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Systematics and evolutionary history of large endemic snails from the Caucasus (*Helix buchii,* and *H. goderdziana*) (Helicidae)

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Abstract. Two species of genus *Helix* Linnaeus, 1758 (Mollusca: Gastropoda: Helicidae) endemic to the Caucasus region are known from Georgia and northeastern Turkey: *Helix buchii* Dubois de Montpereux, 1839 and the recently-described but disputed *Helix goderdziana* Mumladze, Tarkhnishvili and Pokryszko, 2008. The latter species is the largest land snail throughout non-tropical Eurasia. We compared shell shapes and genital morphology of the two species. We analyzed mitochondrial COI and nuclear 18S ribosomal RNA and ITS1 gene fragments in 39 specimens of *H. buchii* and *H. goderdziana* from ten locations from the entire distribution range of these species, together with 13 specimens of the widespread *H. lucorum* Linnaeus, 1758 and *H. pomatia* Linnaeus, 1758. Based on shell morphology alone, most of the individuals of the two species can be discriminated using multivariate approaches. The species have different flagellum/diverticulum ratios, and the foot coloration is a fully diagnostic morphological character. Molecular genetic analysis revealed little variation in 18S+ITS1 fragment, and eleven COI haplotypes. Phylogenetic analyses support reciprocal monophyly of *H. buchii* and *H. goderdziana*. The genetic distances significantly correlate with the geographic and morphological distances; correlation of morphological distances with geography is insignificant. The basal lineages of both species are found within two distinct glacial refugia, a result which matches the separation of eastern and western evolutionary lineages of other relicts of the Western Caucasus. The present distribution of *H. goderdziana* coincides with the expected refugial borders, whereas *H. buchii* is likely to have extended its geographical range since the last glaciation.

Key words: Mollusca, phylogeography, DNA, Caucasus, refugia

Helix Linnaeus, 1758 (Gastropoda: Helicidae) are the largest land snails of northern Eurasia. The genus includes over 25 species (Schütt 2005, Welter-Schultes 2009). *Helix buchii* Dubois de Montpereux, 1839, (Figs. 1A, 1C) until recently known as the largest land snail of the western Palaearctic, is an endemic of the mountain broadleaf forests of the Caucasus ecoregion (Zazanashvili *et al.* 2004), which harbor numerous Tertiary relict species and habitats (Tuniyev 1990, Röhrig 1991, Mai 1995, Veith *et al.* 1998, Kikvidze and Ohsawa 1999, Denk *et al.* 2001, Milne and Abbott 2002, Milne 2004, 2006, Zazanashvili *et al.* 2004, Tarkhnishvili *et al.* 2012).

Another large snail, *Helix goderdziana* Mumladze, Tarkhnishvili and Pokryszko, 2008 (Figs. 1B, 1D), has been recently-described from southwestern Georgia near Goderdzi pass (Mumladze *et al.* 2008). This snail is even larger: the shell diameter in some individuals reaches 68 mm (this paper). The distribution ranges of both species overlap, although *H. goderdziana* is limited to the western Lesser Caucasus and is known from only two localities (Fig. 2). Sysoev and Shileyko (2009) disputed the taxonomic status of *H. goderdziana*, suggesting that the traits used in the original description (foot coloration, shell size, and flagellum length) may vary broadly within a species. Indeed, morphological traits in *Helix* are highly variable, and species-level taxonomy is regularly disputed (Schütt 2005, Neubert and Bank 2006, Sysoev and Shileyko 2009).

Delineating species is a common problem in systematics (De Queiroz 2007, Mallet *et al.* 2007, Hausdorf 2010, Mallet 2010), but distinguishing between similar species is a core step to assess and maintain biodiversity (Bickford *et al.* 2006). There is a lack of comprehensive studies on systematics, distribution and conservation of Caucasian *Helix* species. In order to clarify the evolutionary history and taxonomic status of *H. goderdziana* and *H. buchii* (from here onwards – *Endemic Caucasian Helix*, *ECH*), we applied a combination of molecular genetics and morphometric approaches to the samples collected throughout the range of both species. In addition, we provide brief information on the two known localities of *H. goderdziana* to address its conservation status.

MATERIAL AND METHODS

Sampling

During 2008–2010, we collected adult specimens (individuals with well-developed lip) of *Helix buchii* and *H. goderdziana* from Georgia and NE Turkey (Fig. 2). One to twelve *H. buchii* from eight locations, and two to five *H. goderdziana* from both known locations of this species were sampled. The small samples of *H. goderdziana* reflect its rarity. As outgroups for genetic and morphological studies, the widespread species

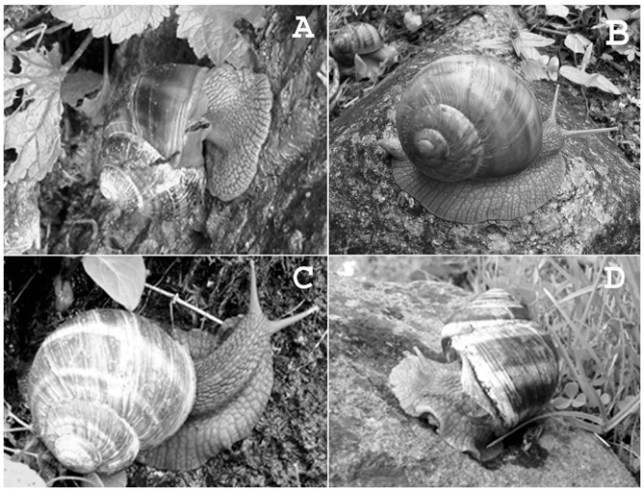

Figure 1. *Endemic Caucasian Helix (ECH).* **A,** subadult *H. buchii*; **B,** subadult *H. goderdziana*; **C,** adult *H. buchii*; **D,** adult *H. goderdziana.*

Helix lucorum Linnaeus, 1758 and *Helix pomatia* Linnaeus, 1758 were used: eight and five adult specimens, respectively (Table 1). Geographic coordinates of each location were recorded with a Garmin Etrex 12 Channel GPS unit (Garmin Corp., Olathe, Kansas, U.S.A.). Live individuals were drowned in water and then preserved and stored in 96% alcohol for further processing. The genitalia were dissected and measured for five *H. buchii*, three *H. goderdziana,* one *H. lucorum,* and one *H. pomatia*. Pieces of muscular tissue of collected individuals were used for DNA extraction and processing. Alcohol-stored specimens and shells are deposited in the collection of Zoological Institute of Ilia State University under accession numbers h1–h59.

