *et al.* (2015). Hybrid zones have also been observed in some landsnail species (e.g. Heller, 1979; Woodruff & Gould, 1987; Chiba, 2005; Hamilton & Johnson, 2015). We believe that hybridization, which must precede introgression, is even more frequent in land snails than the available evidence suggests. Due to patchy small-scale distribution of the interacting species, the actual hybrid distribution may be limited, which makes hybridization difficult to spot in nature. Within the Helicidae, direct evidence of this process remains scarce. Experimental crosses have been successful between closely related species of *Levantina* (Heller, 1979; but see Ketmaier & Glaubrecht, 2015) and partially also between *C. nemoralis* and *C. hortensis* (Lang, 1908). Allegedly, there has been an unsuccessful attempt to cross *H. pomatia* and *H. vladika* (Fechter & Falkner, 1990).

Nuclear data would be helpful in solving unambiguously the relationships between *H. pomatia* and *H. thessalica*. Unfortunately, nuclear markers used recently for gastropod phylogenetics (rDNA: Wade & Mordan, 2000; histones H3 and H4: Armbruster *et al.*, 2005) exhibit little if any variability between closely-related helicid species, as may be observed in the data archived in GenBank (Wade *et al.*, 2007; Cadahía *et al.*, 2014) and (in case of the rDNA gene cluster) supported also by our unpublished data from other *Helix* species. Furthermore, these markers are present in multiple copies in the genome (rDNA) or are members of a rich gene family (histones), causing potential problems with paralogy (Groenenberg, Subai & Gittenberger, 2012; unpublished data of present authors). The field of helicid systematics would certainly greatly benefit from development of nuclear markers

conservative enough to allow cross-species amplification, but suitable for resolving relationships among closely-related species.

Quaternary history of H. pomatia *s. l.*

In central Europe, *H. pomatia* has been a characteristic element of interglacial faunas since the Middle Pleistocene (Prošek & Ložek, 1957; Ložek, 2001). Because of its long presence in the region, its role as an indicator of warm periods, and its present ubiquity, mapping the phylogeographic history of *H. pomatia* may be a significant contribution to the knowledge of processes that shaped the Holocene land snail fauna of Central Europe as a whole. For these considerations, clarifying the taxonomy and phylogenetic relationships of *H. pomatia* is a crucial first step.

The separation of *H. thessalica* restricts the southerly extent of the distribution of *H. pomatia* and narrows its climatic niche towards the colder northern climate, making its survival more probable in northern glacial refugia. The considerable variability of *H. pomatia* in the Carpathians, even after exclusion of *H. thessalica*, suggests that these mountains may have provided opportunities for surviving the glacial periods. The peri-Carpathian range of *H. lutescens*, together with the existence of a clade of *H. thessalica* found so far only along the Carpathian Arc, further support the role of the area as a glacial refugium relevant also for other large helicids.

However, our results also raise some concern regarding the interpretation of the fossil finds of *H. pomatia*, because the usual state of preservation, i.e. small shell fragments, would not allow for distinguishing between *H. pomatia* and *H. thessalica*, and we doubt that even whole shells could be conclusively identified in most cases. For example, this seems true even for two exceptionally preserved, presumably Eemian (Marine Isotope Stage 5e) individuals from western Slovakia (Ložek & Kneblová, 1957) which, to our knowledge, represent the best-preserved interglacial *Helix* from Slovakia. The shell shape and preserved sculpture are insufficient to decide whether they belong to *H. pomatia* or *H. thessalica*, although the former seems more likely. The Carpathians and their vicinity are the home of characteristic elements of some past interglacial landsnail faunas of central Europe, such as *Drobacia banatica* and *Soosia diodonta* (Prošek & Ložek, 1957; Ložek, 2001), which for unknown reasons failed to recolonize their previously occupied ranges in central Europe after the Last Glacial. *Helix thessalica* may share a similar history; caution is therefore needed when interpreting presumed *H. pomatia* fossils.

Quite surprisingly, *H. thessalica* populations from Slovakia and the Czech Republic do not belong to the haplotype group found in Romania and Hungary, but form a distinct branch among those distributed in Serbia, Montenegro, Macedonia and Bulgaria. If the Slovak and Czech populations of *H. thessalica* are indeed of an origin independent from the Carpathian populations, as the data suggest, the species might once have been more widely spread. Interestingly, two of the localities in Slovakia (Nemecká and Ohnište) are the very places where refugia through the Last Glacial Maximum have been proposed based on occurrence of woodland snail species (Juřičková, Horáčková & Ložek, 2014). Survival of *H. thessalica* in the western Carpathians during the glacial is an intriguing hypothesis, contributing to the current debate on the location and extent of northern glacial refugia (e.g. Sommer & Nadachowski, 2006; Schmitt & Varga, 2012; Tzedakis, Emerson & Hewitt, 2013; Wielstra, Babik & Arntzen, 2015) and worth further study.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

ACKNOWLEDGEMENTS

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