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Widespread polyphyly among Alopiinae snail genera: when phylogeny mirrors biogeography more closely than morphology

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Abstract

Consider a group of species that is evenly divided by an easily identifiable complex morphological character. Most biologists would assume that this character should provide better phylogenetic information than, say, the spatial distribution of these species over a fairly continuous 500-km radius area. Paradoxically, this is not the case among terrestrial snail genera in the clausiliid subfamily Alopiinae. Phylogenetic analysis using the nuclear markers ITS1/ITS2 and mitochondrial markers COI/12S reveals widespread homoplasy in the clausilial apparatus (a complex aperture-closing mechanism), and concomitant extensive polyphyly among *Carinigera*, *Isabellaria*, and *Sericata*. In contrast, phylogenetic relationships as revealed by molecular data are closely congruent with biogeography at a relatively small scale. A combination of extremely low vagility and extremely high morphological convergence has conspired to produce this unexpected result. Implications as to the function of the clausilial apparatus are discussed.

Keywords: Clausilial apparatus; Molecular phylogeny; COI; ITS; 12S; Parallel evolution; Biogeography; Clausiliidae

1. Introduction

A unique apomorphic closing mechanism, the clausilial apparatus (CA), characterizes the shells of pulmonate snails in the family Clausiliidae, and modifications of it are used in intra-family classification. The CA is a door-like structure, consisting of a moveable plate and associated lamellae, located in the ultimate whorl of the shell (Fig. 1A). Two types of CA are recognized: (1) the N-type (Fig. 1B) and (2) the G-type (Fig. 1C). In the N-type, the plate cannot close off the aperture entirely, and always leaves open a bypass canal, supported by two parallel lamellae. In the G-type these lamellae are absent and the moveable plate can seal off the aperture completely. The N-type is considered the plesiomorphic condition, from which the G-type is thought to have evolved several times in parallel (Nordsieck, 1963, 1982). Despite the homoplasious nature of this character, the G-type is used as a diagnostic character at the genus level.

Both types of CA are found in a monophyletic (Uit de Weerd and Gittenberger, in press) group of limestone-dwelling genera: *Isabellaria* von Vest, 1867, *Carinigera* Moellendorf, 1873, *Sericata* O. Boettger, 1878, *Albinaria* von Vest, 1867, and *Cristataria* von Vest, 1867. The genus *Isabellaria* (sensu Gittenberger, 1998a) is among others defined as having a G-type CA, in *Albinaria* (sensu Uit de Weerd and Gittenberger, submitted) both types are encountered, whereas all other genera by definition possess an N-type CA. Of the two

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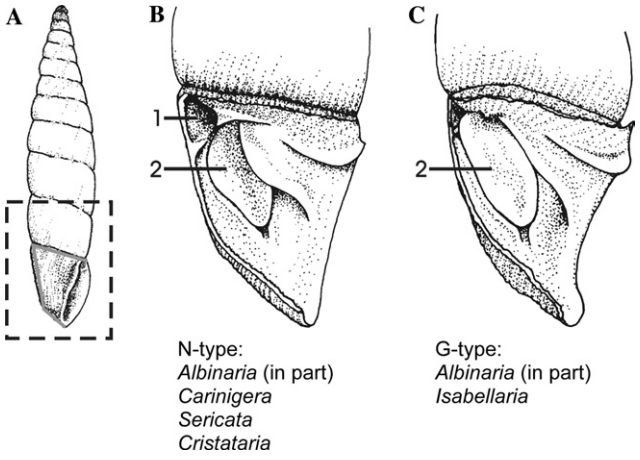

Fig. 1. The two types of CA, and their position in the ultimate whorl of the shell. (A) Side-view of the shell. The palatal shell-wall located within the gray lines was removed to reveal internal shell structures. (B) N-type CA with by-pass canal (1) next to the clausilial plate (2). (C) G-type CA, with complete sealing of the aperture by the clausilial plate (2). Modified after van Moorsel et al. (2000).

genera containing G-type species, *Albinaria* is best studied. Despite its polymorphic CA, *Albinaria* is clearly monophyletic, as demonstrated by two molecular studies (Douris et al., 1998b; van Moorsel et al., 2000).

The monophyly of *Isabellaria*, on the other hand, is controversial. In its present definition, *Isabellaria* can be roughly recognized by its putatively apomorphic G-type CA in combination with a mostly grayish-brown shell surface (Gittenberger, 1998a). Both molecular and morphological studies, however, indicate close affinities between some *Isabellaria* and *Sericata* species (Douris et al., 1998b; van Moorsel et al., 2000). Furthermore, recent molecular studies suggest that *Carinigera* species are closely related to *Isabellaria* species (Uit de Weerd and Gittenberger, in press). Apart from their presumably plesiomorphic N-type CA, the genera *Sericata* and *Carinigera* cannot be distinguished from *Isabellaria* conchologically. *Carinigera* has been set apart from *Isabellaria* and *Sericata* on the basis of some putatively apomorphic genital-anatomical characters, which previous molecular studies, however, revealed as homoplastic (Uit de Weerd and Gittenberger, in press).

Using the present, largely CA-based, criterion for their classification, the so-called *Isabellaria* species show a somewhat disjunct distribution (Fig. 2A). Their combined range extends from the north-eastern Peloponnese in Greece up northwards into the country of Macedonia. In almost this entire area, the ranges of the individual *Isabellaria* species are interspersed with those of so-called *Sericata* and *Carinigera* species (Fig. 2B). *Sericata*, as a whole, has a range roughly coincident with that of *Isabellaria*, whereas *Carinigera* has a more north-eastern, albeit overlapping distribution, occurring in the region from central Greece into Thracia and even into Serbia.

Neighboring *Sericata* and *Isabellaria* species are often highly similar in shell morphology, yet are placed in different genera because of differences in the clausilial apparatus. Four such so-called species pairs, consisting of *Sericata* and *Isabellaria* species, are referred to by Nordsieck (1974, 1977, 1984): *S. stussineri* and *I. lophauchena*; *S. bathyclista* and *I. riedeli*; *S. parnassia* and *I. thermopylarum*; and *S. lutracana* and *I. isabellina*. Two hypotheses have been proposed to explain these occurrences. The species within a species pair are supposed not to be closely related and similarities in external shell morphology exist because of either (a) introgression of these features through hybridization (Nordsieck, 1984, 1997) or (b) convergent evolution (Nordsieck, 1974, p. 132). As an alternative explanation, it was hypothesized that the species in a species pair are, in fact, sister species, requiring either (a) recurrent independent origin of a G- from an N-type CA (van Moorsel et al., 2000) or (b) xenologous transfer of the genetic basis for a G-type CA (Gittenberger, 1998a,b, 2000).

Molecular analyses indicate that the species in comparable N/G-type species pairs within *Albinaria* are closely related, thereby demonstrating that recurrent transformations in CA-type are possible (van Moorsel et al., 2000). The evolution of the CA in *Albinaria* could not be reconstructed unequivocally (see van Moorsel et al., 2000), however. Neither can the possibility of transspecific introgression of the G-type CA (Gittenberger, 1998a,b, 2000) be excluded, since the G-type *Albinaria* species, placed in different clades together with N-type ones, inhabit a more or less continuous combined range in the eastern Peloponnese. In this respect, these G-type *Albinaria* species differ from the G-type *Isabellaria* species, which have a much more disjunct distribution (see Fig. 2A).

The present study aims (1) to recover the phylogenetic relationships—and thus re-evaluate biogeographical patterns—within the assemblage of *Carinigera*, *Isabellaria* and *Sericata* species and (2) to reconstruct the evolution of the CA within this group. As an independent data set we used DNA sequence data, both nuclear and mitochondrial. The nuclear data set consists of the ribosomal internal transcribed spacer 1 (ITS1) and 2 (ITS2), which have proved informative with respect to the relationships between species of *Albinaria*, *Isabellaria*, and *Sericata* (van Moorsel et al., 2000). Of the mitochondrion we sequenced fragments of two genes, viz. the small ribosomal subunit (12S) and the cytochrome *c* oxidase subunit I (COI).