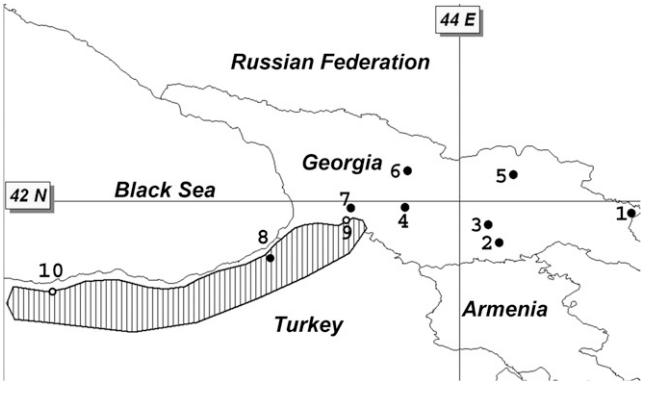

Figure 2. Sampled locations of *Helix buchii* (black dots) and *H. goderdziana* (open circles): **1,** Lagodekhi (eastern Greater Caucasus); **2,** Dmanisi; **3,** Didgori; **4,** Borjomi (central Lesser Caucasus); **5,** Khevsha; **6,** Mokhva (central Greater Caucasus); **7,** Bakhmaro (western Lesser Caucasus); **8,** Jamilikhemshin (Kackar Mountains); **9,** Goderdzi Pass (western Lesser Caucasus, type locality of *H. goderdziana*); **10,** Kovanlyk. Outlined area: borders of the Major Forest Refugium (see discussion), *sensu* van Andel and Tzedakis (1996).

Morphology

The shells of adult specimens (thirteen *Helix buchii*, seven *H. goderdziana*, four *H. pomatia*, and four *H. lucorum*) were scanned using a 3D scanner (Roland PICZA 3D Laser Scanner LPX-600). Nineteen landmarks were selected: L0 = intersection of the main axis and the columellar part of lip; L3 = junction of the lip with the body whorl; L6 = apex; other landmarks were positioned using the junctions of two perpendicular planes, the first crossing the landmarks L0, L3, and L6 and the second adjusted perpendicularly to the first so that landmarks L0 and L6 were common to both (Fig. 3). Placing landmarks and extracting coordinates were performed with software Landmark v2.0 (Wiley *et al.* 2005). Geometric morphometry methods are commonly used for the analysis of snail shells (Conde-Padín *et al.* 2007) when landmark data can be captured. However, if the landmarks do not meet true homology criteria, the interpretation of the analysis results might be misleading (Zelditch *et al.* 2004). Because our landmarks (except L3 and L6) cannot be assumed as homologous, we used a "traditional" Principal Component Analysis (PCA; Joliffe 1992, MacCallum *et al.* 1999) for describing shell shape differences using between landmark distances, which are easier to interpret (Blackith and Reyment 1971, Richtsmeier *et al.* 2002).

To maximally approximate the assumptions of PCA and to maintain sufficiently high sample/ variable ratio, we had to reduce the available set of distance measures to few distance variables. Based on visual observations on *Helix buchii* and *H. goderdziana*, most obvious differences in shell shape are due to the shape of shell spire. Consequently, we used the following eight distance measures describing shell spire: L4–L6, L5–L6, L5–L7, L6–L8, L6–L9, L6–L16, L6–L17, L11–L15, L12–L16, and L13–L17 (Fig. 3). In order to meet a normality assumption and minimize size influence and allometric effect, the distances were log-transformed and then standardized residuals of the regression of each character on the distance between shell apex and most proximate distance of outer lip (L1–L6) were calculated, as recommended by Thorpe and Leamy (1983). Standardized residuals calculated for the 10 variables were subjected to Principal Component Analysis (PCA) with components extracted at eigenvalues over 1.

We dissected five adult specimens of *Helix buchii,* three of *H. goderdziana,* one of *H. lucorum*, and one of *H. pomatia* in order to compare qualitative and quantitative traits of their genital morphology. We measured length of flagellum, length of penis + epiphallus, length of bursa tract, diverticulum, maximum length of mucus gland, and length of dart sac of each dissected individual (Fig. 4). All statistical analysis was

Table 1. Sampling locations with GPS coordinates and number of sampled specimens. Abbreviation in brackets for first column stands for: Geo, Georgia; Tu, Turkey; Pol, Poland.

Sampling location	GPS coordinates	Species	DNA samples	Shell samples	Genital samples
Lagodekhi (Geo)	41.85N, 46.29E	*Helix buchii*	1	1	-
Dmanisi (Geo)	41.33N, 44.35E	*H. buchii*	6	2	-
Didgori (Geo)	41.78N, 44.51E	*H. buchii*	7	2	1
Borjomi (Geo)	41.91N, 43.25E	*H. buchii*	2	1	-
Khevsha (Geo)	42.40N, 44.69E	*H. buchii*	1	1	-
Mokhva (Geo)	42.43N, 43.30E	*H. buchii*	12	2	2
Bakhmaro (Geo)	41.89N, 42.37E	*H. buchii*	2	2	-
Jamilihamshin (Tu)	41.14N, 40.93E	*H. buchii*	3	2	2
Goderdzi (Geo)	42.57N, 41.63E	*H. goderdziana*	2	4	2
Kovanlik (Tu)	38.14N, 40.68E	*H. goderdziana*	3	3	1
Tbilisi (Geo)	41.72N, 44.65E	*H. lucorum*	8	4	1
Wroclaw (Pol)	51.11N, 17.01E	*H. pomatia*	5	4	1

performed using SPSS v.16 for Windows (SPSS Inc. Chicago, Illinois, U.S.A.).

