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Higher than anticipated diversity within an *Albinaria s*pecies (Gastropoda, Pulmonata, Clausiliidae) in southern Turkey

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Disentangling cryptic diversity is important for evolutionary and biogeographical research and conservation planning. In some cases, close examination of the morphology of taxa enables their accurate distinction, but often this is only possible by genetic analysis. In the present study we used geometric morphometrics and genetic analysis of two mitochondrial markers (CO1 and 12S) to explore cryptic diversity within the land snail species *Albinaria lycica* distributed in a limited area in SW Turkey. Our analyses revealed large and higher than anticipated genetic and morphometric differences between and within the two *A. lycica* subspecies, which supports our hypothesis that cryptic diversity in such a small geographical scale can occur. Moreover, the correlation of geographic distances with morphometric and genetic ones suggests an isolation-by-distance pattern of population structure. These results confirm earlier studies showing the power of multidisciplinary approaches to characterizing the enormous, and often cryptic, diversity of clausiliid land snails.

Key words: cryptic diversity, genetics, geometric morphometrics, land snails.

INTRODUCTION

Albinaria Vest 1867 is probably one of the largest land snail genera in terms of species richness, since more than 120 *Albinaria* species have been described so far (Nordsieck, 2007). Moreover, *Albinaria* is considered as an ideal taxon for examining evolutionary hypotheses related with trait divergence, speciation and cryptic diversity (Schilthuizen, 2003). The genus is distributed around the north-eastern coasts of the Mediterranean Sea. The majority of *Albinaria* species have allopatric and often very limited distributions and most of them inhabit southern Greece and the Aegean archipelago. A large number of publications (see Giokas *et al.*, 2006 and references therein) deal with Greek *Albinaria* taxa (taxonomy, phylogeny, evolution, biogeography), but only a few papers focus on the *Albinaria* species distributed exclusively in Turkey (e.g. Neubert *et al.*, 2000; Örstan & Welter-Schultes, 2007), even though there are about 40 *Albinaria* taxa (Schütt, 2010). Several of the aforementioned studies, based primarily on molecular phylogenetic analyses, have changed the taxonomic status of several *Albinaria* taxa either by splitting or by lumping species and subspecies (e.g. Uit de Weerd *et al.*, 2004; Douris *et al.*, 2007), and have disputed the utility of traditional systematics for the delineation of taxa within this genus.

Of special interest is the case of *Albinaria lycica* Nordsieck 1993, a species with a very limited distribution in SW Turkey. Nordsieck (1993) described two forms of *A. lycica* from Southern Turkey, at the western part of Antalya Province: *Albinaria lycica lycica* and *A. lycica phaselis*. The nominotypical subspecies is known from west of Kumluca, in the Demre, Finike, Yanliz Region, whereas *A. lycica phaselis* lives east of Kumluca along the sea coast as far as Göynük (Fig. 1). According to the original description, "subspecies" *phaselis* differs in the relationship of the spiral and the superior (parietalis) lamellae and in the structure of the columellaris (lamella inferior) (Fig. 2). Differences in shell shape and size were not mentioned and some populations belonging to that subspecies have more or

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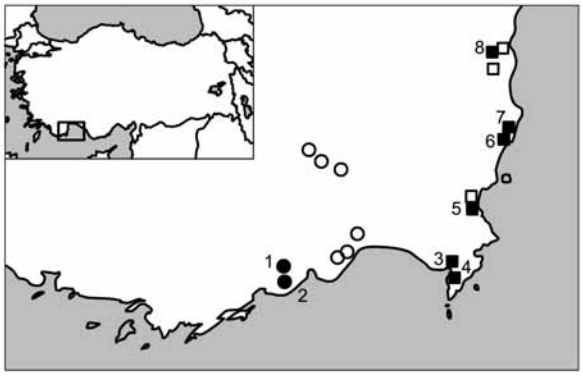

FIG. 1. Geographic distribution of *Albinaria lycica lycica* (circles) and *A. lycica phaselis* (squares). The placemarks signed with numbers correspond to the locality data (for abbreviations see Table 1). Filled object indicate the investigated locations in the present study, whereas the empty objects are literature distributional references.