2. Materials and methods

2.1. Selection of taxa

All species sampled and sources of their sequences are listed in Appendix A. The localities of the samples are shown in Figs. 3A and B. We included all species pres-

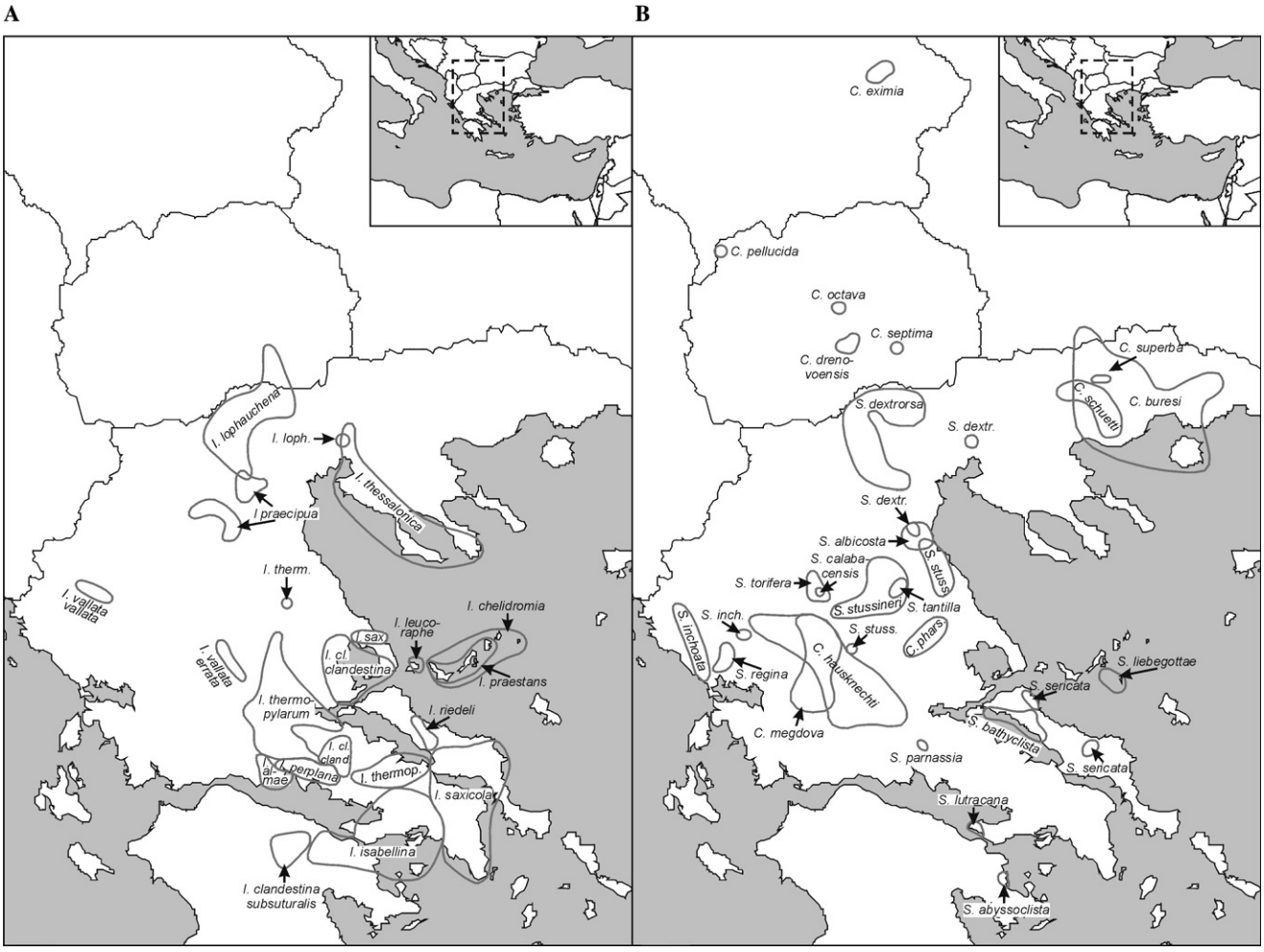

Fig. 2. Schematic distribution of G-type and N-type species. Gray lines indicate borders of distribution areas, and are based on the literature and data in the National Museum of Natural History in Leiden, the Netherlands. (A) G-type species that are classified with the genus *Isabellaria*. (B) N-type species, which are classified with the genera *Carinigera* and *Sericata*.

ently placed in the genera *Isabellaria* (14 species), and nearly all from the genera *Carinigera* (11 species) and *Sericata* (14 species), with the exception of *Sericata calabacensis* Westerlund, 1892, *Sericata parnassia* Boettger, 1888, and *Carinigera pellucida* Dedov and Neubert, 2002. Of the two *Sericata* species, to our knowledge, no living specimens have ever been collected. The third species, *C. pellucida*, had not yet been described at the time of the laboratory analyses. *Isabellaria vallata* is represented by both of its subspecies, since these are widely separated geographically, and so is *I. clandestina*. *Isabellaria clandestina subsuturalis* was originally described as a separate species, and was only recently and guardedly placed in *I. clandestina* by Nordsieck (1974). The *Albinaria* clade and the *Cristataria* clade (see Uit de Weerd and Gittenberger, submitted) are represented by six and four species, respectively. For outgroup rooting, we used four genera that belong together with the ingroup species in the tribe Medorini. In addition, *Montenegrina* represents the related tribe Montenegrinini.

2.2. Sequences and alignment

DNA was extracted from frozen and ethanol-preserved tissue using CTAB. For frozen and fresh ethanol-preserved tissue the protocol by Schilthuizen et al. (1995) was followed. All tissue that had been stored in ethanol for more than one year was extracted using a modified protocol (Uit de Weerd and Gittenberger, in press).

The ITS1, ITS2, 12S, and COI regions were amplified in 35 PCR cycles, using the primers sets listed in Appendix B. The ITS1 and 2 primer sets each amplify the respective spacer as well as adjacent regions of the rRNA genes. The 12S primers span nearly the total length of 12S, with the exception of 79 positions at its 3′ side, and also amplify 24 bases at the 3′ end of the methionine tRNA. The COI primers span 708 bases in the 5′ half of cytochrome *c* oxidase subunit I. PCR product was pooled, gel-purified using spin columns (Qiaquick Gel Extraction Kit by Qiagen), and dye-

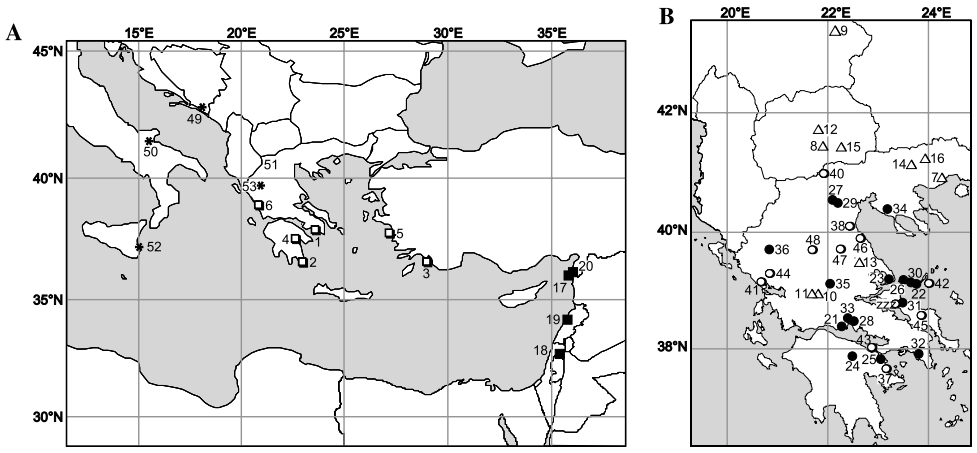

Fig. 3. Collection sites of included species. (A) *Albinaria* species (open squares), *Cristataria* species (closed squares) and outgroup species (asterisks). (B) *Carinigera* species (triangles), *Isabellaria* (closed circles) and *Sericata* species (open circles). Numbers refer to Appendix A.

terminator cycle-sequenced (Big Dye by PE Biosystems) in both directions. Electrophoresis was performed on an ABI 377 automated sequencer (PE Biosystems). Forward and reverse sequences were assembled and edited in Sequencher (Gene Codes).

