

The Caucasus is neither a cradle nor a museum of diversity of the land snail genus *Helix* (Gastropoda, Stylommatophora, Helicidae), while Crimea is home to an ancient lineage

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<https://zoobank.org/7F4E305A-A732-470C-AEB5-58F5E58605BF>

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Academic editor: Frank Köhler ♦ Received 6 August 2023 ♦ Accepted 10 October 2023 ♦ Published 15 November 2023

Abstract

The Caucasus and the adjacent Pontic Mountains in north-eastern Anatolia are home to numerous endemic land snail genera and species. The diversity of the region is the result of both intra-regional speciation and the persistence of relict lineages. The same seemed to be true for the genus *Helix*, which has been present in the Greater Caucasus since the Miocene. In the Caucasus region, there are three *Helix* species. *Helix buchii* (Pontic Mountains and Georgia) and *Helix albescens* (southern Ukraine to northern Lesser Caucasus) are both separated by deep splits from the major *Helix* clades in the mitochondrial phylogeny. In contrast, *Helix lucorum* belongs to the Anatolian radiation of *Helix*. At least part of its intraspecific diversification may have occurred in north-eastern Anatolia and the adjacent parts of the Caucasus. Here, we report new evidence suggesting that the Caucasus and the Pontus regions were less important as a refugium of ancient *Helix* lineages or as a diversification centre than previously hypothesised. *Helix lucorum* probably diversified more westwards, while *H. buchii* is a less ancient lineage than previously thought. *Helix albescens* had its long-term refugium on the Crimean Peninsula in southern Ukraine, not in the Caucasus. The Caucasus is close to the eastern limit of the distribution range of the genus and, although the fossil record shows that *Helix* was present there as early as the Miocene, the current diversity of the genus there is the result of much later colonisation.

Key Words

Anatolia, fossil, land snail, phylogeography, refugia, Ukraine

Introduction

The Caucasus ecoregion (Zazanashvili et al. 2020) is one of the global biodiversity hotspots, areas of high endemic diversity threatened by ongoing habitat loss (Myers et al. 2000; Marchese 2015). Mountainous topography and climatic stability can explain accumulation of diversity (Harrison and Noss 2017; Rahbek et al. 2019) and these two factors combine in the region. There are high mountains and there are climatic refugia as well. For example, some representatives of the late Neogene woody flora (Browicz 1989; Denk et al. 2001; Nakhutsrishvili

et al. 2015; Magri et al. 2017; Maharramova et al. 2018), as well as some endemic land snails (Pokryszko et al. 2011; Walther et al. 2014; Neiber and Hausdorf 2017; Neiber et al. 2017; Mumladze et al. 2023) and other taxa (Tarkhishvili et al. 2000), survived in the Colchis refugium in the eastern part of the Euxine floristic province of humid temperate forests, which extends along the southern Black Sea coast to the western Greater Caucasus (Browicz 1989). Quaternary glacial forest refugia extended from the western Caucasus along northern Anatolia approximately to Ordu in northern Turkey (Tarkhishvili et al. 2012).

Phylogenetic relationships between some Pontic land snail taxa on the one hand and those from southern Anatolia on the other hand suggest that the Pontic region contains relicts of lineages that were eliminated in central Anatolia by the uplift and aridification of the Anatolian Plateau and its surrounding mountain ranges (Neiber and Hausdorf 2017; Neiber et al. 2021). The uniqueness of the land snail fauna of the Caucasus and the adjacent Pontus Mountains (north-eastern Turkey) is demonstrated by the many species and genera that occur only in the easternmost part of the Pontic Mountains, especially on the northern seaward side around Artvin (Cook 1997; Schütt 2005). Other unique lineages live in the Greater Caucasus (e.g. Walther et al. (2018)). In light of this, the presence of two species of the genus *Helix* without close relatives in the Caucasus and Pontus regions seemed to add to the list of ancient lineages preserved in this long-term refugium (Korábek et al. 2015), the more that there are Micoene and Pliocene *Helix* fossils in the Greater Caucasus (Steklov 1966). The first, *Helix buchii* Dubois de Montpéroux, 1840, is mainly distributed in the Pontus Mountains and in Georgia. The available data suggest that the diversity of mitochondrial lineages is greatest in the western part of its range (Korábek et al. 2022). The other species, *Helix albescens* Rossmässler, 1839 is widely distributed in the Greater Caucasus and southern Ukraine, as far west as Odesa Oblast. Until recently, the species was thought to live as far west as Bulgaria (e.g. Irikov and Erőss (2008)), but this was based on misidentifications (Korábek et al. 2022) and the confusion with *Helix philibinensis* Rossmässler, 1839 (as in Schileyko 1978). The distribution of its intraspecific genetic diversity is unknown.