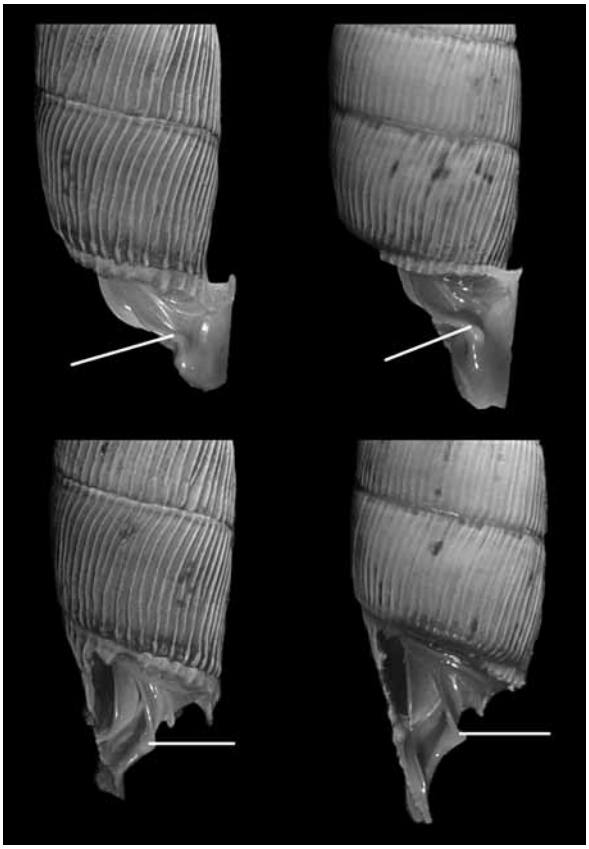

FIG. 2. Differences in the shape of inferior lamella (columellaris). Left: *Albinaria lycica phaselis*, right: *A. lycica lycica*. Upper two pictures: left ventral view, lower two pictures: dorsal view. Body whorl was opened in order to make inner lamellae visible.

less smooth shell surface. Nordsieck (1993) suggested that the two forms belong to one species, although intermediate forms that would support a subspecies classification have not been found.

The importance of exploring cryptic diversity at any level lies in its implications for evolutionary theory, biogeography and conservation planning. Therefore, the main goal of our study is to explore molecular, morphological and geographical variation of the *A. lycica* forms in order to examine (1) if the hypothesis of cryptic intra-subspecific diversity in such a small geographical scale is possible and valid, (2) how this diversity is expressed at the morphological and molecular levels, and (3) if there is a spatial structure of morphological and genetic diversity.

MATERIALS AND METHODS

Sample collection

Albinaria specimens were collected from eight populations (two belonging to *A. lycica lycica* and six to *A. lycica phaselis*) (Table 1, Fig. 1). The specimens were identified using comparative material deposited by Nordsieck in the SMF (Senckenberg Forschungsinstitut und Naturmuseum, Frankfurt am Main, Germany) and following the original descriptions in Nordsieck (1993).

Geometric morphometrics

The studied populations were tested for morphological diversification using a landmark-based method of acquiring geometrical data of shape and size. Shell variation has been traditionally quantified through straight-line measurements and ratios, to distinguish between individuals and populations, among and within snail species. Recently, geometric morphometrics (GM) has been employed for examining shells, both to provide direct size-free analyses of shell shape (Conde-Padin *et al.*, 2007a; Hayes *et al.*, 2007) and to answer broader evolutionary questions (Pfenninger & Magnin, 2001; Conde-Padin *et al.*, 2007b; Haase & Misof, 2009). GM has the advantage over traditional morphometrics in being, theoretically, free of effects due to size, position, rotation, and scale (Rohlf & Marcus, 1993).