All sequences were imported in MegAlign 4.03 (DNASTAR, 1999) and aligned using the Clustal V method with default settings. The ITS and 12S alignments were further adjusted manually in MacClade 4.0 (Maddison and Maddison, 2000). A secondary structure model of domain III of 12S of *Albinaria caerulea* was calculated using the RNAlign Server (Page, 2000), to be used in alignment refinements and in the selection of positions to be included in the analyses. Since secondary structure models can be misleading for ITS, at least in the group studied here (Armbruster, 2001; Uit de Weerd and Gittenberger, in press), we instead compared the alignment to previously identified conserved regions that had been shown to be informative (van Moorsel et al., 2001c; Uit de Weerd and Gittenberger, in press). All regions within ITS1, ITS2 and 12S that were ambiguously aligned within the ingroup were excluded. The COI datamatrix was checked for gaps and stopcodons. The resulting alignments were deposited with TreeBASE (http://www.treebase.org).

2.3. Bayesian analyses

Since rates and modes of evolution were expected to differ between ITS, 12S, and COI, and even between sites within COI (see Li, 1997, pp. 177–193), we decided to use a model-based approach to reconstruct the phylogeny of the group. We performed Bayesian inference, using MrBayes 3.0 (Ronquist and Huelsenbeck, 2003), as this approach allows optimization of parameters within data partitions, while at the same time assuming a similar underlying phylogenetic tree for each partition. We recognized six data partitions: ITS1, ITS2, 12S, COI position 1, COI position 2, and COI position 3. Using PAUP* 4.0b10, we calculated for each data partition the likelihood scores of different models superposed on a JC69 NJ tree calculated for that partition. These likelihood scores were subsequently evaluated using MrModeltest 1.1b (Nylander, 2002). Whenever the likelihood ratio test and the Akaike information criterion favored different models, we chose to use a more inclusive model that combines both models.

All Bayesian analyses were performed twice, starting with random trees. Four Markov chains were run 4×10^6 generations, and sampled once every 100 generations. We used three incrementally ($T = 0.20$) heated chains and a cold one. All analyses were started under default priors. Likelihood settings were changed to the preferred substitution model, and unlinked across partitions.

Bayesian inference was first performed independently for two subsets: (1) mitochondrial (12S and the three codon positions of COI) and (2) nuclear (ITS1 and 2) sequences. We determined the burn-in period by graphically checking for stationarity. When the outcomes of the two parallel Bayesian analyses within each subset were consistent, the trees of both analyses of each data set were combined, discarding those from within the burn-in period. Tree and bipartition probabilities of these combined trees, were calculated in MrBayes 3.0. The 95% credibility intervals of trees obtained from the mitochondrial and the ITS data set were checked for overlap, and we compared the probabilities of the individual clades. This was done (1) by identifying clades supported in the majority rule consensus tree of both data sets; (2) by discriminating within each data set between clades significantly (>95%) and those not significantly supported by the other data set, and comparing the probabilities of both groups (one-tailed Mann–

Whitney *U* test, SPSS); and (3) by tracing incompatible clades, significantly supported by each data set. Depending on their congruence, all data partitions were analyzed together in MrBayes 3.0, using the same procedure as before and using the same model for each partition as in the previous analyses.

2.4. Reconstruction of character evolution

Since the reconstruction of CA-type evolution is based both on the topology inferred as well as on the states of the terminal nodes, we randomized each parameter to test for significant deviations. The trees obtained through Bayesian inference allowed a probabilistic inference of character evolution. Of all 76,000 trees of a respective data set, the minimum number of CA-type transformations required was calculated in MacClade 4.0 (Maddison and Maddison, 2000). These numbers were then compared to the number of transformations required in an equal number of random trees generated in PAUP* 4.0b10 (Swofford, 2002). The distribution of states on the terminal nodes, given the 50% majority rule consensus tree, was tested using a T-PTP test as implemented in PAUP* 4.0b10 (Swofford, 2002).

Apart from the total number, we also investigated the inferred relative contribution of each type of transformation, i.e., from N-type to G-type and reverse, using both ACCTRAN and DELTRAN optimization. As different equally parsimonious reconstructions are possible for the same tree, we counted unambiguous changes only, and did so over all trees from a given Bayesian analysis.

3. Results

3.1. Sequences and alignment

The alignment of the sequences was not problematic. The secondary structure calculated for domain III of 12S, matched that of *Aplysia cervina* (Medina et al., 2001). Nearly all positions included in our study were situated in regions marked as conserved by these authors, mostly—though not exclusively—in stem regions. Our ITS1 and 2 alignment contained all previously identified conserved cores (van Moorsel et al., 2001c). No gaps or stop codons were found in the COI data set. Since positional homology within the COI sequences was beyond question, even the most variable positions could be aligned among ingroup species. These COI positions represent the larger part of the variation in the mitochondrial data set (67%), since only relatively slowly evolving and therefore less variable regions could be unambiguously aligned between the 12S sequences. In the ITS data set many of the most variable positions also had to be excluded due to alignment difficulties. Consequently, in the ingroup a larger sequence divergence, both uncorrected and GTR corrected, was observed within COI than within 12S and within ITS1 and 2 (one-tailed Wilcoxon signed ranks test, $P < 0.001$). Pairwise uncorrected and GTR-corrected sequence divergence within both mitochondrial genes was larger than in ITS (one-tailed Wilcoxon signed ranks test, $P < 0.001$) for the ingroup.

3.2. Comparison of the nuclear and mitochondrial tree

Based on the outcome of MrModeltest 1.1b, the GTR + I + Γ model was used for all data partitions in the MrBayes 3.0 analyses. This model was selected both by the likelihood ratio test and by the Akaike information criterion for all data partitions except COI position 2 (LRT: F81 + Γ; AIC: GTR + I) and COI position 3 (LRT: GTR + Γ; AIC: GTR + I + Γ). In both cases the GTR + I + Γ model was chosen for further analyses, being the least inclusive model to combine both alternatives. The likelihood scores within all subsequent Bayesian analyses levelled off within 200,000 generations.

Although their 95% credibility intervals did not overlap, the trees obtained through separate Bayesian analysis of nuclear (ITS) and mitochondrial (12S and COI) DNA often supported identical clades (see Appendix C). Still, since such a comparison ignores clades that are less than 50% supported by either one of the data sets, it provides a rather conservative estimate of the degree of congruence between both data sets. Overall, well-supported clades in one data partition are also supported in the other. Clades significantly (>95%) supported by mitochondrial DNA had an average support of 60% in the ITS analyses, whereas the not significantly supported clades from the mitochondrial analyses had an average support of 0.04% in the ITS analyses (one-tailed Mann–Whitney *U* test, $P < 0.001$). Similarly, the average probabilities were, respectively, 64% and 0.05% for the mitochondrial analyses (one-tailed Mann–Whitney *U* test, $P < 0.001$). As a third measure of degree of topological congruence, we traced conflicting signal between the two data sets, i.e., significantly (>95%) supported clades found in one data set result that are incompatible with significantly supported clades from the other. No such conflicting clades were found, although the data sets place *I. praecipua* in different highly supported clades. The ITS data set groups this species with *S. tantilla*, *S. stussineri*, and *I. lophauchena* (99% supported), whereas the mitochondrial data set places *I. praecipua* with *S. dextrorsa* and *S. torifera* (94% supported).

Most of the topological differences between the mitochondrial and the ITS tree are found near the base of each tree. The two data sets differ in their degree of resolution of apparently deeper divergences. These can be highly supported by ITS, but always receive low proba-

bilities in the mitochondrial data set. Thus, in the analyses of the mitochondrial data set even the monophyly of the ingroup is poorly supported. In 64% of the trees, *Montenegrina* is placed as a sistergroup to *S. inchoata* and *S. regina*, whereas only 28% of the trees group *Montenegrina* with the other outgroup taxa.