In addition to the two possibly relictual species, there is a third *Helix* species in the Caucasus, *Helix lucorum* Linnaeus, 1758. It is distributed all over the Caucasus region, Anatolia and the southern Balkan Peninsula. The occurrence of most of the main intraspecific mitochondrial clades in the Caucasus region or adjacent north-eastern Anatolia, with endemic clades in the south-eastern Caucasus (Korábek et al. 2022) may indicate an origin of the species in north-eastern Anatolia or the Caucasus. It was hypothesised that the Pontus region could be a source region of its Holocene expansion to the western Caucasus as well as to the Balkans (Korábek et al. 2018).

Based on our expanded sampling, we tested the hypothesis that the Caucasus region and the Pontic Mountains were the area where the intraspecific diversity of *H. lucorum* originated and that the other two species are old, phylogenetically isolated lineages that persisted in the Caucasus (*H. albescens*) or the Pontic Mountains (*H. buchii*).

Methods

Partial sequences of the mitochondrial genes for cytochrome c oxidase subunit I (*coxI*), 16S rRNA (*rrnL*) and 12S rRNA (*rrnS*) were analysed. Laboratory methods were as described in Korábek et al. (2022) with some

modifications. DNA was extracted as described by Scheel and Hausdorf (2012). The primers 16Sar, 16Sbr (Palumbi et al. 1991), 16S_MN3R (Neiber et al. 2017), 16Scs1 and 16Scs2 (Chiba 1999) were used for amplification and sequencing of the *rrnL* gene, a modified pair LCO1490 and HCO2198 (Hausdorf et al. 2003) for *coxI* and 12SGast_fwd2 and 12SGast_rev3 (Cadahía et al. 2014) for the *rrnS*. Internal primers were designed for PCR and sequencing of *coxI* in a number of *H. buchii* samples: COI_buchii_F: TATTTGGCGTGTGATGTGGC, COI_buchii_R: TAATAGCTCCGGCCAAAACA.

The new data were combined with previously published sequences (Korábek et al. 2022 and references therein). Only seven samples of *H. albescens*, one of them from outside its autochthonous distribution range (Bulgaria), have been analysed previously (Korábek et al. 2022); we added 40 more from 15 additional localities within the presumed native range and two from introduced populations in Ukraine (Kyiv, Poltava). For *H. buchii*, we analysed 57 (27 new) individuals from 32 (13 new) localities (Fig. 1B). Finally, 91 newly-added individuals from 88 sites were analysed for *H. lucorum*, mainly from south-eastern Balkans and the Caucasus region. All the sequences used are listed, along with locality information, in Suppl. material 1: table S1.

Alignments were done with MAFFT 7.487 (E-INS-i algorithm; Katoh and Standley (2013)). Substitution model selection was performed with IQ-TREE 1.6.12 (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017; partitioning into 3 *coxI* codon positions and the two rRNA genes). Concatenated alignments of *coxI*, *rrnL* and, in *H. buchii*, *rrnS* sequences were analysed with BEAST 1.10.4 (Suchard et al. 2018) for each species separately. *Helix nicaeensis* Férussac, 1821, the sister species of *H. lucorum*, was used as an outgroup for *H. lucorum*; the other two species lack close relatives. BEAST was used to produce ultrametric trees where comparably old clades may be defined. Tree prior was set to Bayesian Skyline (a flexible prior not imposing strong constraints on the distribution of branch lengths), using a lognormal relaxed clock and no calibration. However, we specified the same mean rate for all species for approximate comparability. We ran the analyses in two replicates and collected a total of 10,000 post-burn-in trees for each of the three analysed species, making sure that effective sample sizes were > 800 for all parameters, except the skyline model.