All undamaged shells of adult specimens were used. The specimens were set down on a graph-paper all in the same plane to avoid distortion (optical distortions were controlled for with the graph paper), with the aperture facing up and being in the starting

TABLE 1. *Albinaria lycica* populations included in the study, geographic data of the sampling localities, sample sizes of the populations used in geometric morphometrics (N1) and molecular analysis (N2), and GenBank accession numbers of sequences. Locality codes refer to Fig. 1

Locality code	Taxon name	Locality	Coordinates	N1	N2	Accession numbers (12S/CO1)
1	*A. lycica lycica*	Lycia, Basilica in Demre, sea level	36˚14'40" N, 29˚59'06" E	9	3	1.1 JQ797617/JQ797626 1.2 JQ797618/JQ797627 1.3 JQ797619/JQ797628
2	*A. lycica lycica*	Lycia, Eastern necropolis of Myra, near Demre, 30 m a.s.l.	36˚15'45" N, 29˚59'13" E	2		
3	*A. lycica phaselis*	Lycia, Korsanköy, S of Karaöz, ESE of Kumluca, sea level	36˚15'18" N, 30˚24'25" E	8		
4	*A. lycica phaselis*	Road between Karaöz and Melanippe, 50 m a.s.l., from bridge supporting road	36˚15'30" N, 30˚24'41" E	2	1	4.1 JQ797620/JQ797629
5	*A. lycica phaselis*	Lycia, Olympus, sea level, cliffs just behind beach	36˚23'49" N, 30˚28'32" E	6	3	5.1 JQ797614/JQ797623 5.2 JQ797615/JQ797624 5.3 JQ797613/JQ797622
6	*A. lycica phaselis*	Lycia, N end of Phaselis, 40 m a.s.l., by ruins	36˚31'40" N, 30˚33'02" E	3	1	6.1 JQ797616/JQ797625
7	*A. lycica phaselis*	Lycia, Peninsula N of Phaselis, 20 m a.s.l.	36˚31'57" N, 30˚33'42" E	10		
8	*A. lycica phaselis*	Lycia, Sarıpınar Hayı, W. of Güynük, 375 m a.s.l., mostly on cliffs in steep sided valley	36˚42'09" N, 30˚31'10" E	3	1	8.1 JQ797612/JQ797621

point of a y-axis set as the shell axis. Geometric morphometric variables of the shells were obtained with 16 landmarks (LM) representing the outline of the shell and of the aperture as shown in Figure 3. These 16 landmarks were digitized using tpsDig (Rohlf, 2005). LM1 represents the apex and LM2, LM4, LM6 and LM3, LM5, LM7 the left and right lower sutures of the second, third and forth whorl, respectively. LM8, LM10 and LM9, LM11 represent the left and right upper and lower sutures of the penultimate whorl, LM12, LM13, LM14 and LM15 cover the height and width of the aperture, while the last landmark (LM16) marked the end of the lamella superior. The selected landmarks occupy, as accurately as possible, repeatedly the same positions over the sum of the specimens. Not all landmarks are homologous from a developmental aspect, but the necessity for coverage of the whole shape and keeping as much information for analysis as possible, prevailed. Two landmarks (LM1 and LM16) were of type I, ten landmarks (LM2-LM11) represented suture lines and were considered type II (geometrically homologous). The remaining landmarks were either extreme points on surfaces or were placed halfway between other landmarks, making them type III (Bookstein, 1991; Zelditch *et al.*, 2004).

All geometric morphometric analyses were performed with MorphoJ (Klingenberg, 2011). The coordinates of shape to be used for further statistical

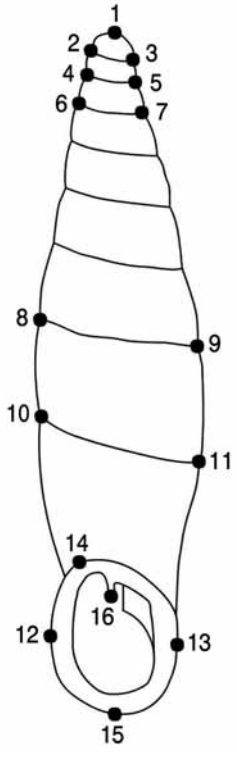

FIG. 3. An *Albinaria* specimen positioned with the axis of the shell on the y-axis and the aperture in the same plane as the objective, showing the location of the 16 landmarks used in the study.