Since both data sets often similarly resolved relatively recent divergences, but were less informative with respect to older divergences, we pooled them to extract any remaining phylogenetic signal that could resolve these early divergences. The observation that both data sets are largely congruent with respect to the more shallow divergences, where both show good resolution, in our opinion justifies this concatenation of the data sets.

The analyses of both data sets combined, with a graphically checked burn-in of 200,000 generations, produced a well-resolved tree (Fig. 4), even with respect to most of the basal ingroup divergences. This increased clade support of the combined data set demonstrates that the separate data sets are not incongruent but rather are inconclusive with respect to deeper divergences. The concordance of the inferred deeper divergences with distributional data provides further support for the tree inferred from the combined data set. We consequently based inferences of phylogenetic relationships and character evolution on this tree.

3.3. Phylogenetic relationships

Three well-supported main clades are found within the ingroup (Fig. 4): (1) an *Albinaria* clade, with both CA-types; (2) a clade consisting purely of N-type species, viz. the genus *Cristataria* together with its sister clade (clade 4), consisting of *S. inchoata*, *S. regina*, *C. hausknechti*, and *C. megdova*; and (3) a clade with the remaining *Carinigera* and *Sericata* species together with the *Isabellaria* species, thus consisting of both N- and G-type species. The interrelationships between these three main clades are poorly resolved: 44% of the trees support a sistergroup relation between clades 1 and 2, 27% between clade 1s and 3, whereas 29% of the trees show clades 2 and 3 as sister groups. Clade 3 is confined

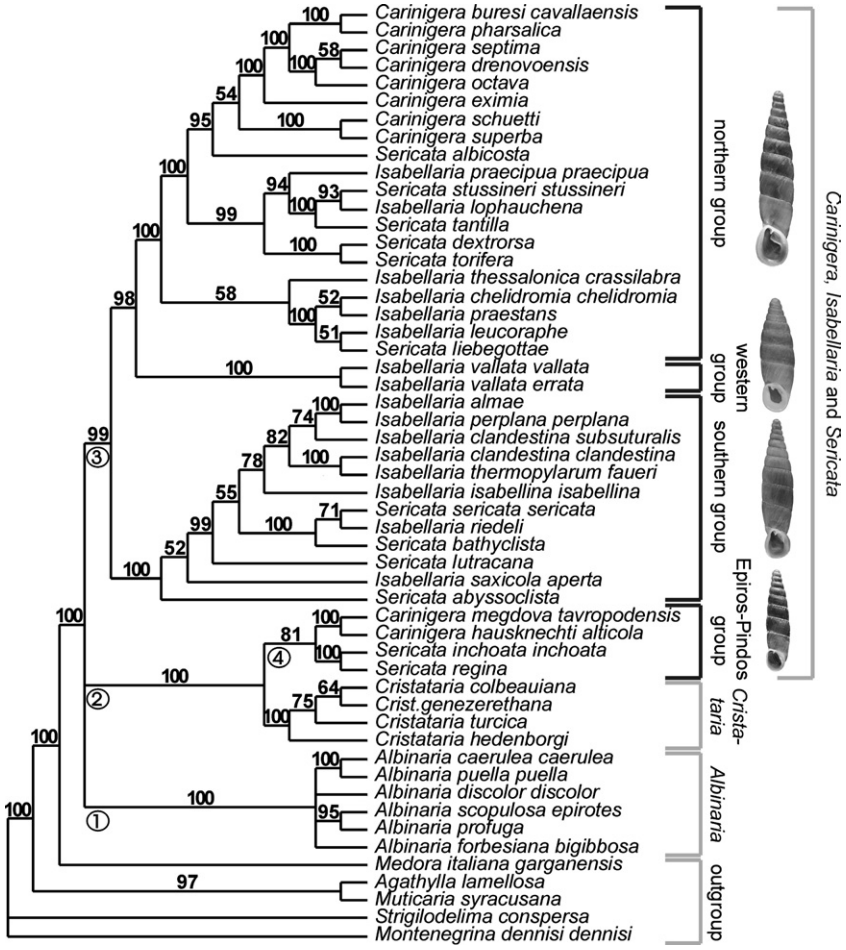

Fig. 4. The 50% majority rule consensus tree from the Bayesian analysis of the combined data set. Numbers above branches represent posterior probabilities; the encircled numbers indicate clades referred to in the text.

to central and northern Greece, Bulgaria, Macedonia and Serbia; the other two clades have a larger, more southern, area of distribution. Our tree thus places all *Isabellaria* species in a single mixed clade (clade 3), but distributes *Sericata* and *Carinigera* species across two clearly separated clades, viz. clade 3 and clade 4. Both clades are more or less geographically delimited. Clade 4 inhabits the western part of the Greek mainland (Fig. 5A), whereas the remaining species placed in clade 3 are found in the eastern part (Figs. 5B–D).

The inferred relationships within clade 3 show a strong geographic pattern, but do not correspond to the CA-based classification. Three well-supported geographically confined subclades are found, each containing so-called *Isabellaria* species: a southern group (Fig. 5B), a western group (Fig. 5C) and a northern group (Fig. 5D). Excepting *C. pharsalica* (see Section 4), a species from the northern group occurring in the southern part of Thessaly, the three groups are roughly separated by the Thessalian plains. The southern subclade consists of both *Isabellaria* and *Sericata* species, the western subclade is formed by a single *Isabellaria* species, viz. *I. vallata*, and its sister group, the northern subclade, contains *Isabellaria* and *Sericata* species, as well as a