PCR and sequencing with the primer pair LCO1490 and HCO219 targeting the “barcoding” fragment of *coxI* produced for two *H. buchii* individuals an incomplete pseudogene sequence containing an in-frame stop codon and frame-shift indels. Two phylogenetically distant mitochondrial lineages are known from *H. buchii*, one of which was probably acquired from an unknown, possibly extinct species (Korábek et al. 2015). A phylogenetic analysis of the pseudogene could reveal which of the mitochondrial lineages is more likely to be the original one for *H. buchii*. We used a representative set of *Helix* and *Maltzanella* Hesse, 1917 *coxI* and *rrnL* sequences

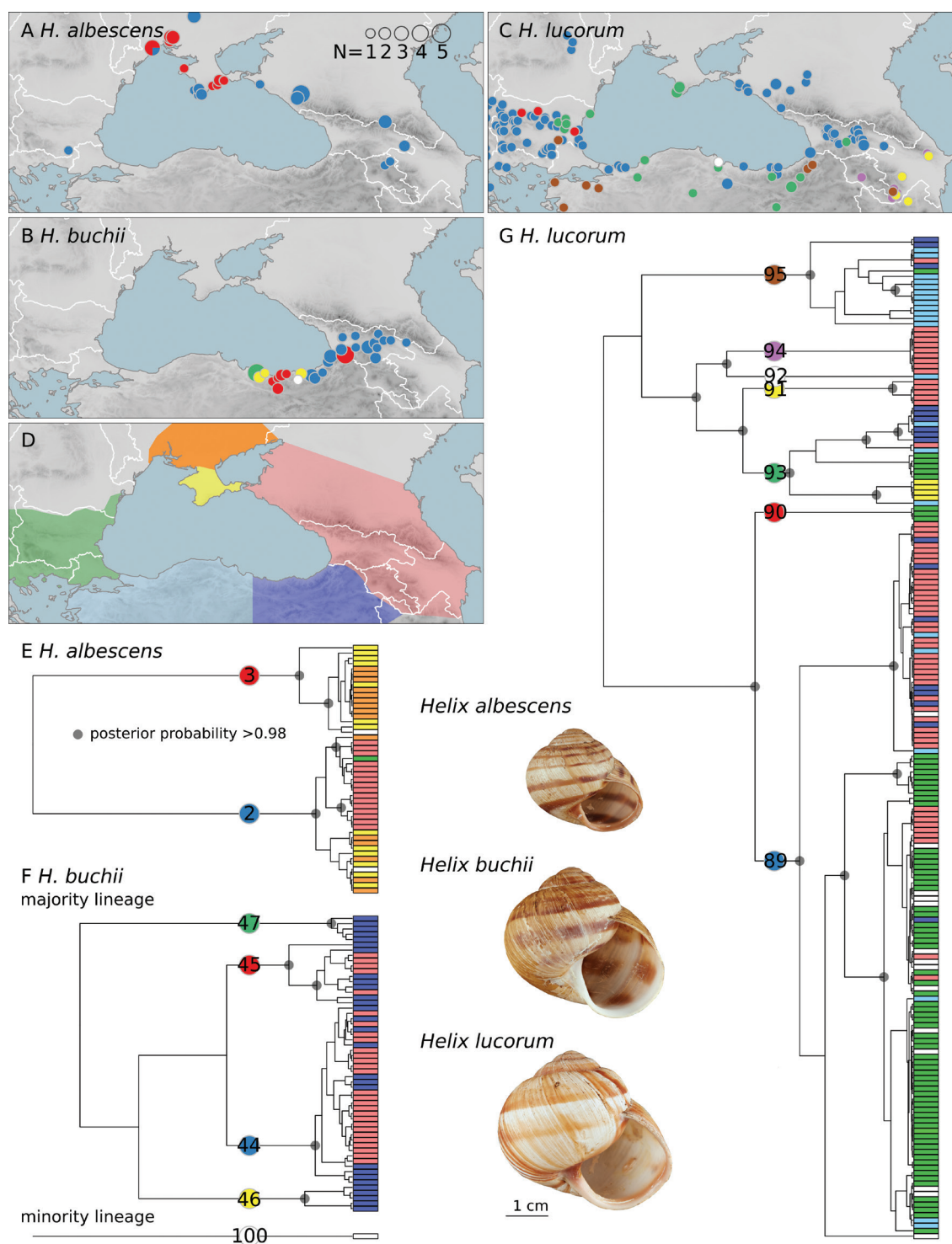


Figure 1. Intraspecific mitochondrial lineages of the Caucasian *Helix* species and their distribution. **A–C.** Distribution of major clades (i.e. the branches labelled with correspondingly coloured circles in the trees). Non-native or potentially non-native occurrences are not distinguished. **A.** *H. albescens*; **B.** *H. buchii*; **C.** *H. lucorum*; **D.** Classification of the discussed regions: southern Ukraine (mainland, orange), Crimea (yellow), the Caucasus region (red), eastern and western part of Anatolia (dark and light blue) and the Balkans (green). The east-west division of Anatolia is for plotting purposes only and coincides with the western distribution limit of *H. buchii*, which also marks the limit of the wettest area of the Pontic Mountains; **E–G.** Bayesian mitochondrial trees based on concatenated alignments of partial *rrnL*, *rrnS* (only in *H. buchii*) and *cox1* sequences. Supported nodes are indicated by small circles at nodes (omitted from the shallowest nodes), large circles on branches denote the clades used for plotting the maps along with the corresponding numbers of the clades as given in Korábek et al. (2022). Tips