analysis where obtained with Procrustes generalized least squares superimposition. This method excludes the impact of size on the shape of the shell and, theoretically, variations independent of shape are removed using this analysis (Zelditch *et al.*, 2004). Size variables (centroid size) for each specimen were also generated. We examined, using pooled within-group regression analysis, the effect of centroid size on shape, regressing the resulting Procrustes coordinates on centroid size. That regression score was significant (Permutation test, 10000 rounds against the null hypothesis of independence, % predicted = 39.608%, $p < 0.0001$). The residuals from this procedure were used to standardize each group to mean centroid size, and thus to remove allometric shape variation related to size. Shape data corrected for size (regression residuals) were analyzed with Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA) in order to explore morphological variation among the populations of the two *A. lycica* subspecies. The reliability of the discrimination was assessed by leave-one-out cross-validation.

From the CVA, pairwise Mahalanobis distances among the studied populations were extracted. These distances were compared with their corresponding genetic and geographical distances (minimum geographic distances) using the Mantel test with 5000 permutations as implemented in PAST (Hammer *et al.*, 2001).

DNA extraction, amplification and sequencing

A total of nine *A. lycica* specimens belonging to populations 1 (three specimens), 4 (one specimen), 5 (three specimens), 6 (one specimen), and 8 (one specimen) were analyzed and included in the molecular marker analyses, along with additional sequences of *Sericata sericata sericata* (Leach 1819) retrieved from GenBank (AY382080 – 12S, Uit de Weerd & Gittenberger, 2005, and AY425590 – CO1, Uit de Weerd *et al.*, 2004). *Sericata sericata* was employed as outgroup. Specimen data and GenBank accession numbers are given in Table 1.

Total genomic DNA was extracted using the NucleoSpin Tissue DNA extraction kit (MACHEREY-NAGEL). Partial sequences of the 12S rRNA (12S) and the cytochrome c oxidase subunit I (COI) were PCR-amplified using primers $tRNA^{met}$ and 12S (Uit de Weerd & Gittenberger, 2005), and LCO1490 and HCO2198 (Folmer *et al.*, 1994), respectively. Amplification of the target sequences involved an initial cycle of denaturation at 94°C for 5 min, and 35-40 subsequent cycles of 94°C for 1 min, 63°C for 1 min (12S) or 48°C for 1min (CO1) and 72°C for 1 min, in the presence of 3.0 mM $MgCl_2$.

PCR products were purified with the NucleoSpin Extract II DNA purification kit (MACHEREY-NAGEL). Single-stranded sequencing was conducted on an ABI PRISM 3100 capillary sequencer (Applied Biosystems, VBC Biotech, Austria) using the primers of the amplification procedure as sequencing primers.

Alignment and genetic divergence

DNA sequences were aligned using ClustalX v.2.0.12 (Larkin *et al.*, 2007) with default parameters. The genetic divergences, as percentage of genetic distance values, under the Kimura 2-parameter model of evolution (Kimura, 1980), between the clades of our phylogeny were estimated in MEGA v 4.0 (Tamura *et al.*, 2007).

Phylogenetic analyses

The selection of the most suitable model of DNA substitution was done using Modeltest 3.7 (Posada & Crandall, 1998), under the Bayesian Information Criterion. The most suitable models for 12S and COI, were K81uf+G and HKY+I+G (Pinvar=0.63), respectively.