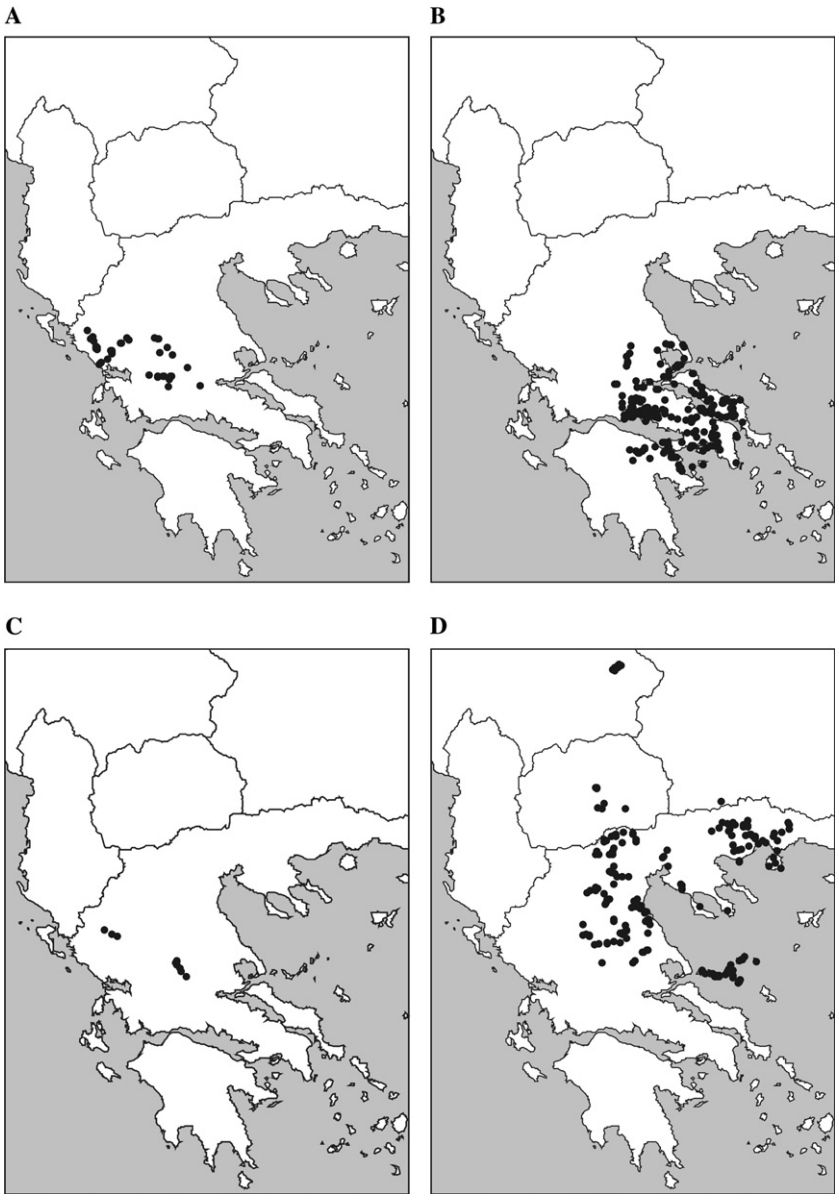

Fig. 5. The geographical distribution of the clades that were identified by the Bayesian analysis of the combined data set: (A) clade 4, from Epiros and the Pindos mountains; (B) the southern group within clade 3; (C) the western group, viz. *Isabellaria vallata*, within clade 3; and (D) the northern group within clade 3. Localities are based on the literature and data in the National Museum of Natural History in Leiden, the Netherlands. Localities of species that remain unstudied molecularly, i.e., *C. pellucida*, *S. calabacensis*, and *S. parnassia* are not shown.

Carinigera clade. Relationships within each of the subclades again follow a geographic pattern. For instance, all species from the Northern Sporades, viz. *I. chelidromia*, *I. leucoraphe*, *I. praestans*, and *S. liebegottae*, constitute a well-supported clade.

3.4. CA-type evolution

Our results demonstrate that transformations in CA-type have occurred frequently. The consistency index for CA-type, when plotted on the majority rule consensus tree of all data partitions combined (Fig. 6), has a value of only 0.11 (re-scaled CI: 0.056). At least eight instances of parallelism and one reversal are required under DELTRAN optimization. When using ACCTRAN optimization, seven cases of parallelism and two reversals are found. Nevertheless, the G- and N-types are clearly non-randomly distributed across the terminal nodes (T-PTP test: $P < 0.05$). Taking into account uncertainties in tree topology, our results are still significantly more concordant with the distribution of G- and N-type species than would be expected by chance. All 76,000 DNA-derived trees required fewer than 12 transformations in CA-type, compared to 2% of the random trees ($P < 0.02$). Although species within each of the three sampled species pairs (*S. lutracana* and *I. isabellina*; *S. stussineri* and *I. lophauchena*; and *S. bathyclista* and *I. riedeli*) are shown as relatively closely related in the 50% majority rule consensus tree, only *S. stussineri* and *I. lophauchena* are identified as sister species.

The transformations from N-type to G-type were found more common than the reverse. In 99% of the trees from the Bayesian analysis, the number of inferred N to G transformations exceeds that of the inferred G to N transformations (see Appendix D).

4. Discussion and conclusions

Our results confirm the hypothesis that the CA-type is a highly homoplasious character at the systematic level addressed in this study. Although the CA-types are

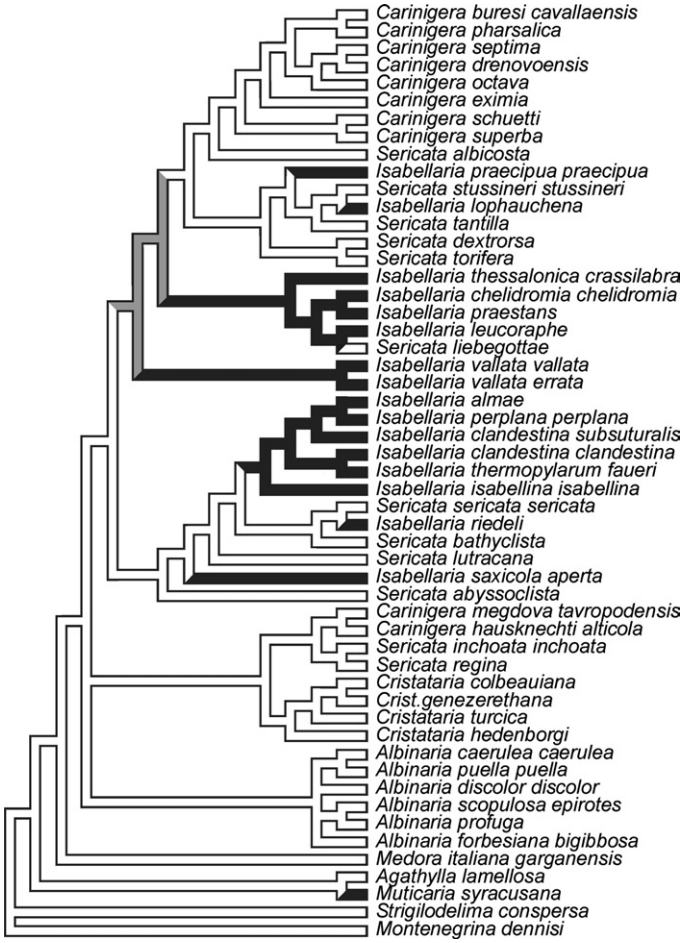

Fig. 6. Reconstruction of CA-type evolution, plotted on the 50% majority rule tree of the combined data set. White branches represent an inferred N-type, black lines an inferred G-type. The gray-colored branches indicate an equivocal reconstruction, ACCTRAN optimization preferring the G-type, DELTRAN optimization the N-type.

non-randomly distributed across the tree, the recurrent evolution of the G-type and the plesiomorphism of the N-type preclude their use as diagnostic character states in the genus definitions. In contrast to the CA-type, biogeographic patterns are more indicative of relatedness. These observations necessitate a generic revision of the species actually classified with *Carinigera*, *Isabellaria*, and *Sericata*. Since this implies critical analyses of the anatomical and conchological characters of the various species, and a formal nomenclatorial review, such a revision will be presented separately. On the basis of the framework provided by the present paper, the species will be classified into two genera, corresponding with our clades 3 [*Isabellaria*, with three subgenera] and 4 [needs a new name] (Gittenberger and Uit de Weerd, in press). Here we will focus on the evolutionary implications.

4.1. Mitochondrial compared to nuclear DNA evolution

The mitochondrial sequences examined lend high support to relatively recent divergences only. The lack of deep-level phylogenetic resolution in the mitochondrial data set is probably due to saturation effects, since the less divergent ITS sequences resolve older divergences reasonably well. Even though 12S and COI rank among the most conserved regions within the mitochondrion (Palumbi, 1996, pp. 235–236), mitochondrial DNA generally has a higher evolutionary rate than has nuclear DNA (Li, 1997, p. 192), and mitochondrial evolution may even have accelerated within land snails. Comparatively high rates of mitochondrial DNA evolution have been inferred within the land snail genus *Mandarina* (Chiba, 1999), and a high diversity of mitochondrial DNA has been observed even at species level in land snails (e.g., Hayashi and Chiba, 2000; Thomaz et al., 1996; Watanabe and Chiba, 2001). The comparatively high divergence between COI sequences probably further contributed to the noise in the mitochondrial data set.

The increased resolution of the combined data set relative to the separate data sets nevertheless demonstrates that at least some phylogenetic information even for the oldest divergences is still present in the mitochondrial DNA. Studies incorporating the inferred amino acid sequences of all mitochondrial protein-coding genes of *A. caerulea* show that deep-level phylogenetic information can indeed be retrieved from mitochondrial DNA, given sufficient sampling (see Grande et al., 2002; Tomita et al., 2002).