DNA analysis and inferring relations between haplotypes

Total cellular DNA was extracted from a small piece of the hind part of the foot of individual snails. Extraction was performed using QIAGEN® QIAamp DNA Mini Kit followed by a slightly modified standard protocol provided by QIAamp DNA Mini Kit Handbook (QIAGEN, Hilden, Germany). Partial sequences of mitochondrial gene COI and fragments of nuclear 18S ribosomal RNA gene and internal transcribed spacer 1b (18S+ITS1) were amplified and sequenced for 34 *Helix buchii,* five *H. goderdziana,* eight *H. lucorum,* and five *H. pomatia*. Amplification conditions and temperature profiles are given in Appendix 1. The amplicons were sequenced on the automatic sequencer ABI 3130 (Applied Biosystems, Foster City, California). Single-stranded sequencing was performed with polymerase chain reaction primers, using the Big-Dye Terminator v3.1 (Applied Biosystems, Foster City, California). DNA sequences were edited using SEQSCAPE v2.5 (Applied Biosystems Inc. Foster City, California); only unique COI and 18S+ITS1 haplotypes were deposited in GenBank (accession # GU784797–GU784807). The alignment of the sequences was performed with BioEdit v7.0 (Hall 1999). Phylogenetic analyses were performed for high-quality sequence fragments including 364 bp for COI (the obtained sequences of COI were not readable in the end of 3′ direction) and 473 bp for 18S+ITS1.

The sequences were aligned with the six most similar GenBank sequences, as shown by BLAST output *Lozekia deubeli* (Kimakowicz, 1890) (COI; GenBank accession # EU182503), *Marmorana scabriuscula* (Deshayes, 1830) (COI; # EU189930), *Arianta arbustorum* Linnaeus, 1758 (both genes; # AF296946 and AY546455), species of *Satsuma* H. Adams, 1868 (both genes; #AB242535 and AB481049), and *Iberus* Montfort, 1810 spp. (both genes; # EF440266 and EU446026), and *Caucasotachea calligera* (Dubois de Montpereux, 1840) (18S+ITS1; # GU784810 – sequenced by authors specifically for this manuscript). Unfortunately, no homologous DNA fragments of other *Helix* are available from GenBank). Phylogenetic relationships between the individual COI haplotypes were inferred

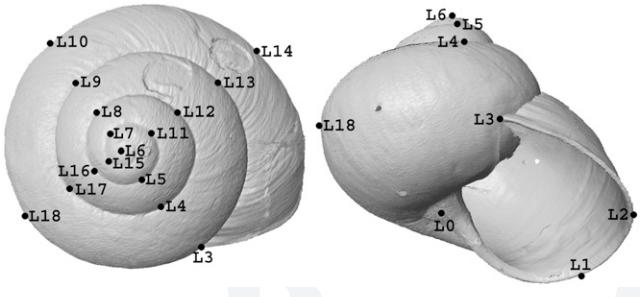

Figure 3. The position of the landmarks used for morphometric analysis of shells of the studied species.

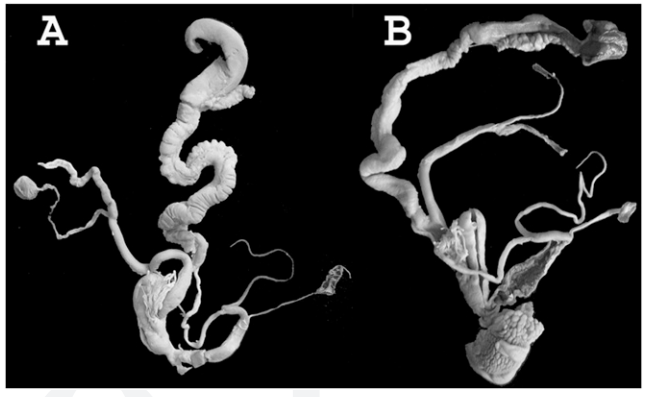

Figure 4. Overall view of genital organs. **A,** *Helix goderdziana*; **B,** *H. buchii*.

with neighbor-joining (NJ), maximum parsimony (MP), and Bayesian algorithms. NJ and MP trees were inferred using MEGA v5 (Tamura *et al.* 2011) with applying default settings (all positions included, 1000 bootstrap replications, Max-mini branch-and-bound for MP). Bayesian phylogenetic analysis was performed using the software BEAST v1.5.1 (Drummond and Rambaut 2007). Posterior distributions of parameters were approximated using Markov Chain Monte Carlo (MCMC) with length of chain 3×10^7 that harvested effective sample size (ESS) > 100 for each parameter. The best model was identified by the model comparison procedure based on the marginal likelihood, using a code written for BEAST (Suchard *et al.* 2001). Prior to this analysis, we tested the molecular clock hypothesis (Hasegawa *et al.* 1985) and found the best model of nucleotide substitution using Bayesian Information Criterion (BIC) using software MEGA v5 (Tamura *et al.* 2011). All possible evolutionary pathways among the obtained haplotypes of *H. buchii* and *H. goderdziana* were reconstructed using Median-Joining (MJ) algorithm (Donnelly and Tavare 1986, Bandelt *et al.* 1999) using the software Network 4.6.1 (Bandelt *et al.* 1999). The GenGIS software (Parks *et al.* 2009) was used for plotting the phylogenetic tree on a geographic map (Fig.7).

Because 18S+ITS1 sequences were identical for three out of four studied species (see results), they were not subjected to the detailed phylogenetic analyses.