Bayesian analysis of each of the markers and the combined dataset was performed in MrBayes v3.1 (Ronquist & Huelsenbeck, 2003) with the model parameters of each gene partition. Four incrementally heated Markov chains, with the default heating values, were used for 2×10^6 and 3×10^6 generations for each marker and the combined dataset, respectively. The current tree was saved to file every 100 generations. After verifying that stationarity had been reached, both in terms of likelihood scores and parameter estimation, and using TRACER v1.5.0 (http://tree.bio.ed.ac.uk/software/tracer/) (Rambaut & Drummond, 2004), the first 2×10^3 and 3×10^3 trees (10% "burnin" in Bayesian terms) were discarded, and a majority-rule consensus tree was generated from the remaining trees. During tree search, full parameter estimation was performed, and the posterior probabilities (pp) were calculated as the percentage of samples recovering any particular clade (Huelsenbeck & Ronquist, 2001), where pp ≥ 0.95 indicate significant support (Wilcox *et al.*, 2002). Four independent Bayesian analyses were run so that global likelihood scores, individual parameter values, topol-

ogy and nodal support could be compared to check for local optima.

RESULTS

Geometric morphometrics

Geometric morphometrics analysis revealed a clear size and shape distinction between the two *A. lycica* subspecies. *Albinaria lycica phaselis* was significantly larger than *A. lycica lycica* ($F = 17648$, $p < 0.0001$, Fig. 4A). The landmark-based analyses of individual shells confirmed the shape distinction of the two subspecies. In PCA, which aimed at finding linear combinations maximizing the total variance, the first three principal components (PC1-PC3) explained 71% of the total variance. In the CVA, which maximized differentiation among populations (or predefined groups), the first three canonical variates (CV1-CV3) explained 88% of total variation, and showed a much clearer separation between the two morphotypes along the first CV axis (Fig. 4B). Clear shape discrimination was also found between *A. lycica lycica* and *A. lycica phaselis* shells after a Discriminant Function Analysis ($t^2 = 978.8529$, $p < 0.0001$). After a permutation test with 1000 replicates, 90.7% of "unknown" individuals (leave-one-out cross-validation) were correctly classified. Shape changes along the CV1 are shown in Figure 4C and portray the tendency of *A. lycica phaselis* to be slimmer compared to *A. lycica lycica*.

Genetic analyses

Sequence divergence between the *A. lycica lycica* and *A. lycica phaselis* clades, estimated as genetic distance values, ranged from 14.6% to 15.7% for COI and from 18.1% to 20.5% for 12S. Within *A. lycica phaselis*, COI genetic distances were higher (6.7%) than 12S dis-