4.2. Distribution

In finding geographically confined clades, our tree demonstrates the limited dispersal abilities of these snails. A previous molecular study on *Albinaria* in Crete revealed a comparable geographic coherence of clades (Douris et al., 1998a). Direct measurements on dispersal velocity, carried out on *Albinaria* in Crete, confirm that this is low for at least this genus and this region. Field observations on marked *Albinaria* specimens suggest a mean dispersal velocity of 1–2 m/y per snail, with dispersal mainly following limestone boulders or outcrops (Schilthuizen and Lombaerts, 1994). Dispersal appears to be similarly restricted in *Cristataria* (see Bar, 1977). The spread of a species as a whole may be even lower, especially in areas already inhabited by other species (Welter-Schultes, 2000). Based on its presumed date of introduction and its present distribution, Welter-Schultes (2000, p. 79) estimates the dispersal of *A. praeclara* at the Knossos ruins at 0.5–0.6 m/y. On a larger spatial scale, across the area of distribution of *Isabellaria*, *Carinigera* and *Sericata*, suitable habitats, viz. limestone and marble outcrops, at least presently have a highly fragmented distribution (see Bornovas and Rondogianni-Tsiambaou, 1983) and should hamper dispersal even further. Our results suit the observations of slow dispersal better than does the traditional classification, which implies either many instances of long-distance dispersal or extinction following extensive spread of ancestral taxa.

The most notable exception to the pattern of geographically confined clades is *C. pharsalica*, which is one of the southernmost distributed species of the northern clade. Our analyses group this species among the other *Carinigera* species of the northern clade, all of which occur far more to the North, near the Greek-Bulgarian border in Macedonia, and in Serbia. Further studies (Uit de Weerd et al., in prep.) indicate that the southern distribution of *C. pharsalica* can only be explained as a relatively recent long-distance dispersal event. The other southerly distributed clade within the so-called northern group, viz. the species of the Northern Sporades (*I. chelidromia*, *I. leucoraphe*, *I. praestans*, and *S. liebegottae*), may have colonized these islands from the nearby Chalkidiki peninsula, where their supposed sistergroup, viz. *I. thessalonica*, still occurs (Fig. 2A).

4.3. CA-type evolution

Our results refute the hypothesis that *Isabellaria* is monophyletic and demonstrate that instead the G-type CA was acquired several times in parallel. Although we made no a priori assumptions about character state polarity within the CA-type, both the ACCTRAN and DELTRAN reconstruction (Fig. 6) show the N-type as ancestral, which is in conformity with the unanimous view in the literature. Several lineages independently acquired the G-type CA, which subsequently reversed to the N-type in one lineage or possibly two lineages. It has been hypothesized that such transformations in

CA-type may be caused by a relatively simple change early in CA ontogeny (Gittenberger, 2000).

The inferred polarity of the CA-type appears to be insensitive to the limited sampling of *Albinaria* species. While the vast majority of *Albinaria* species have an N-type CA, in some a G-type is present. None of the latter species is represented in this study. The large number of *Albinaria* species and their relatively rapid divergence complicate phylogeny reconstruction within this genus (van Moorsel et al., 2001b). However, even when all *Albinaria* species were coded as possessing a G-type, in a nearly significant 94% of the trees the transformations to the G-type exceeded those to the N-type, whereas only 0.9% favored the transformations to the N-type.

The previous study by van Moorsel et al. (2000), which included only a small subset of the species placed in clade 3, markedly differs from ours in the character reconstruction within this clade and consequently in its conclusions. That study showed: (1) the G-type as ancestral to this clade and (2) only limited homoplasy, in the form of a parallel development of the N-type CA. Our results refute both these findings. It is our opinion that these different inferences are due to a severe underrepresentation of N-type *Sericata* and *Carinigera* species in the study by van Moorsel et al. (2000), with only two of these N-type species being included.

The inferred parallel evolution of the G-type CA is concordant with the view that the G-type is a last step in a process of increased sealing of the aperture (Nordsieck, 1982; Wagner, 1919, pp. 88–89). Clearly, in the family Clausiliidae as a whole, the G-type evolved several times in parallel, long after the main groups had diverged. Fossils of N-type clausiliid snails are known from the Paleocene onwards (Nordsieck, 2000), whereas the first known G-type fossils date from the upper Pliocene (Nordsieck, 1982, 2000). Our data confirm this pattern of evolution and show that this parallel evolution occurs even at much lower systematic levels than previously thought. Moreover, our results are concordant with the postulated post-Miocene parallel evolution of the G-type CA within the Clausiliidae. The split between the *Albinaria* clade and *I. saxicola* is estimated to have occurred minimally 9.3 MYA, early in late Miocene (van Moorsel et al., 2001b), and clearly predates the evolution of a G-type CA within clade 3.

The recurrent evolution of a G-type clausilium within clade 3, is suggestive of local selective advantages conferred by this type of CA. These supposed advantages apparently outweigh the improved ventilation through the by-pass canal when the snail is retracted (Gittenberger, 1996; Rees, 1964; von Vest, 1867). Selection in favor of the G-type may still be operating today, reducing the ranges of N-type species. In the southern part of the Greek mainland in particular, ranges of *Sericata* species are relatively small as compared to those of the *Isabellaria* species, and are more or less surrounded by those G-type species. At its type locality, where it was collected in 1887 (Boettger, 1888), *S. parnassia* is nearly absent, whereas the G-type species *I. thermopylarum* and *Idyla bicristata* appear to be thriving there (Uit de Weerd and Gittenberger, pers. obs.).

Two advantages of a G-type CA have been proposed. The G-type, in lacking the by-pass canal, may (1) limit evaporation (Nordsieck, 1982; von Vest, 1867) or (2) more effectively protect the snail against predation (Gittenberger and Schilthuizen, 1996; Gittenberger, 1997; Schmidt, 1868). According to the first view (Nordsieck, 1982; von Vest, 1867), the G-type clausilium is an adaptation to promote survival in a dry climate. So far, studies on the effect of the presence (Christelow, 1992) or shape (Warburg, 1972) of the clausilial plate on evaporation have been inconclusive, however. The impact of a G-type CA, during aestivation, is questioned by Gittenberger and Schilthuizen (1996), who state that G- and N-type species alike effectively seal off the aperture, by 'gluing' their shell to the substratum. Evaporation through the aperture region is impossible then, and the permeability of the entire shell wall becomes the dominant factor. Indeed, differences in shell thickness may have contributed to the differential survival during aestivation of two *Albinaria* species (Giokas et al., 2000). In addition, studies on the genus *Cristataria*, which dwells in a relatively arid environment, suggest that behavioral adaptations such as aestivation in aggregates in crevices may be more crucial than the clausilium in preventing desiccation (Arad et al., 1995; Heller and Dolev, 1994). A G-type may, however, be advantageous in winter, when the snails are most active and cannot as easily escape desiccation by other means. Precipitation in winter is comparatively low in the eastern half of the Greek mainland, where all G-types occur (see Nellestein and Dekker, 1998).

The second hypothesis states that the G-type serves as a protection against predation, from—most notably—*Drilus* larvae (Coleoptera, Drilidae). *Drilus* predation varies between localities and can exceed 50% in *Albinaria* populations (Welter-Schultes, 2000, pp. 126–127). The larvae presumably attack the snails when these are glued to the rock during aestivation (Schilthuizen et al., 1994) by boring holes in the ultimate or the penultimate whorl of the shell (Roth, 1855; see also Gittenberger, 1999). van Moorsel et al. (2001a) found a majority of these drilid entrance holes located in front of the clausilium. The small drilid larvae may subsequently pass the clausilial barrier through the by-pass canal in N-type species, whereas in G-type species the clausilial plate blocks the entrance to the shell (Gittenberger and Schilthuizen, 1996; Gittenberger, 1997). The presence of *Drilus* larvae inside N-type shells with only a hole in front of the clausilium, suggests that the by-pass canal can be used by the predators indeed (A. and E. Gittenberger, pers. obs.), although the occurrence in

G-type specimens of drill-holes through the clausilial plate (A. and E. Gittenberger, pers. obs.) indicates that, even in these snails, the apertural devices are not completely impenetrable.

