

Phylogeography of the land snail *Albinaria hippolyti* (Pulmonata: Clausiliidae) from Crete, inferred from *ITS-1* sequences

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The polytypic Cretan land snail *Albinaria hippolyti* has a range that is partly fragmented and partly subdivided by hybrid zones. For this reason, it has served as a model species for investigating speciation and radiation in Mediterranean Clausiliidae. The first internal transcribed spacer (*ITS-1*) of the nuclear ribosomal DNA was sequenced in 20 populations of *A. hippolyti* and phylogenetically analysed using maximum parsimony. We employed a novel method involving logarithmic weighting of gaps and topological constraints based on bootstrap values. The resulting phylogeography suggests that the species has undergone a recent cycle of range expansion and range reduction. Speciation cannot be linked to major geological vicariance events in the Miocene and Pliocene, as has been suggested previously. The subspecies *A. h. arthuriana* appears unrelated to other *A. hippolyti* subspecies, which supports recent suggestions, based on morphology, to regard it as a separate species. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, 83, 000–000.

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INTRODUCTION

Several areas in the eastern Mediterranean are well known for their high biodiversity, particularly in sessile organisms. Examples include plants (Medail & Quezel, 1997), isopods (Sfenthourakis, 1996) and various groups of terrestrial molluscs (Riedel, 1992;

Maassen, 1995; Welter-Schultes & Williams, 1999). Most prominent among the latter group is *Albinaria*, a clausiliid genus represented by c. 120–130 species (Nordsieck, 1999; Welter-Schultes, 2000b). Species are mainly characterized by diagnostic combinations of shell (and sometimes genital) traits. They dwell on limestone rocks where they feed on microflora, and little niche differentiation is apparent (Gittenberger, 1991). Sympatry is rare; most species occupy small (500 km² on average) parapatric ranges (for a fine-grained distribution map of some Cretan species, see Welter-Schultes, 1998a). Nevertheless, they cannot be considered geographical forms of a much smaller number of biological species, given the strong genetic differentiation (Schilthuisen & Gittenberger, 1996) and

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reproductive isolation in areas where their ranges overlap (Schilthuizen, 1994a).

Studies to elucidate the modes of speciation and radiation in *Albinaria* have recently focused on the central Cretan species *Albinaria hippolyti* (Boettger). This species was selected as a model because it shows a fortuitous combination of biogeographical characteristics. First of all, it is subdivided into six morphologically and allozymatically well-defined subspecies (Schilthuizen, Welter-Schultes & Wiese, 1993; Schilthuizen, 1994b). Two of these occupy isolated ranges, while the remaining four are interconnected by narrow hybrid zones, in which many characters intergrade concordantly (Schilthuizen, 1995). Besides the sharp changes that characterize hybrid zones, several traits show gradual geographical or altitudinal variation within (and sometimes surpassing) the subspecific boundaries. Moreover, outside of the 'main range' of the species, many small to very small populations exist which can be attributed to one of the six subspecies (Schilthuizen, 1994b). In short, *A. hippolyti* has the taxonomic and biogeographical characteristics that Endler (1977) considered particularly attractive for the study of population structure, geographical variation and speciation.

Given that ecological differentiation seems to be limited, speciation in *Albinaria* is likely to be allopatric, rather than sympatric or parapatric. This is supported by studies of the hybrid zones in *A. hippolyti*, which appear to be the result of secondary contact between already differentiated subspecies, and usually are not associated with obvious habitat transitions (Schilthuizen, 1995). The geological history of the Aegean region (and of Crete in particular) has been turbulent for millions of years (Rögl & Steininger, 1983; Jacobshagen, 1986). Tectonic movements have resulted in vertical shifts locally of more than 700 m; Meulenkamp, 1971; Peters & Huson, 1985), and in historical times, vertical movements along the south coast of 1–2 m per 1000 years have been documented (van Andel & Shackleton, 1982). In combination with sea-level changes, these movements must have fragmented and reconnected islands frequently, giving ample opportunities for vicariance events. To some extent, however, vicariant biogeographical patterns may have been modified or complicated by recent human-induced dispersal, as suggested by Welter-Schultes (1998b, 2000b).