To explore to what extent morphological variability among *ECH* individuals is associated with their evolutionary differentiation we applied partial Mantel test (Manel *et al.* 2003) with 10,000 permutations, using IBD software (Bohonak 2002). All 20 studied *ECH* individuals were included in the analysis, without *a priori* attribution to *Helix buchii* or *H. goderdziana*. To perform Mantel test genetic distances between individual COI sequences were estimated according to Kimura (1980) using MEGA v5.

Morphological distances (shell shape) were estimated as Euclidean distances based on individual scores from all PCA axes with eigenvalues exceeding unity. We explored whether: (I) genetic distances between the individuals of *Helix buchii* and *H. goderdziana* significantly correlated with geographic distances between the locations; (II) morphological distances significantly correlated with (a) genetic distances between the individuals, and (b) geographic distances between the locations.

RESULTS

Morphometry

The output of PCA based on the shell measurements is shown in Table 2 and Fig. 5. Two PCA axes were extracted with eigenvalues > 1. All included variables had a high communality values (> 0.8), indicating that the result can be used in a meaningful way (Table 2). The first PCA axis (72% of the total variation and 10% for second PCA axis) had similar positive loading for all the variables which implies that increasing score values along the axis marks higher shells with broader spire (wider apical whorls) relative to the shell size. Adult individuals of *Helix lucorum* have the highest scores along this axis, and *H. buchii* and *H. pomatia* have the lowest scores. *Helix goderdziana* keeps an intermediate position between *H. buchii* and *H. lucorum*, but the overlap is higher with the latter species (Fig. 5). The interspecific differences in the average values of the first PCA scores are significant

Table 2. Loadings of individual shell dimensions on the PCA axes. PCs with eigenvalues exceeding unity are shown. All variables are standardized residuals of the corresponding measurements from the regression line on lnL1–L6. Last column contains Communality (indicating a percent of variance accounted by the PCs) values for each distance variable.

Distances	PC1	PC2	Communalities
L4–L6	0.91	-0.27	0.894
L5–L7	0.88	-0.20	0.811
L5–L6	0.82	-0.34	0.779
L12–L16	0.83	0.50	0.932
L6–L8	0.90	0.09	0.813
L6–L9	0.91	-0.05	0.828
L6–L15	0.65	-0.21	0.465
L6–L16	0.88	-0.26	0.837
L6–L17	0.90	-0.18	0.838
L11–L15	0.88	0.34	0.897
L13–L17	0.75	0.63	0.956

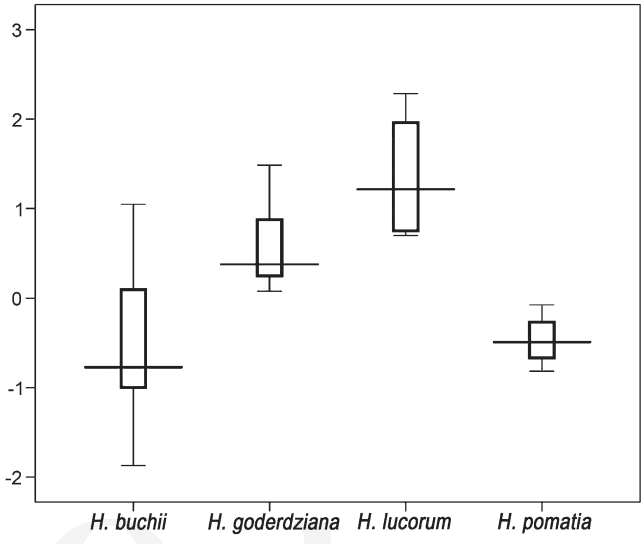

Figure 5. Box plots of individual scores of the four studied *Helix* species along the first PCA axis defined by shape of the shell spire.

Table 3. Multiple pairwise comparison (with Bonferroni adjustment) after One-way ANOVA based on individual scores for first PCA axis. Numbers indicate the mean differences. Numbers in bold represent significant results at 0.05 significance level.

	H. goderdziana	*H. lucorum*	*H. pomatia*
Helix buchii	**-1.14**	**-1.87**	0.42
H. goderdziana		-0.76	1.07
H. lucorum			**1.82**

(One-way ANOVA, $F_{3,26} = 8.9$, $P < 0.001$). Mean differences are significant ($P < 0.05$ after Bonferroni adjustment) between *H. lucorum* and *H. buchii*, *H. lucorum* and *H. pomatia, H. buchii* and *H. goderdziana*; the differences are not significant ($P > 0.05$) between *H. goderdziana* and *H. lucorum*, *H. pomatia* with *H. buchii*, and *H. pomatia* with *H. goderdziana* (Table 3).

Most of the genitalia measurements did not show obvious differences neither between *Helix buchii* and *H. goderdziana*, nor among *ECH* and the two other *Helix* species (Fig. 6). However, the flagellum/diverticulum ratio in the studied individuals of *H. goderdziana* was significantly lower than in *H. buchii* and much shorter in *ECH* than in either *H. lucorum* or *H. pomatia*.

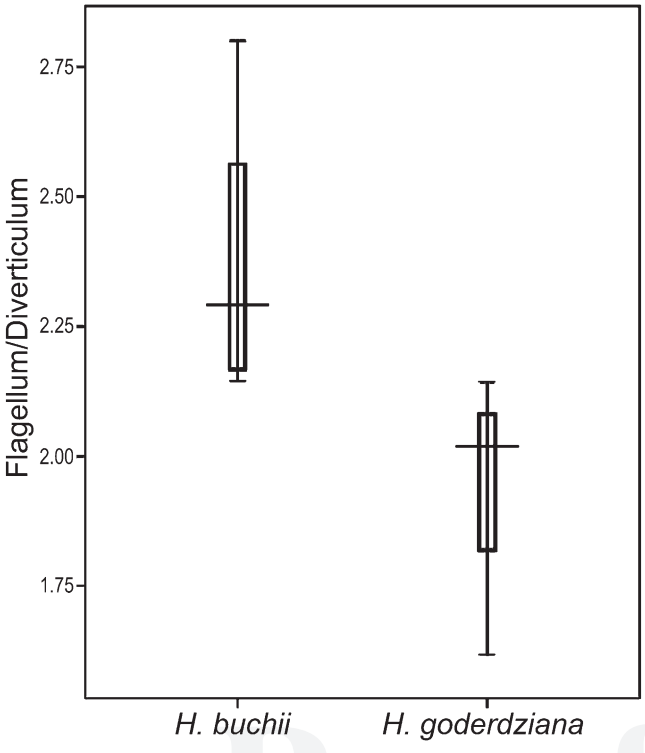

Figure 6. Box plots of flagellum/diverticulum ratios for *Helix buchii and H. goderdziana*.