Ideally, a hypothesis on the adaptive value of the G-type should explain the relatively recent occurrence of the G-type CA after the divergence of the main geographic clades. If the evaporation hypothesis is correct, the evolution of a G-type CA could have been triggered by a progressively dryer climate. Palynological and palaeobotanical studies indeed indicate a more humid climate in north-eastern Greece in late Miocene (±6.5 MYA) than at present (Kloosterboer-van Hoeve et al., 2000a, p. 81; Velitzelos and Gregor, 1986). It is around that time that a general gradual change towards a dryer climate is first observed (Karistineos and Ioakim, 1989), albeit with superimposed cyclical fluctuations (Kloosterboer-van Hoeve et al., 2000b). This timescale matches the time span of 9.3 MYA, in which the G-type is thought to have evolved. Alternatively, one can speculate that the iterative acquisition of a G-type CA within separate lineages from the eastern Greek mainland was facilitated by an increase in *Drilus* predation there. This defense mechanism may not yet have evolved and subsequently been selected for in other regions, presumably more recently invaded by *Drilus*. This hypothesis is concordant with studies on *Drilus* larvae in *Albinaria*, which appear to be in an intermediate state in a process of host specialization (Örstan, 1999).

Asymmetric though the transformation rate may be, our results indicate that the transformations to a G-type are not irreversible. Such irreversibility had been assumed (Nordsieck, 1982), because the transformation from N- to G-type involves the loss of two lamellae. One should bear in mind, however, that these lamellae are formed by folds of the mantle, which can fold only in a restricted number of positions because of internal organs. Moreover, this transformation may be directed by the position of a single lamella earlier in the development of the shell (Gittenberger, 2000). Interestingly, the only unambiguous reversal inferred occurs in the ancestor to *S. liebegottae*, a species that is found only on a few small remote islands in the Aegean Sea. As *Drilus* females are flightless (Lawrence, 1991), it would be interesting to know whether these islands are inhabited by *Drilus*.

Until selective forces acting on the G- and N-type CA have been better identified, we can only speculate about the causes of the parallel evolution of a G-type CA in the past. Obviously, comparative field studies on *Drilus* predation and desiccation pressures should be conducted. Candidate species for such studies are *I. lophauchena*, and *S. dextrorsa*, two species shown as closely related in this study, having large overlapping areas of distribution, that offer various sample sites where both G- and N-type snails occur syntopically.

4.4. Geography versus morphological evolution

Our data suggest that in land snails even supposedly complex characters may arise in parallel in geographically confined clades, probably as a result of a common selection pressure. A similar phenomenon has been reported in several land snail genera from oceanic islands, viz. *Samoana* (Johnson et al., 2000), *Samoana* and the allied genus *Partula* (Goodacre and Wade, 2001) and the genus *Mandarina* (Chiba, 1999). Molecular studies of these genera revealed clades, confined to islands or island groups, in which a similar suite of characters had evolved independently, in presumably ecologically adapted morphotypes. Even in the absence of geographical barriers, the limited range of dispersal of land snails may prevent selectively advantageous traits from spreading rapidly, leaving opportunity for parallel evolution of these traits as a response to changing or highly localized selective regimes. Given that isolation by distance is observed in many land snails (e.g., Pfenninger et al., 1996; Ross, 1999; Schilthuizen et al., 1999), such iterative parallel evolution of morphological traits may be much more common than currently thought. Here it remained undetected because these traits were used in systematics as diagnostic characters.

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Appendix A

Species sampled in this study with their locality codes, accession number and the origin of their sequence when not determined in this study

Sample information			GenBank accession numbers and sources			
No.	Species	Co-ordinates	ITS1	ITS2	12S	COI
1	*Albinaria caerulea caerulea*	Unknown	Not submitted[c]	Not submitted[c]	X83390[a]	X83390[a]
2	*Albinaria discolor discolor*	36°41′N 23°03′E	AF254589[b,**]	AF254589[b,**]	AY382064[e]	AY425547
3	*Albinaria forbesiana bigibbosa*	36°39′N 29°06′E	Not submitted[c]	Not submitted[c]	AY382065[e]	AY425548
4	*Albinaria profuga*	37°38′N 22°44′E	AY382088[e]	AY382089[e]	AY382067[e]	AY425549
5	*Albinaria puella puella*	37°52′N 27°16′E	Not submitted[c]	Not submitted[c]	AY382068[e]	AY425550
6	*Albinaria scopulosa epirotes*	38°59′N 21°23′E	AF254588[b,**]	AF254588[b,**]	AY382070[e]	AY425551
7	*Carinigera buresi nordsiecki*	40°54′N 24°14′E	AY382124[d]	AY382099[d]	AY425514	AY425552
8	*Carinigera drenovoensis*	41°25′N 21°54′E	AY382125[d]	AY382100[d]	AY425515	AY425553
9	*Carinigera eximia*	43°19′N 22°08′E	AY382126[d]	AY382101[d]	AY425516	AY425554
10	*Carinigera hausknechti alticola*	38°57′N 21°48′E	AY382127[d]	AY382102[d]	AY425517	AY425555
11	*Carinigera megdova tavropodensis*	38°57′N 21°41′E	AY382128[d]	AY382103[d]	AY425518	AY425556
12	*Carinigera octava*	41°42′N 21°46′E	AY382129[d]	AY382104[d]	AY425519	AY425557
13	*Carinigera pharsalica*	39°29′N 22°37′E	AY382130[d]	AY382105[d]	AY425520	AY425558
14	*Carinigera schuetti*	41°07′N 23°38′E	AY382131[d]	AY382106[d]	AY425521	AY425559
15	*Carinigera septima*	41°24′N 22°15′E	AY382132[d]	AY382107[d]	AY425522	AY425560
16	*Carinigera superba*	41°13′N 23°54′E	AY382133[d]	AY382108[d]	AY425523	AY425561
17	*Cristataria colbeauiana*	36°14′N 36°07′E	AY382134[d]	AY382109[d]	AY382072[e]	AY425562
18	*Cristataria genezerethana*	32°48′N 35°31′E	AY382135[d]	AY382110[d]	AY382073[e]	AY425563
19	*Cristataria hedenborgi*	34°17′N 35°54′E	AY382094[e]	AY382095[e]	AY382074[e]	AY425564
20	*Cristataria turcica*	36°07′N 35°56′E	AY382096[e]	AY382097[e]	AY382075[e]	AY425565
21	*Isabellaria almae*	38°24′N 22°16′E	AY425486	AY425487	AY425524	AY425566
22	*Isabellaria chelidromia chelidromia*	39°08′N 23°43′E	AY425488	AY425489	AY425525	AY425567
23	*Isabellaria clandestina clandestina*	39°12′N 23°12′E	AY382137[d]	AY382112[d]	AY425526	AY425568
24	*Isabellaria clandestina subsuturalis*	37°53′N 22°29′E	AY425490	AY425491	AY425527	AY425569
25	*Isabellaria isabellina isabellina*	37°50′N 23°02′E	AY382138[d]	AF254618[b,*]	AY382076[e]	AY425570
26	*Isabellaria leucoraphe*	39°11′N 23°29′E	AY425492	AY425493	AY425528	AY425571
27	*Isabellaria lophauchena*	40°32′N 22°05′E	AY425494	AY425495	AY425529	AY425572
28	*Isabellaria perplana perplana*	38°29′N 22°31′E	AY382139[d]	AF254614[b,*]	AY425530	AY425573