are labelled with colour indicating the area of origin as in D (samples from outside the covered area are coloured white). The species are illustrated by shells from Georgia: Patara Dmanisi (*H. buchii*, ZMH 100320), Armenia: Gegha Valley by Kavchut (*H. lucorum*, ZMH 101308) and Russia: Kamennomostkiy in Adygea (*H. albescens*, ZMH 86704).

(from a dataset used by Korábek et al. (2022): fig 3) to analyse the phylogenetic position of the *cox1* nuclear pseudogene sequences obtained from *H. buchii* (merged for the analysis into a single sequence at their 404 bp overlap). The sequences used are listed in Suppl. material 1: table S2. The merged pseudogene sequence was added manually to the alignment from Korábek et al. (2022) and the data were analysed with Maximum Likelihood phylogenetic analysis in IQ-TREE following partitioning and automated model selection as above. The support was assessed with 500 bootstraps.

The sequence data underlying this article are available from the NCBI Nucleotide database (available at: www.ncbi.nlm.nih.gov/Genbank). Accession numbers are provided in the online supplementary material along with sampling localities and voucher information.

Results

Helix albescens Rossmässler, 1839

We found two major clades, each of them with only shallow internal divergences (Fig. 1E). Only one was found in the Caucasus, but both are present in southern Ukraine. In the Crimean Mountains, the two clades are parapatric, one occurring in the west and the other in the east. The eastern clade has its basal-most haplotype there. The western clade is surprisingly the one found also in the Caucasus. We observed three groups of haplotypes in this clade, one (without strong support) in southern Ukraine inclusive of Crimea and the other two on the southern (Georgia, Armenia) and northern (Russia) side of the Greater Caucasus, respectively.