Phylogenetic relations of the studied species

The sequenced fragment of nuclear 18S+ITS1 was identical for *Helix goderdziana, H. buchii* and *H. lucorum*. Five substitutions separate these species from *H. pomatia.* The sequenced COI fragment had 92 informative sites for all 52 obtained sequences of four *Helix* species. The lowest BIC value was shown for Hasegawa-Kishino-Yano model (HKY) with gamma distribution (HKY+G). Five haplotypes of *H. buchii*, two of *H. goderdziana,* three of *H. lucorum* and one of *H. pomatia* were identified. Individual haplotypes of *ECH* differed by 1–15 substitutions. NJ, Bayesian, and MP consensus tree (Fig. 7) supported (1) monophyletic origin of *ECH*, with respect to the outgroup taxa (*H. pomatia, H. lucorum,* one hygromiid and four helicids downloaded from the GenBank) and (2) reciprocal monophyly of *H. buchii* and *H. goderdziana*. The MJ network (Fig. 8) showed a single possible path connecting *H. goderdziana* and *H. buchii.* Six out of the seven unique haplotypes inferred within *ECH* are geographically distinct. Two haplotypes of *H. goderdziana* are attributed to NE Turkey (Kovanlyk) and SW Georgia (Goderdzi), respectively; two haplotypes of *H. buchii* are attributed to the Central Greater Caucasus (Mokva, Khevsha) and to the Lesser and Eastern Greater Caucasus (Borjomi, Didgori, Dmanisi, Lagodekhi) respectively. Three remaining basal haplotypes of *H. buchii* mark individual locations in the Western Lesser Caucasus (Jamili, Bakhmaro). Only the latter location had two closely-related haplotypes, individuals from other studied ECH locations did not differ genetically. The hypothesis of a molecular clock was supported (LRT = 56.8, $P < 0.001$) for the sequenced fragment of COI, without considering the codon position.

Relationships between morphology, genetics, and geography

A Mantel test showed significant correlation between genetic and geographic distances for *ECH* samples ($r_{xy} = 0.41$, $P < 0.001$). The morphological distance (distance between the individuals based on the first two PCA axes for shell measurements) significantly correlates with genetic distance (COI sequence) between the corresponding individuals, if controlled for geographic distance ($r_{xy} = 0.22$, $P = 0.02$) between the locations but no correlation of morphological distance with geography was detected.

DISCUSSION

Systematics and Taxonomic inference

This study suggests that *Helix buchii* and *H. goderdziana* are two distinct, reciprocally-monophyletic evolutionary lineages. Morphological differences between these species are slight but obvious. Foot coloration, albeit variable in most

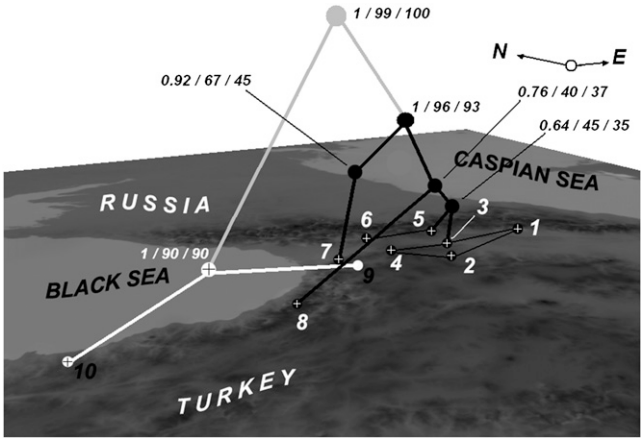

Figure 7. Phylogenetic relations between the *ECH* from different parts of the range; consensus tree based on the BA, NJ, and MP analyses. *Helix buchii*: black lines and circles; *H. goderdziana*: white lines and circles. The numbers attributed to individual nodes are Bayesian posterior probabilities / bootstrap supports for NJ tree nodes / bootstrap supports for MP tree nodes. Numbering of the sites (smaller crossed circles on the map) as in Fig. 2. The sites with identical haplotypes of *H. buchii* (1–4 and 5–6) are connected with narrower lines. Note that site 7 unites two haplotypes (see results).

land snails (Sysoev and Schileyko 2009), is the fully diagnostic character. In over 100 observed live individuals of *H. buchii*, the foot is dark, from grey to black, whereas over 20 adult and juvenile *H. goderdziana* found in both natural locations had light-colored yellowish foot, similar to that of the widespread *H. lucorum* (not all the observed specimens were used in the analysis, see Table 1). *Helix goderdziana* have in average larger shells with relatively broader spires than *H. buchii*, being more similar in shell shape to *H. lucorum* than to its sister species, if size and allometry factors are assumed. At last, *H. goderdziana* have lower flagellum/diverticulum ratio than *H. buchii*, and both *ECH* species have substantially lower diverticulum/flagellum ratio than *H. lucorum* or *H. pomatia.*

Long-running debates on the species criteria focus on some questions, on which an expert consensus perhaps never will be achieved (*e.g.*, Mayden 1977, Hey 2001, Avise 2004, de Queiroz 2007, Hausdorf 2010, Mallet 2010). Incipient species commonly exchange genes for millions of years, although this might not prevent progressive divergence (Mallet *et al.* 2007, Hausdorf 2010). We follow the suggestion of Mallet (2010) and refrain from the puritanical approach to species definition, deciding the nomenclatural questions dependent on the practical appropriateness. *Helix goderdziana* and *H. buchii* are morphologically, ecologically and geographically distinct and they are marked with reciprocally-monophyletic mitochondrial haplo-groups. These facts convince the authors that the differential species names are practically applicable to the studied taxa.