29	*Isabellaria praecipua praecipua*	40°29′N 22°12′E	AF254601[b,*]	AF254601[b,*]	AY425531	AY425574
30	*Isabellaria praestans*	39°07′N 23°42′E	AY425496	AY425497	AY425532	AY425575
31	*Isabellaria riedeli*	38°48′N 23°28′E	AF254619[b,*]	AF254619[b,*]	AY425533	AY425576
32	*Isabellaria saxicola aperta*	37°56′N 23°47′E	AF254613[b,*]	AF254613[b,*]	AY425534	AY425577
33	*Isabellaria thermopylarum faueri*	38°32′N 22°23′E	AF254620[b,*]	AF254620[b,*]	AY425535	AY425578
34	*Isabellaria thessalonica crassilabra*	40°23′N 23°10′E	AY425498	AY425499	AY425536	AY425579
35	*Isabellaria vallata errata*	39°07′N 22°03′E	AY382140[d]	AY382113[d]	AY382077[e]	AY425580
36	*Isabellaria vallata vallata*	39°42′N 20°51′E	AF254604[b,*]	AF254604[b,*]	AY425537	AY425581
37	*Sericata abyssoclista*	37°40′N 23°08′E	AY425500	AY425501	AY425538	AY425582
38	*Sericata albicosta*	40°05′N 22°25′E	AY382146[d]	AY382119[d]	AY382078[e]	AY425583
39	*Sericata bathyclista*	38°46′N 23°19′E	AY425502	AY425503	AY425539	AY425584
40	*Sericata dextrorsa*	40°58′N 21°55′E	AY425504	AY425505	AY425540	AY425585
41	*Sericata inchoata inchoata*	39°09′N 20°41′E	AY382147[d]	AY382120[d]	AY382079[e]	AY425586
42	*Sericata liebegottae*	39°07′N 23°59′E	AY425506	AY425507	AY425541	AY425587
43	*Sericata lutracana*	38°02′N 22°51′E	AF254609[b,*]	AF254609[b,*]	AY425542	AY425588
44	*Sericata regina*	39°17′N 20°51′E	AY382148[d]	AY382121[d]	AY425543	AY425589
45	*Sericata sericata sericata*	38°35′N 23°50′E	AY382149[d]	AF254612[b,*]	AY382080[e]	AY425590
46	*Sericata stussineri stussineri*	39°53′N 22°38′E	AY425508	AY425509	AY425544	AY425591
47	*Sericata tantilla*	39°42′N 22°14′E	AY425510	AY425511	AY425545	AY425592
48	*Sericata torifera*	39°41′N 21°41′E	AY425512	AY425513	AY425546	AY425593
Outgroup						
49	*Agathylla lamellosa*	42°39′N 18°05′E	AY382123[d]	AY382098[d]	AY382081[e]	AY425594
50	*Medora italiana garganensis*	41°40′N 15°53′E	AY382142[d]	AY382115[d]	AY382082[e]	AY425595
51	*Montenegrina dennisi dennisi*	40°01′N 21°17′E	AY382143[d]	AY382116[d]	AY382084[e]	AY425596
52	*Muticaria syracusana*	37°04′N 15°18′E	AY382144[d]	AY382117[d]	AY382083[e]	AY425597
53	*Strigilodelima conspersa*	39°40′N 20°57′E	AY382150[d]	AY382122[d]	AY382085[e]	AY425598

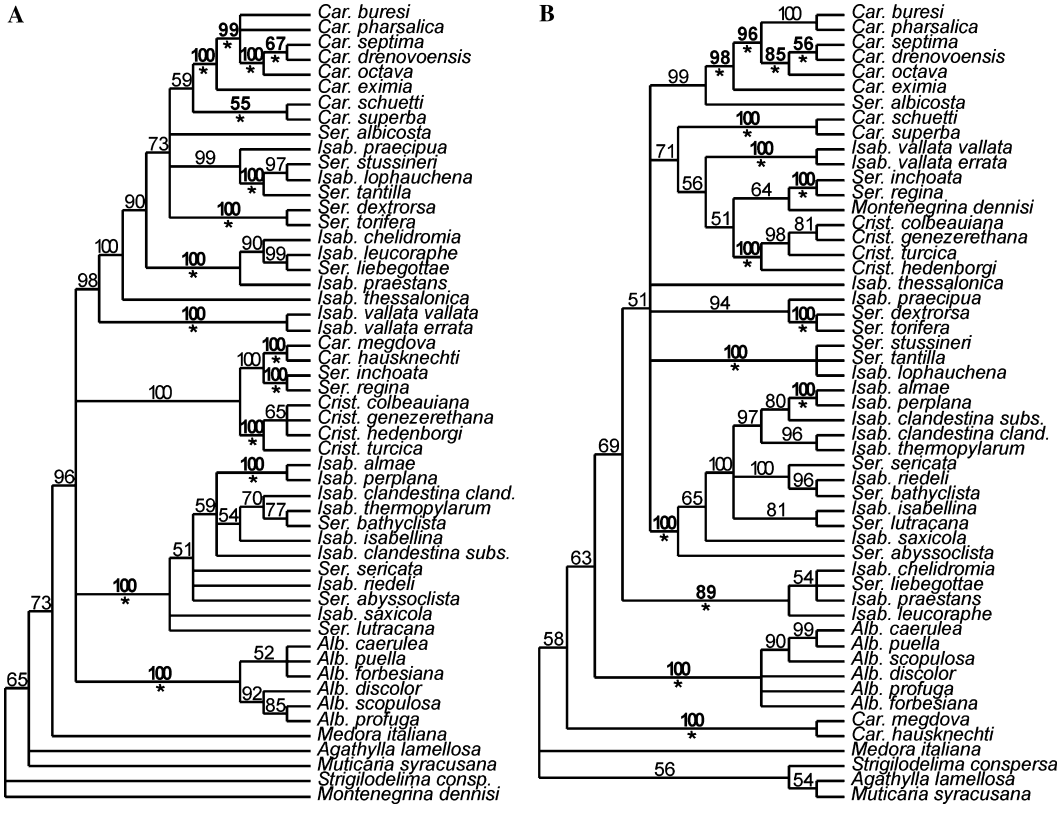
[a] Hatzoglou et al. (1995).
[b] van Moorsel et al. (2000).
[c] van Moorsel et al. (2001c).
[d] Uit de Weerd and Gittenberger (in press).
[e] Uit de Weerd and Gittenberger (submitted).
[*] Different specimen from the same population as sampled in the present study.
[**] Different specimen from another population, not shown on the map.

Appendix B

Primers used for amplification of the DNA regions analyzed

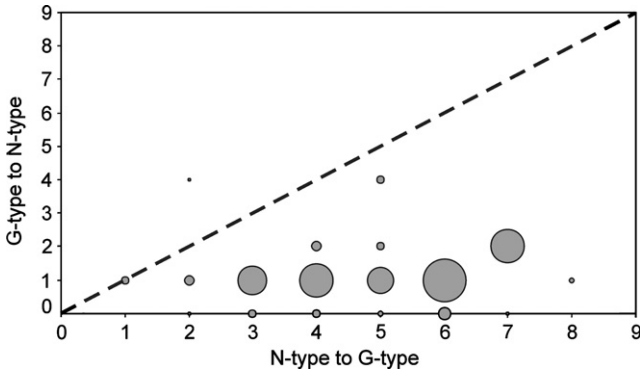
Region	Primer (5′ to 3′)	Origin	*T* (°C)
ITS1	Forward: CAC ACC GCC CGT CGC TAC TAC C Reverse: ATG CGT TCA AGA TGT CGA TGT TCA A	Modified from Hillis and Dixon (1991); Uit de Weerd and Gittenberger (in press)	61
ITS2	Forward: GGC GGC CTC GGG TCC ATC C Reverse: TTC CCG CTT CAC TCG CCG TTA CTG	Uit de Weerd and Gittenberger (in press)	61
COI (5′ half)	Forward: ACT CAA CGA ATC ATA AAG ATA TTG G Reverse: TAT ACT TCA GGA TGA CCA AAA AAT CA	Gittenberger et al. (2004), modified from Folmer et al. (1994)	47
3′ $tRNA^{Met}$ and 5′ 12S	Forward: TAA GCT GTA GGG CTC ATA AC Reverse: GAG AGT GAC GGG CGA TTT G	Uit de Weerd and Gittenberger (submitted)	47

Appendix C



The 50% majority rule trees from the Bayesian analysis of the separate data sets. Numbers represent posterior probabilities, asterisks indicate clades supported by a majority of trees in both data sets. (A) Tree obtained from the ITS1 and 2 data set. (B) Tree obtained from the mitochondrial data set, viz. 12S and COI.

Appendix D



Distribution of unambiguous transformations in the 76,000 trees obtained from the Bayesian analysis of the combined data set. The hatched line indicates the 1:1 relationship.

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