Helix buchii Dubois de Montpéreux, 1840

Two divergent lineages have been found in *H. buchii*. All but one of the analysed individuals yielded haplotypes belonging to the ‘majority lineage’, which is deeply separated from the major *Helix* radiations (Figs 1F, 2). The data show generally low intraspecific diversity in Georgia, whereas multiple lineages including the ‘minority lineage’, which is likely the sister lineage of *Helix pathetica* Mousson, 1854 within the Anatolian radiation of *Helix* (Fig. 2), were found in north-eastern Turkey. The oldest split of the majority lineage separated a clade (47 as numbered by Korábek et al. (2022); Fig. 1F) from the westernmost sampling site from all other haplotypes. Almost all Georgian samples are closely related to each other and the adjacent eastern Turkish samples. An exception to these are samples from the Goderdzi Pass in south-western Georgia (Mumladze et al. 2013), which were described as *Helix goderdziana* Mumladze, Tarkhishvili & Pokryszko, 2008, sharing a mitochondrial lineage (45) with some individuals from the west of the species range.

Partial, but overlapping *cox1* pseudogene sequences, were obtained from two individuals (GenBank accessions: isolate SP2 [OQ148366](https://www.ncbi.nlm.nih.gov/nuclot/OQ148366), isolate 6916 [OQ148367](https://www.ncbi.nlm.nih.gov/nuclot/OQ148367)). These samples otherwise yielded haplotypes of the clades 44 and 45 of the majority lineage. Phylogenetic analysis placed the pseudogene sequences into the Anatolian clade of *Helix* (Korábek et al. 2015), along with the sole sample of the minority mitochondrial lineage collected near Sümela Monastery in Turkey (Fig. 2).

Helix lucorum Linnaeus, 1758

In Armenia and Azerbaijan, all but one sample (from Tatev Monastery, Armenia) yielded haplotypes from two co-occurring clades (Fig. 1G), which as yet have not been found elsewhere, while almost all samples from Georgia and Russia belong to an unrelated clade that is otherwise distributed in northern Anatolia and accounts also for the majority of the occurrences in the Balkans, as well as introduced populations in various parts of Europe. This widespread intraspecific clade (number 89 in Korábek et al. (2022), blue in the map in Fig. 1C), is associated with conical to flattened conical shells in contrast to more globular ones typical for more southerly populations in Anatolia and the Lesser Caucasus (Korábek et al. 2018).

The haplotypes of the clade 89 form four well-supported groups. One of them was found only in a non-native sample from Spain, but one was recorded so far only in the eastern Balkans and none of the remaining two is unique to the Caucasus and Pontus. Furthermore, there are nearly identical haplotypes (1 bp difference over 1469 bp of the *rrnL+cox1* alignment) in the Caucasus on the one hand and the eastern Balkans on the other hand. The sister lineage of the whole clade (red in Fig. 1C) was found exclusively in the eastern Balkans.

Populations with globular shells, which share mitochondrial clade 93 (green in Fig. 1C), are widespread in northern Anatolia, but are also found in Crimea and north-eastern Bulgaria. The geographically isolated populations of this lineage in Bulgaria differ in shell shape and colouration from the majority of Bulgarian *H. lucorum*.

Discussion

Helix albescens Rossmässler, 1839

Both major mitochondrial clades within *H. albescens* occur in the Crimean Mountains and the lowlands of southern Ukraine, while only one of them occurs in the Caucasus (Fig. 1A). It is possible that *H. albescens* was present in the Caucasus already before the Last Glacial, as we found some differentiation of Caucasian populations on both sides of the Greater Caucasus ridge. However, it is unlikely that *H. albescens* has persisted in the Caucasus for an extended period of time. Instead, the Crimean Mountains, where its two main clades appear