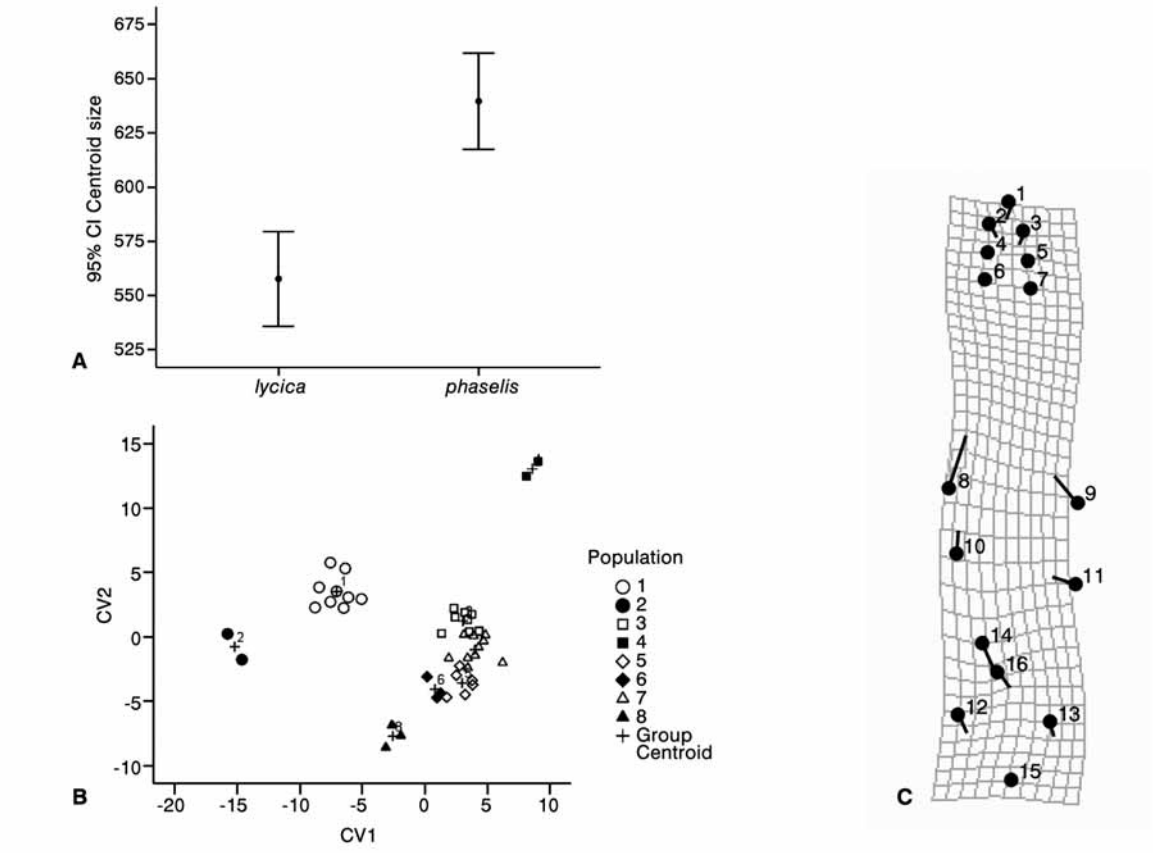

FIG. 4. (A) Means ± 95% Confidence Intervals of centroid size for the two *Albinaria lycica* morphotypes, and their ordinations based on landmark data. (B) Canonical Variates Analysis, and (C) shape changes along the CV1. In C the shifts of landmark positions are indicated with straight lines. Each line starts with a dot at the location of the landmark in the starting shape (i.e. *lycica*). The length and direction of the line indicates the movement of the respective landmark towards the final shape (i.e. *phaselis*).

tances (3.9%). The values of genetic divergence, within and between defined units of *A. lycica*, for the mitochondrial markers separately, are shown in Table 2.

Phylogenetic analyses for both markers gave very similar results, distinguishing the same monophyletic groups, and showed only minor differences, concerning their respective statistical support values. The phylogenetic tree of the BI analysis of the combined mtDNA dataset is presented in Figure 5.

TABLE 2. Genetic distances (%) among mtDNA clades for COI (above diagonal) and 12S (below diagonal). Values in diagonal are genetic divergences within each clade (12S/COI).

	Sericata	*lycica*	*phaselis*
Sericata	-	17.5	18.0
lycica	32.1	0.2/0.0	12.0
phaselis	26.5	17.2	3.9/6.7

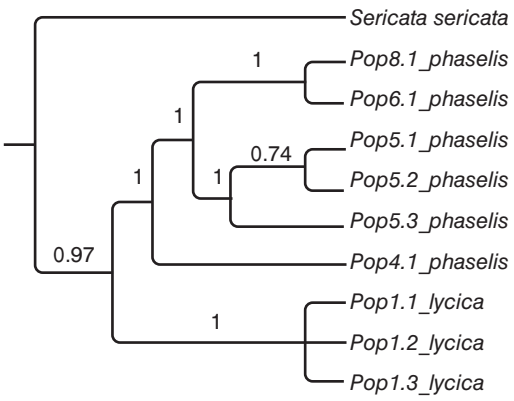

FIG. 5. Bayesian Inference (BI) tree produced by the combined mtDNA dataset showing the phylogenetic relationships among the *Albinaria lycica* specimens of the present study. Numbers above branches are BI posterior probabilities.

Correlations between morphometric, genetic and geographical distances

The Mantel test detected a moderate, yet significant, correlation between the matrices of Mahalanobis morphometric distances and the geographic distances for the eight studied populations ($r = 0.36, p = 0.026$). Similarly, there was significant correlation between the COI and 12S genetic distances ($r = 0.844, p = 0.03$) for the five populations (1, 4, 5, 6 and 8), from which we had available genetic data.