In this study we aimed to infer the evolutionary and distributional history of *A. hippolyti* on the basis of an analysis of DNA sequences for the first internal transcribed spacer of the ribosomal DNA (*ITS-1*). This region has the disadvantage that its population genetic behaviour is as yet far from clear (Hillis *et al.*, 1991; Rich *et al.*, 1997), making quantitative analyses tentative at best. Also, given that it is a multicopy

region with sometimes imperfect concerted evolution, intraindividual variation is common in certain groups (see, for example, Harris & Crandall, 2000; von der Schulenburg *et al.*, 2001). This may result in serious problems for phylogenetic analyses at low taxonomic levels such as this one. However, in land snails, presumably due to their low rates of mobility, such problems appear to be minimal (Schilthuizen *et al.*, 1999; van Moorsel, Dijkstra & Gittenberger, 2000). In spite of these possible complications, we preferred to choose this nuclear marker rather than a mitochondrial one, because of the presence of hybrid zones between the majority of our taxa. Due to its unlinked nature, organellar DNA, unlike most nuclear DNA markers, might easily pass through hybrid zones, which could confound the determination of the phylogeographical relationships between subspecies (Rieseberg & Soltis, 1991).

MATERIAL AND METHODS

SAMPLING

A. hippolyti snails were collected by M.S. in Crete in 1991 and 1997. In total, 20 populations were sampled, covering all six subspecies, most of the isolated populations and multiple representatives for geographically variable subspecies. This geographical variation has been formalized by recognizing intrasubspecific categories, named after the largest town or mountain top in the area where the population occurs (Schilthuizen *et al.*, 1993). In the text, these categories are referred to as, for example, *A. h. hippolyti* f. [forma] 'Máráthos'. We also included an isolated *A. hippolyti* population from Agía Pelagía, which has been variously attributed to *A. h. harmonia* (M. Schilthuizen, unpubl. data) or *A. h. hippolyti* (Welter-Schultes, 2000c). *A. h. arthuriana* was the only subspecies for which sampling was limited, because its exact distribution and variability were not yet known when the fieldwork for this study was carried out (see Welter-Schultes, 1998a, 2000c; for more distributional details for this subspecies). As subspecies attributed to *A. hippolyti* may not be assumed to form a monophyletic group, 13 other clausiliid species were included in the analysis. These were: from Crete and its satellite islet Día, *A. candida* (Pfeiffer), *A. corrugata* (Bruguère), *A. cretensis* (Rossmässler), *A. spratti* (Pfeiffer), *A. teres* (Olivier), *A. torticollis* (Olivier), *A. ulrikae* Schilthuizen & Gittenberger, and *A. wiesei* Gittenberger. As the Cretan *Albinaria* fauna contains elements more closely related to non-Cretan species (Nordsieck, 1999), we also selected from other Greek localities: *A. arcadica* (Pfeiffer), *A. caerulea* (Deshayes), *A. olivieri* (Roth), and *Isabellaria saxicola* (Pfeiffer). These non-*hippolyti* snails were gathered by

C.v.M., E.G. and other collectors between 1986 and 1998. All individuals were transported alive and frozen at -80°C upon arrival in the laboratory. Voucher specimens (both dry shells and ethanol-preserved snails) have been deposited in the collection of the National Museum of Natural History 'Naturalis', Leiden, the Netherlands. Cretan collection localities are indicated in Figure 1.

DNA TECHNIQUES

DNA was extracted from 1–2 mm³ of the foot muscle of single individuals with a CTAB-based protocol as described earlier (Schilthuizen, Gittenberger & Gulyaev, 1995). DNA from the non-*hippolyti* samples, which derived from an unrelated study, was extracted

using a sucrose-based protocol (van Moorsel, van Nes & Megens, 2000). DNA samples were stored in 25 μL 10 mM Tris, 1 mM EDTA buffer.

The target DNA was amplified with primers 18d ('fruitfly') and 5.8c ('Silkworm') from Hillis & Dixon (1991), to produce fragments including the entire *ITS-1* region. Halfway through the study, primer 18d was replaced by the oligonucleotide 18Sb (5'TTCCGTAGTGAACCTGCGG3'), which was designed to attach to a position closer to the 5' end of the spacer. Reactions were performed in 25- μL volumes, using 1 μL undiluted DNA sample, 10 pmol of each primer, a final $[\text{Mg}^{2+}]$ of 4.0 mM, a final dNTP concentration of 0.4 mM and otherwise standard conditions. The following PCR program was run on either a Gene Cyclor (BIO-RAD) or a PTC-200 (MJ Research):