Evolutionary history of Endemic Caucasian Snails

If we consider morphological similarity, geographic closeness, and monophyly (based on COI sequence) of *ECH* relative to the analyzed widespread *Helix* species, *H. buchii* and *H. goderdziana* are likely to be sister taxa, although this assumption needs additional genetic data for more representatives of the genus.

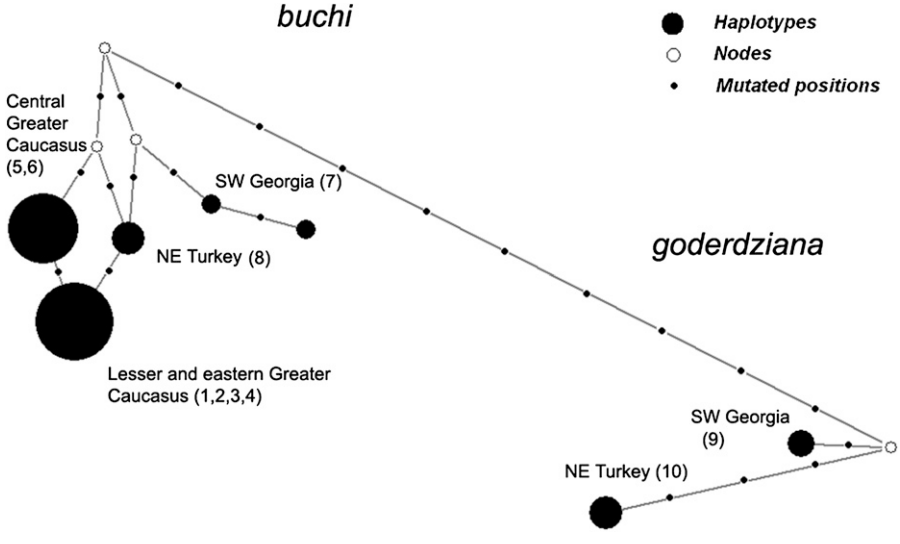

Figure 8. Median-joining network connecting inferred haplotypes of *Helix goderdziana* (Goderdzi and Kovanlyk) and *H. buchii* (all others). Numbers in parenthesis represent location numbers (see Fig. 2). Size of the circles marking haplotypes is proportional to the number of respective individuals.

Helix lucorum (and not the superficially more similar *H. pomatia*) is genetically closer to the *ECH* clade. This is supported by both phylogenetic inference based on the mitochondrial COI and structural identity of the sequenced fragment of nuclear 18S+ITS1. As opposed to the suggestion of Steinke *et al.* (2004), the fragment is less variable among the included outgroup of Helicidae than the sequenced fragment of COI: the mean proportion of pairwise differences among *H. buchii*, *H. lucorum*, and species of the outgoup reach 0.12 for the homologous 18S+ITS1 fragment but 0.23 for homologous fragment of mitochondrial COI.

The outcome of the partial Mantel test suggests that size and shape of shell correlates with genetic distance for *ECH* rather than by short-term/reversible

adaptations to local climates. The extant range of *Helix goderdziana* is restricted to the western Lesser Caucasus in SW Georgia and NE Turkey. Paleontological data suggests that this area supported a major forest refugium (MFR) during the last glacial maxima (Zeist and Bottema 1988, Van Andel and Tzedakis 1996). Molecular genetic study of the salamander *Mertensiella caucasica* (Waga, 1876) (Tarkhnishvili *et al.* 2000) revealed presence of two distinct evolutionary lineages of the salamanders isolated since pre-glacial time. The range of the western lineage coincides with the MFR and, hence, with the distribution range of *H. goderdziana*; the range of the eastern lineage is restricted to a small area in central Georgia. This finding supports the hypothesis of existence of multiple forested refugia east of MFR (Velichko and Kurenkova 1990, Tarkhnishvili *et al.* 2012). The geographic line separating MFR from the habitats supporting the eastern lineage of the salamander and the basal haplotype of *H. buchii* coincides with a belt of dry climate crossing the Meskheti Mountains in SW Georgia (Tarkhnishvili *et al.* 2008). The present geographic distribution of the climates was shaped ca. 6 MYBP (million years before present) (Fortelius *et al.* 2002, but see Micheels *et al.* 2009). Data on the rates of molecular evolution in COI in mollusks are controversial. Marko (2002) suggests 1.21% substitution rates per MY, but later studies of snail divergence in Europe (Gittenberger *et al.* 2004, Haase and Misof 2008) indicate that the molecular evolution can be much faster. If Marko's calibration is considered, the average split time between *H. buchii* and *H. goderdziana* may be 3.36 MYBP (95% confidence interval 1.7–4.5 MYBP). However, one cannot exclude that the lineages have been separated much later, in middle or even late Pleistocene. One can suppose that the "dry belt", limiting the eastern range of *H. goderdziana*, was an insuperable barrier for the spread of mesophylic species with limited dispersal ability during glacial maxima. This may have triggered the original split between the two snail lineages. The ancestral lineage of *H. buchii* survived in the refugia far from the Black Sea with a more continental climate, and the ancestors of *H. goderdziana* survived in MFR.

Habitat preferences and conservation

There are remarkable ecological differences between the two *ECH* species. *Helix buchii* is found in a wide habitat spectrum, mainly broadleaf forest litter away from the water sources but never in coniferous forest. This species is relatively common in primary forests of Caucasian mountain, whereas both known locations of *H. goderdziana* lay in exceedingly damp habitats along the brooks in mixed or broadleaf forest (*Alnus barbata* and *Picea orientalis*). The only known Georgian locality of *H. goderdziana* is currently under intensive anthropogenic pressure. In the last 5 years, the habitat was repeatedly littered and damaged (most of trees were cut down), and water in the brooks was polluted by sawdust and waste. We were unable to find *H. goderdziana* in 2010 and 2011 at the type locality. The disappearance of the species may be related either to the changing of microclimatic conditions at the brooks or the water pollution. The potential solution for the future is creation of a mini-reserve in the area, but this needs immediate attention from the relevant governmental bodies and international conservation community.