However, we did not find significant correlations between Mahalanobis distances and genetic distances ($r = 0.143, p = 0.366$ for COI, and $r = 0.001, p = 0.569$ for 12S). Geographical distances were correlated with the 12S genetic distances ($r = 0.928, p = 0.025$), but not with the COI genetic distances ($r = 0.741, p = 0.088$). Finally, when we considered only the five populations, the Mahalanobis morphometric distances and the geographic distances were found to be uncorrelated ($r = 0.093, p = 0.505$).

DISCUSSION

According to the molecular analyses, the two *A. lycica* forms constitute discrete and highly supported entities. Moreover, the level of mtDNA divergence for both markers, recorded between the two *A. lycica* forms (Table 2) is extremely high and genetic differentiation turns out to be greater than anticipated, falling within the range reported for interspecific genetic differentiation in other land-snail genera (Thomaz *et al.*, 1996; Chiba, 1999; Goodacre & Wade, 2001; Watanabe & Chiba, 2001; Parmakelis *et al.*, 2003; Parmakelis & Mylonas, 2004; Johnson *et al.*, 2010). Compared to the divergence levels of other clausiliid rock-dwelling genera distributed in the Mediterranean Basin, the levels reported in the present study are well above or very similar to those estimated for *Albinaria*, *Sericata*, *Isabellaria* and *Cristataria* species distributed in Greece (Douris *et al.*, 1998; Uit de Weerd *et al.*, 2004; Giokas *et al.*, 2006) or in Turkey (Uit de Weerd & Gittenberger, 2005). An alternative cause of such divergence between the two *A. lycica* subspecies could be the introgression of mt DNA from another *Albinaria* species. Nordsieck (1993) reports that *A. lycica lycica* is found sympatrically with *A. anatolica* in several localities. However, this explanation is less probable since in the localities of our sampling, no specimens of another *Albinaria* species were found. Furthermore, genetic distances within *A. lycica phaselis* are also quite high (Table 2) and support the hypothesis that cryptic intra-subspecific diversity in such a small geographical scale is possible.

Moreover, geometric morphometric analysis supports the view that the two *A. lycica* forms are quite distinguishable in terms of shell shape and size. We must note here that in the original description of the two taxa (Nordsieck, 1993) shell size and shape were not reported as significantly different. The intra-subspecific morphometric variation is also high and the populations of both subspecies are quite distinct in

shape (Fig 4B). More interestingly, shape variation is well correlated with geographic distance between populations when all population samples were considered. This probably means that in the case of *A. lycica* morphology keeps a good track of evolutionary processes driven by selection. However, this hypothesis is still unconfirmed because we have not yet enough data to support that this morphological variation is along an obvious environmental gradient. Similarly, at least for 12S, genetic distances were found correlated with geographical ones suggesting an isolation-by-distance (IBD) pattern at this spatial scale. We must mention the absence of any evident geomorphological borders separating the two subspecies or their populations. Another result worthnoting is the small geographical scale of that phenomenon. It seems that the two *A. lycica* forms were capable to diverge rapidly and in small spatial scale, even though we do not have sufficient data to support that this eventually resulted in speciation.

In conclusion, both outcomes revealed higher than anticipated inter and intra-subspecific variation at the molecular and morphological level, even though we did not find a significant association between morphometric and genetic distances. Perhaps the two subspecies constitute cryptic species, i.e. two or more distinct species that are erroneously classified (and hidden) under one species name. Of course, a more detailed analysis that would include additional samples will further clarify that hypothesis.

Most remarkable was the fact that both morphological and molecular approaches arrived to a similar conclusion in our case, even though using phenotypes to explore and describe biological diversity has become less popular than using genetics to do so. Results from the two approaches often conflict at the species level and below, since phenotypic divergence is probably driven mostly by selection and genetic divergence by stochastic processes. However, in the "*lycica*" case morphometrics has proved a useful tool for exploring patterns of diversity. Our findings remind of the original questions regarding the debate between molecular and traditional taxonomy, and call for synergies between these approaches.

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