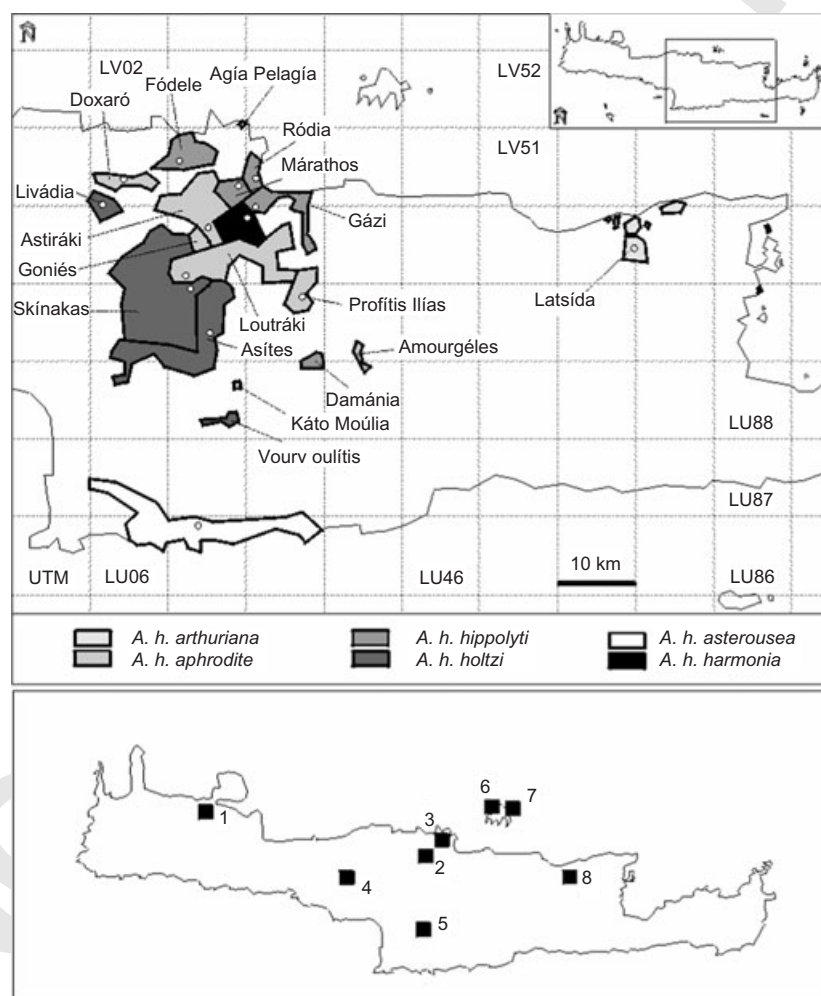


Figure 1. A, distribution of *A. hippolyti* in Crete; in the larger ranges, sample sites have been indicated as open circles. Names refer to intrasubspecific categories (see text), and the codes refer to the UTM grid. B, Collection localities for Cretan outgroup species; numbers refer to the following taxa: 1. *A. candida*; 2. *A. spratti*; 3. *A. ulrikae*; 4. *A. cretensis*; 5. *A. corrugata*; 6. *A. teres*; 7. *A. torticollis*; 8. *A. wiesei*. The non-Cretan outgroup species *A. arcadica* (Peloponnese), *Isabellaria saxicola* and *A. caerulea* (both Attica), *A. olivieri* (Karpathos) and *Medora italiana* (Italy) have not been indicated.

4 min at 94 °C, then 30 cycles of 60 s at 94 °C, 60 s at 55 °C and 90 s at 72 °C. The program was terminated with an extension step of 5 min at 72 °C.

PCR products were excised from agarose gels and purified using the freeze-squeeze technique (Tautz & Renz, 1983). Subsequently, they were ligated into pCR 2.1 (Invitrogen) or the pGEM-T (Promega) vectors, and used to transform *E. coli*. The presence of the correct insert was checked by PCR. Plasmids were isolated on QIAPREP spin columns (QIAGEN) and sequenced on an ABI PRISM 373 automated DNA sequencer, using universal primers located on the vector. Inserts were sequenced in one direction, or, in the case of ambiguities, in both directions. For each *A. hippolyti* sample, two sequences were determined, derived from different individuals, with the exception of the *A. h. arthuriana* sample (6 sequences from 6 individuals), *A. h. hippolyti* f. 'Márathos' (1 sequence), *A. h. aphrodite* f. 'Ámourgéles' (1 sequence), *A. h. aphrodite* f. 'Astiráki' (1 sequence), *A. h. aphrodite* f. 'Loutráki' (3 sequences from 3 individuals), *A. h. holtzi* f. 'Asítes' (4 sequences from 4 individuals), and *A. h. holtzi* f. 'Livádia' (4 sequences from 3 individuals; see Results). Non-*hippolyti* species were represented by one sequence each.