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REFERENCES

Armbruster, G. F. J., C. H. M. Van Moorsel, and E. Gittenberger. 2000. Conserved sequence patterns in non-coding ribosomal ITS1 of distantly related snail taxa. *Journal of Molecular Studies* **66**: 570–573.

Bandelt, H., P. Forster, and A. Rohl. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37–48

Bickford, D., D. J. Lohman, N. S. Sodhi, P. K. L. Ng, R. Meier, K. Winker, K. K. Ingram, and I. Das. 2006. Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution* **22**: 148–155.

Blackith R. and R. A. Reyment. 1971. *Multivariate Morphometrics*. Academic Press, New York.

Bohonak, A. J. 2002. IBD (Isolation By Distance): A program for analyses of isolation by distance. *Journal of Heredity* **93**: 153–154.

Conde-Padín, P., J. W. Grahame, and E. Rolán-Alvarez. 2007. Detecting shape differences in species of the *Littorina saxatilis* complex by morphometric analysis. *Journal of Molluscan Studies* **73**: 147–154.

Denk, T., N. Frotzler, and N. Davitashvili. 2001. Vegetational patterns and distribution of relict taxa in humid temperate forests and wetlands of Georgia (Transcaucasia). *Biological Journal of the Linnean Society* **72**: 287–332.

Donnelly, P. and S. Tavare. 1986. The age of alleles and a coalescent. *Advances in Applied Probability* **18**: 1–19.

De Queiroz, K. 2007. Species concepts and species delimitation. *Systematic Biology* **56**: 879–886.

Drummond, A. J. and A. Rambaut. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**: 214. doi:10.1186/1471-2148-7-214

Folmer, O., M. Black, W. Heah, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294–299.

Fortelius, M., J. Eronen, J. Jernvall, L. Liu, D. Pushkina, J. Rinne, A. Tesakov, I. Vislobokova, Z. H. Zhang, and L. Zhou. 2002. Fossil mammals resolve regional patterns of Eurasian climate change over 20 million years. *Evolutionary Ecology Research* **4**: 1005–1016.

Gittenberger, E., W. H. Piel, and D. S. J. Groenenberg. 2004. The Pleistocene glaciations and the evolutionary history of the polytypic snail species *Arianta arbustorum* (Gastropoda, Pulmonata, Helicidae). *Molecular Phylogenetics and Evolution* **30**: 64–73.

Haase, M. and B. Misof. 2009. Dynamic gastropods: Stable shell polymorphism despite gene flow in the land snail *Arianta arbustorum*. *Journal of Zoological Systematics and Evolutionary Research* **47**: 105–114.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**: 160–174.

Hausdorf, B. 2010. Progress toward a general species concept. *Evolution* **64**: 923–931.

Joliffe, I. T. and B. J. Morgan. 1992. Principal component analysis and exploratory factor analysis. *Statistical Methods in Medical Research* **1**: 69–95.

Kikvidze, Z. and M. Ohsawa. 1999. Adjara, East Mediterranean refuge of tertiary vegetation. *In*: M. Ohsawa, W. Wildpret, and M. D. Arco, eds., *Anaga Cloud Forest, a Comparative Study on Evergreen Broad-leaved Forests and Trees of the Canary Islands and Japan*. Chiba University Publications, Chiba, Japan.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111–120.

MacCallum, C. R., K. F. Widaman, S. Zang, and S. Hong. 1999. Sample size in factor analysis. *Psychological Methods* **4**: 84–99.

Mai, D. H. 1995. *Tertiäre Vegetationsgeschichte Europas*. Gustav Fischer Verlag, Jena, Germany. [In German]

Mallet, J. 2010. Why was Darwin's view of species rejected by twentieth century biologists? *Biology and Philosophy* **25**: 497–527.

Mallet, J., M. Beltrán, W. Neukirchen, and M. Linares. 2007. Natural hybridization in heliconiine butterflies, the species boundary as a continuum. *BMC Evolutionary Biology* **7**: 28. doi:10.1186/1471-2148-7-28

Manel, S., M. K. Schwartz, G. Luikart, and P. Taberlet. 2003. Landscape genetics: Combining landscape ecology and population genetics. *Trends in Ecology and Evolution* **18**: 189–197.

Marko, P. 2002. Fossil calibration of molecular clocks and divergence times of geminate species pairs separated by the Isthmus of Panama. *Molecular Biology and Evolution* **19**: 2005–2021.

Micheels, A., A. Bruch, and V. Mosbrugger. 2009. Miocene climate modeling sensitivity experiments for different CO_2 concentrations. *Palaeontologia Electronica* **12**: 1–20.

Milne, R. I. and R. J. Abbott. 2002. The origin and evolution of Tertiary relict floras. *Advances in Botanical Research* **38**: 281–314.

Milne, R. I. 2004. Phylogeny and biogeography of *Rhododendron* subsection *Pontica*, a group with a Tertiary relict distribution. *Molecular Phylogenetics and Evolution* **33**: 389–401.

Milne, R. I. 2006. Northern hemisphere plant disjunctions: A window on Tertiary land bridges and climate change? *Annals of Botany* **98**: 465–472.

Mumladze, L., D. Tarkhnishvili, and B. M. Pokryszko. 2008. A new species of the genus *Helix* from the Lesser Caucasus (SW Georgia). *Journal of Conchology* **39**: 483–485.

Neubert, E. and A. R. Bank. 2006. Notes on the species of *Caucasotachea* C. Boettger 1909 and *Lindholmia* P. Hesse 1919, with annotations to the Helicidae. *Archiv für Molluskenkunde* **135**: 101–132.