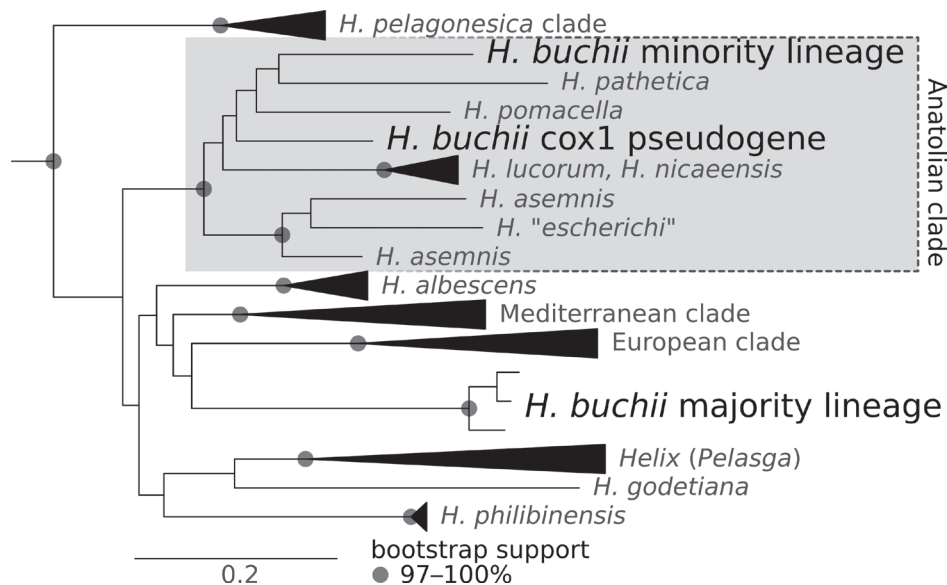


Figure 2. Maximum Likelihood tree based on *rrnL* and *cox1* sequences showing the relationships of *H. buchii*. Both mitochondrial lineages and the *cox1* pseudogene (presumed NUMT) sequence obtained from two of its individuals with the majority lineage mitochondrion are included. The samples listed in Suppl. material 1: table S2 were used; the tree is rooted with *Maltzanella* (not shown). Support values were obtained by standard bootstrapping.

to be parapatric (in contrast to the rest of Ukraine) are the most probable long-term refugium. The Crimean Mountains show some biogeographic affinities with the western Caucasus (Cameron et al. 2013; Turbanov and Balashov 2015; Neiber and Hausdorf 2017), but were a glacial refugium in their own right (e.g. Kukushkin et al. (2020)) and host a number of endemic land snail species and genera (Hausdorf 1994; Balashov and Baidashnikov 2013; Cameron et al. 2013; Balashov 2014, 2016; Neiber et al. 2019). The long-term stronghold of *H. albescens* in southern Crimea supported by our data, therefore, corresponds to known patterns. Furthermore, within Ukraine, the variability in shell shape was found to be the greatest in Crimea (Kramarenko 2016) and the diversity of banding patterns is greater in the Crimean Mountains than in the Crimean lowlands (Kramarenko and Leonov 2011).

The extent of the native distribution of *H. albescens* on the East European Plain is unclear. It is relatively continuously distributed across the steppe ecoregion of southern Ukraine and in adjacent regions of Russia south to the Caucasus, both in natural and transformed habitats. In Ukraine, there are no Quaternary fossil records of this species outside Crimea (Balashov 2016: 85). Since at least 2006, *H. albescens* has been present in Kyiv, central Ukraine, marking the first case of an evident invasion outside its native range far from its continuous distribution. Since then, it has also reached several other settlements in central and eastern Ukraine. Thus, *H. albescens* is now spreading northwards and it is possible that much of its distribution in the East European Plain is the result of recent expansion, possibly largely human-assisted. Similarly, several other stylommatophoran gastropods that originated or were isolated for a long time in the Crimean Mountains have relatively recently spread to the

southern part of the East European Plain. To what extent this range extension is anthropogenic remains unclear. In particular, this applies to *Brephulopsis cylindrica* (Menke, 1828) (Enidae), *Helicopsis filimargo* (Krynicky, 1833) (Geomitridae) and *Monacha fruticola* (Krynicky, 1833) (Hygromiidae) (Balashov 2016; Neiber and Hausdorf 2017; Balashov et al. 2021). All these species, along with *H. albescens*, form a group of inhabitants of dry open and semi-open habitats that spread to the south of the East European Plain postglacially from Crimea and anthropogenic factors allow for continuation of this expansion.