ALIGNMENT AND PHYLOGENETIC ANALYSIS

Electropherograms were checked and, where obvious reading errors had been made, corrected. All 58 sequences were then initially aligned with the Clustal (Higgins & Sharp, 1988) algorithm of Sequence Navigator 1.0 (ABI), using default settings, and subsequently corrected by hand. The alignment was then converted into a data matrix for phylogenetic analysis by removing the conserved 18S and 5.8S regions, and also highly variable regions, which could not be aligned unambiguously. In addition, unalignable sequence regions from the most divergent outgroup species (*I. saxicola*) were replaced by missing data. This resulted in a matrix of 58 sequences and 388 characters.

Initial inspection of the data matrix suggested that even in the ingroup a considerable degree of homoplasy was present, and that genetic distances were large, (up to 5% within the ingroup) making it unlikely that ancestral sequences persist in present-day populations. Therefore, a tree-like phylogenetic history was assumed. Because of the high number of taxa (which would mean prohibitively long computation times using maximum likelihood methods), and the many insertions and deletions in the alignment (which would result in the loss of potential phylogenetically important information using distance or maximum-likelihood methods), maximum parsimony was chosen as the method for reconstructing this phy-

logeny. A maximum parsimony analysis was done using PAUP*4.0b10 (Swofford, 2002), with uninformative characters removed. Gaps were included as a fifth character state. However, rather than treating each insertion/deletion position individually, a weighting scheme was used according to the respective insertion/deletion length, using the formula $w = a + b \ln k$, where w is the weight of a gap with length k , and a and b are constants. This logarithmic weighting procedure is based on Gu & Li (1995) and makes use of the empirical frequency distribution of insertions and deletions of different lengths in animal pseudogenes. For constants a and b , we used 3.49 and 1.81, respectively. The former is the average of the values given by Gu & Li (1995) for insertions (2.93) and deletions (3.85), whereas the latter is the average of the values that we found in this study. One hundred bootstrap replicates were done on this dataset, using ten replicates of a heuristic search with random addition sequence at each bootstrap replicate. Branches were swapped under the tree bisection-reconnection (TBR) algorithm, the number of trees saved in each replicate was limited to 10 000, and the characters were sampled with equal probability but with weights applied. A combination of the clades that had the strongest (= 70%) support in the bootstrap analysis was then used as an enforced constraint in a second search, in which sequence regions that were unalignable for the full dataset, but alignable for the ingroup, were reintroduced. This search was done by performing a heuristic search with 100 random-addition replications, branch swapping by TBR, and with the maximum number of trees set to 20 000. To test robustness of the result, 100 bootstrap replicates were carried out, using the same heuristic search settings. Characters were sampled with equal probability, with weights applied.

RESULTS

Sequences for *A. hippolyti* ranged in length from 461 bp for *A. h. asterousea* to 513 bp for *A. h. f. 'Agía Pelagía'*. On the agarose gels, intrapopulational length variation was observed in some samples, and in one PCR product of *A. h. holtzi* f. 'Livádia', intraindividual length variation was observed. Since concerted evolution is a slow and inaccurate process, such polymorphisms are not unexpected in *ITS* studies (Buckler, Ippolito & Holtsford, 1997). In such cases, all length variants were sequenced. All sequences have been submitted to GenBank (accession numbers AF136011–AF136069). The full alignment and the PAUP file can be viewed in TreeBASE (<http://www.treebase.org/treebase/index.html>, accession SN161). The alignment, which had a total length of 738 positions, contained 496 positions where homol-

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ogy was unambiguous. After exclusion of the 18S and 5.8S regions, 388 positions remained.

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The first stage of the maximum parsimony analysis, based on 58 informative characters, produced 482 distinct trees 16 958 in length. A bootstrap analysis for these trees gave seven clades with percentages = 70%. These seven clades were: (1) all sequences of *A. h. asterousea*; (2) all sequences of *A. h. hippolyti*; (3) *A. h. holtzi* f. 'Livádia' A, C, and D, *A. h. holtzi* f. 'Ski-nakás', *A. h. holtzi* f. 'Vourvoulitis' B, and *A. h. holtzi* f. 'Asítes' C and D; (4) *A. h. f. 'Agía Pelagía'*; (5) *A. h. arthuriana*; (6) *A. h. arthuriana* f. 'Látsida' D and E; (7) *A. h. harmonia*. These clades were combined into a constraint tree, which was then enforced in the second stage of the search with 61 positions of the alignment re-included (see Methods). This second stage, based on 78 informative characters, resulted in 20 000 trees 22 277 in length, with a rescaled consistency index of 0.40. A randomly selected tree is presented in Figure 2, and the majority-rule consensus in Figure 3. The trees showed that *A. hippolyti* is not monophyletic: it fell into two groups, one composed of *A. h. arthuriana*, and the other composed of the remaining five subspecies. Four of the six subspecies (*A. h. hippolyti*, *A. h. harmonia*, *A. h. asterousea*, and *A. h. arthuriana*) were monophyletic with high bootstrap support of 70–99%. The two subspecies with the largest distribution areas, however (*A. h. holtzi* and *A. h. aphrodite*), formed polyphyletic assemblages. The unclassified *A. h. f. 'Agía Pelagía'* appeared separately, although closely related to *A. h. harmonia*.