Parks, D. H., M. Porter, S. Churcher, S. Wang, C. Blouin, J. Whalley, S. Brooks, and R. G. Beiko. 2009. GenGIS: A geospatial information system for genomic data. *Genome Research* **19**: 1896–1904.

Richtsmeier, J. T., V. B. Deleon, and S. R. Lele. 2002. The promise of geometric morphometrics. *Yearbook of Physical Anthropology* **45**: 63–91.

Röhrig, E. 1991. Deciduous forests of the Near East. *In*: E. Röhrig and B. Ulrich, eds., *Ecosystems of the World.* Elsevier, Amsterdam, Netherlands. Pp. 165–174.

Schütt, H. 2005. *Turkish Land Snails*. Verlag Natur and Wissenschaft, Solingen, Germany.

Suchard, M. A., E. W. Robert, and J. S. Sinsheimer. 2001. Bayesian selection of continuous-time Markov chain evolutionary models. *Molecular biology and Evolution* **18**: 1001–1013.

Sysoev, A. and A. Schileyko. 2009. *Land Snails and Slugs of Russia and Adjacent Countries.* Pensoft, Sofia-Moscow, Russia.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA v5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution.* **28**: 2731–2739.

Tarkhnishvili, D., A. Gavashelishvili, and L. Mumladze. 2012. Palaeoclimatic models help to understand current distribution of Caucasian forest species. *Biological Journal of the Linnean Society* **105**: 231–248.

Tarkhnishvili, D., U. Kaya, A. Gavashelishvili, and I. Serbinova. 2008. Ecological Divergence between two Evolutionary Lineages of the Caucasian Salamander: Evidence from the GIS analysis. *Herpetological Journal* **18**: 155–163.

Tarkhnishvili, D. N., R. S. Thorpe, and J. W. Artnzen. 2000. Pre-Pleistocene refugia and differentiation between populations of the Caucasian salamander (*Mertensiella caucasica*). *Molecular Phylogenetics and Evolution* **14**: 414–422.

Thorpe, R. S. and L. Leamy. 1983. Morphometric studies in inbred and hybrid house mice (*Mus* sp.): Multivariate analysis of size and shape. *Journal of Zoology* **199**: 421–432.

Tuniyev, B. S. 1990. On the independence of the Colchic center of amphibian and reptile speciation. *Asiatic Herpetological Research* **3**: 67–84.

Van Andel, T. H. and P. C. Tzedakis. 1996. Palaeolithic landscapes of Europe and environs, 150,000–25,000 years ago: An overview. *Quaternary Science Reviews* **15**: 481–500.

Veith, M., S. Steinfartz, R. Zardoya, A. Seitz, and A. Meyer. 1998. A molecular phylogeny of 'true' salamanders (family Salamandridae) and the evolution of terrestriality of reproductive modes. *Journal of Zoological Systematic and Evolutionary Research* **36**: 7–16.

Velichko, A. A. and A. A. Kurenkova. 1990. Landscapes of the Northern Hemisphere during the Late Glacial Maximum. *In*: O. Soffer and G. Gamble, eds., *The World at 18,000 BP*. Unwin Hyman, London, United Kingdom. Pp. 255–265.

Welter-Schultes, F. 2009. Species in genus *Helix*. Available from www.animalbase.uni-goettingen.de (version 21-01-2009), accessed 2 June 2012.

Wiley, D. F., N. Amenta, D. A. Alcantara, D. Ghosh, Y. J. Kil, E. Delson, W. Harcourt-Smith, F. J. Rohlf, K. St. John, and B. Hamann. 2005. Evolutionary morphing. *Proceedings of the IEEE Visualization* (VIS'05). 431–438.

Zazanashvili, N., G. Sanadiradze, A. Bukhnikashvili, A. Kandaurov, and D. Tarkhnishvili. 2004. Caucasus. *In*: R. A. Mittermaier, P. G. Gil, M. Hoffmann, J. Pilgrim, T. Brooks, C. G. Mittermaier, J. Lamoreux, and G. A. B. Da Fonseca, eds., *Hotspots Revisited, Earth's Biologically Richest and Most Endangered Terrestrial Ecoregions*. CEMEX/Agrupación Sierra Madre, Mexico.

Zeist, W. V. and S. Bottema. 1988. Late Quaternary vegetational and climatic history of southwest Asia. *Proceedings of the Indian National Science Academy* **54**: 461–480.

Zelditch, M. L., D. L. Swiderski, H. D. Sheets, and W. L. Fink. 2004. *Geometric Morphometrics for Biologists: A Primer*. Elsevier Academic Press, San Diego, California.

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Appendix 1.

Source	Primer sequence	Amplification conditions	Temperature profile
COI universal (Folmer *et al.* 1994)	5′-GGTCAACAATCATAAAGATATTGG-3′ 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	20µl total volume, with: 2 µl template DNA 1.5U of Taq polymerase (Promega) 1x Promega buffer 1.5 µm of $MgCl_2$ 0.1 µm of each dNTP, 0.1 µm primer concentrations	1 cycle of 3 min @ 95 °C 25 cycles: 40s @ 94 °C 40s @ 50 °C 1min @ 72 °C 1 cycle of 10 min @ 72 °C
18S+ITS1 mollusc-specific (Armbruster *et al.* 2000; van Moorsel *et al.* 2000)	5′-TAACAAGGTTTCCGTAGGTGAA-3′ 5′-GCTGCGTTCTTCATCGATGC-3′	20 µl total volume, with: 3 µl template DNA 1U of Taq polymerase(Promega) 1x romegabuffer 1.5 µm of $MgCl_2$ 0.1 µm of each dNTP, 0.1 µm primer concentrations	1 cycle of 3 min @ 95 °C 25 cycles: 40s @ 94 °C 30s @ 56 °C 0.3 °C each cycle) 1min @ 72 °C 1 cycle of 10 min @ 72 °C