Helix buchii Dubois de Montpéroux, 1840

Korábek et al. (2015) found two divergent mitochondrial lineages within *H. buchii*. One sample from Sümela (Trabzon Province of Turkey) yielded a lineage belonging to a group of largely Anatolian species (“Anatolian clade” sensu Korábek et al. (2015)). However, all other samples yielded a different, highly divergent lineage with no relatives and unaffiliated with any major clade within the genus. The majority lineage indicates that *H. buchii* could be an old species persisting in a long term refugium from the Neogene. One or the other lineage might be the result of an introgression from another species, but the source of the introgression remained unclear because neither lineage is closely related to other known extant species. It is unlikely that one of them is a nuclear pseudogene (NUMT), because the results for the *rrnL* and *cox1* genes were replicated here also with the *rrnS* gene, which is in *Helicidae* located in a different part of the mitogenome and coded on the other strand (Terrett et al. 1996; Groenenberg et al. 2012; Gaitán-Espitia et al. 2013; Korábek et al. 2019).

Based on the distribution of individual clades from the majority lineage, *Helix buchii* did not originate in the Caucasus hotspot or the centre of land snail diversity in the mountains around Artvin in Turkey. The data show generally low intraspecific diversity in Georgia, whereas multiple lineages (including the minority lineage) were found in north-eastern Turkey. Our results obtained with improved sampling thus confirm earlier observations (Mumladze et al. 2013; Korábek et al. 2022). The origins of *H. buchii* can be traced to the western part of the Eastern Pontic Mountains and the additional data confirm that its current presence in the Caucasus is the result of a recent range expansion from the west.

The phylogenetic placement of the *cox1* pseudogene (presumed NUMT) sequenced here in the Anatolian clade indicates that the minority mitochondrial lineage is the original mitochondrial lineage of *H. buchii*. This means that *H. buchii* is younger and less isolated than suggested by the sequence data representing the majority mitochondrial lineage. The origin of the majority lineage remains unclear. Perhaps the inferred early branching is an artefact caused by its long branch. In any case, the closest relatives of *H. buchii* appear to be species from the north-west of Anatolia.

Helix lucorum Linnaeus, 1758

With two sympatric lineages restricted to the south-eastern Caucasus region (Azerbaijan, Armenia, adjacent Iran), the species is not a complete newcomer to the area, although it is still only a very young group in the context of the *Helix* phylogeny (Korábek et al. 2022). However, the phylogenetic relationships of these two lineages suggest that they originated in Anatolia. The sister lineages of both are found there, one of them being distributed all over the north of Anatolia. As the sister group of *H. lucorum*, *Helix nicaeensis*, lives in north-western Anatolia, the likelihood that the species diversified near the Caucasus region seems low and the two endemic lineages are likely the result of an earlier, pre-Holocene colonisation.

The geographic origin of the widespread clade 89 (see Fig. 1C, G), which with one exception accounts for the distribution of *H. lucorum* in Russia and Georgia, is unclear, as sampling of Anatolia remains inadequate and the distribution ranges of the *H. lucorum* lineages are likely to have been affected by anthropogenic translocations. Mumladze (2015) raised reasonable doubts as to whether *H. lucorum* is native to Georgia and the new data add to these. The fact that the sister clade of clade 89 has, so far, been found only in Bulgaria suggests that the origin of this clade is located more westerly than the Pontus region, which was previously suggested as its area of origin (Korábek et al. 2018). Korábek et al. (2018) found one haplotype group in this clade to be unique to the Pontus Mountains and Georgia (apart from an introduced population in Moscow), but it is now also known from localities in the Provinces of Tokat and Sakarya, i.e. westwards

up to the westernmost Anatolia. Very similar haplotypes at the western and eastern extremes of the range of clade 89 (i.e. eastern Balkans versus the western Caucasus), given the distance, suggest human-mediated dispersal in at least one direction, although natural dispersal cannot be excluded at present.

The current distribution of different *H. lucorum* lineages within the species' range is influenced by anthropogenic translocations (Korábek et al. 2018). The occurrence near the Tatev Monastery in Armenia could be such a case, as the lineage present there differs from the regional background. Similarly, globular-shelled populations of clade 93 (green in Fig. 1C, G) were found in north-eastern Bulgaria in an area disconnected from the main range of the clade in Anatolia. The presence of clade 93 in Crimea (a globular form previously separated as *H. lucorum taurica* Krynicki, 1833; see Korábek et al. (2018)) is also most easily explained by introduction (Korábek et al. 2018). The most conchologically similar individuals have so far been found in north-western Anatolia in the provinces of Bolu and Kastamonu. It is likely that translocations have also occurred within Anatolia. The co-occurrence of several lineages in a region must, therefore, be interpreted with caution, as they may have been brought together by humans, especially if samples come from synanthropic habitats.

Conclusion

It is likely that *Helix buchii* is not an ancient and isolated lineage of *Helix* in the Caucasus region, but part of a group of species that diversified in Anatolia (the 'Anatolian clade' of Korábek et al. (2015)). Our new data suggest that the species originated in the Pontus region in northern Anatolia and colonised the Caucasus region relatively recently. *Helix albescens* has also not been present in the Caucasus region for a long time, as its crown group probably originated in Crimea. The third Caucasian species, *H. lucorum*, is another member of the Anatolian clade of *Helix*. Populations of *H. lucorum* from Armenia and Azerbaijan have mitochondrial lineages that may be endemic to the region, suggesting that this species may have been present there for a longer period of time than the other two species. However, the more northerly populations in Georgia and Russia belong to a recently-spread clade, for which the new data suggest an origin elsewhere, possibly as far west as the Balkans, and which probably colonised the region only with human help.

The hypothesis of the Caucasus and the adjacent Pontus region of Turkey as a place where ancient lineages of *Helix* (not belonging to the European, Mediterranean or Anatolian clades of *Helix*) persisted for a long period of time (Korábek et al. 2015) can thus be rejected. Within the wider region, deeper intraspecific diversity was preserved or endemic species exist in the western Pontus, in the Lesser Caucasus and in Crimea, but the Greater Caucasus and the territory of Georgia were colonised relatively recently

(comparison with the central-European species *Helix pomatia* suggests as late as Late Pleistocene or Holocene; Korábek et al. (2023)). There appears to be no direct link between the *Helix* species that lived in the northern foothills of the Greater Caucasus (Chechnya, Dagestan) from the Middle Miocene and Late Pliocene to the Early Pleistocene (Steklov 1966; Beluzhenko 2014, 2015) and the *Helix* species currently living in the region. This is bad news for the potential use of the remarkable fossil record of this region for evolutionary analyses of *Helix*. Whether there is a link between Miocene *Helix* fossils from Crimea (Mayer 1856; Andrusov 1896; Wenz 1923) and *H. albescens* remains to be established, but it would be consistent with a previous estimate of the divergence time of *H. albescens* and the European clade of *Helix* at around 10 million years (Neiber and Hausdorf 2015).

Acknowledgements

We thank colleagues who provided us samples for analysis or helped with collecting, especially S. Kramarenko (Mykolayiv National Agrarian University, Ukraine), M. Son (Institute of Marine Biology NAS Ukraine, Odesa, Ukraine), E. Rybalchenko (Poltava, Ukraine) and P. Romanov (Kryvyi Rih, Ukraine). The work was funded by the Alexander von Humboldt Foundation. The publication of this article was funded by the Open Access Fund of the Leibniz Association.

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Supplementary material 1

List of sequences

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Data type: xlsx

Explanation note: **table S1.** List of sequences used for intraspecific phylogenetic analyses presented in Fig. 1. **table S2.** List of sequences used for inferring the phylogenetic position of a *cox1* pseudogene in Fig. 2.

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