DISCUSSION

Our phylogenetic analysis strongly suggests that *A. hippolyti* is a polyphyletic taxon: the east Cretan subspecies *A. h. arthuriana* is not related to the other five subspecies, but appears more closely related to other *Albinaria* species from eastern Crete, such as *A. wiesei* and *A. teres*. Consequently, Nordsieck's (1999) decision, on the basis of shell characters, to treat it as a separate species, *A. arthuriana* (Boettger), is supported. The remainder of this discussion, therefore, is restricted to the five central Cretan subspecies *A. h. hippolyti*, *A. h. harmonia*, *A. h. aphrodite*, *A. h. holtzi* and *A. h. asterousea*.

The *ITS-1* tree suggests that *A. h. holtzi* and *A. h. aphrodite* (which are not well separated in the tree) represent the ancestral, paraphyletic stock of the species, with the remaining three, clearly monophyletic, subspecies (*A. h. hippolyti*, *A. h. harmonia* and *A. h. asterousea*) branching off from this stock independently. The fact that *A. h. holtzi* and *A. h. aphrodite* are also the subspecies that occupy the highest altitudes on the Idi-massif suggests

that *A. hippolyti* originated there. *A. h. aphrodite* and *A. h. holtzi* were molecularly highly variable, although the molecular variation showed little geographical structuring: sequences from the same locality ended up in very different parts of the tree, sometimes supported by high bootstrap values (e.g. *A. h. holtzi* f. 'Vourvoulitis' and *A. h. aphrodite* f. 'Loutraki'), whereas sequences from very different localities (e.g. *A. h. holtzi* f. 'Vourvoulitis' A and *A. h. aphrodite* f. 'Amourgeles') were placed very close to each other. This would suggest either long-distance gene flow, or retained ancestral polymorphisms, or a combination of both. However, since no model exists for the population genetic behaviour of ribosomal DNA (a multicopy region with concerted evolution), further quantification is not possible.

Previous attempts to explain the distributional and evolutionary history of *A. hippolyti* have relied mostly on the history of recurrent cycles of transgressions and regressions in this part of Crete over the past 15 million years (e.g. Schilthuizen, 1994b; Welter-Schultes, 2000a,b). The area was mostly uninterrupted continental land during the Langhian and Serravallian (16.8–11.8 Mya). A transgression during the Tortonian fragmented it into at least two islands, roughly corresponding with the present-day mountains of Idi and Asteroúsia, and probably a third at the site of a cluster of hills south of Iráklion (Gioúchtas, Monodéndri, Oxi Kefáli and Megáli Korifi; Meulenkamp, 1985; Welter-Schultes, 2000b), hereafter referred to as the Gioúchtas-Palaeoisland. A brief regression during the Messinian salinity crisis (6.0–5.5 Mya) probably reconnected the palaeoislands, but the saline conditions may have prevented dispersal of land snails. The palaeoislands were thus effectively isolated until, during the Pliocene (5.4–1.9 Mya), the region experienced uplifting, the palaeoislands were connected and Crete gained its present coastlines. [The geological history presented here (Fig. 4), is treated more fully in Welter-Schultes, 2000b; comprehensive summaries can also be found in Jacobshagen, 1986; Higgins & Higgins, 1996.]

An evolutionary scenario for *A. hippolyti* based on distribution and geology alone (Welter-Schultes, 2000a), and assuming no dispersal over sea, would suggest a pre-Tortonian origin for the subspecies *A. h. hippolyti*, *A. h. holtzi*, and *A. h. aphrodite*, given that each of these occurs disjunctly on two former palaeoislands. A similar age would have to be postulated for *A. h. asterousea*, which is the sole occupant of the third palaeoisland. The age of *A. h. harmonia* cannot be reconstructed on the basis of geological data. Populations living in areas that became exposed only as recently as the Pleistocene, on the other hand, would have to be relatively young in origin (e.g. *A. h. aphrodite* f. 'Profítis Ilías').

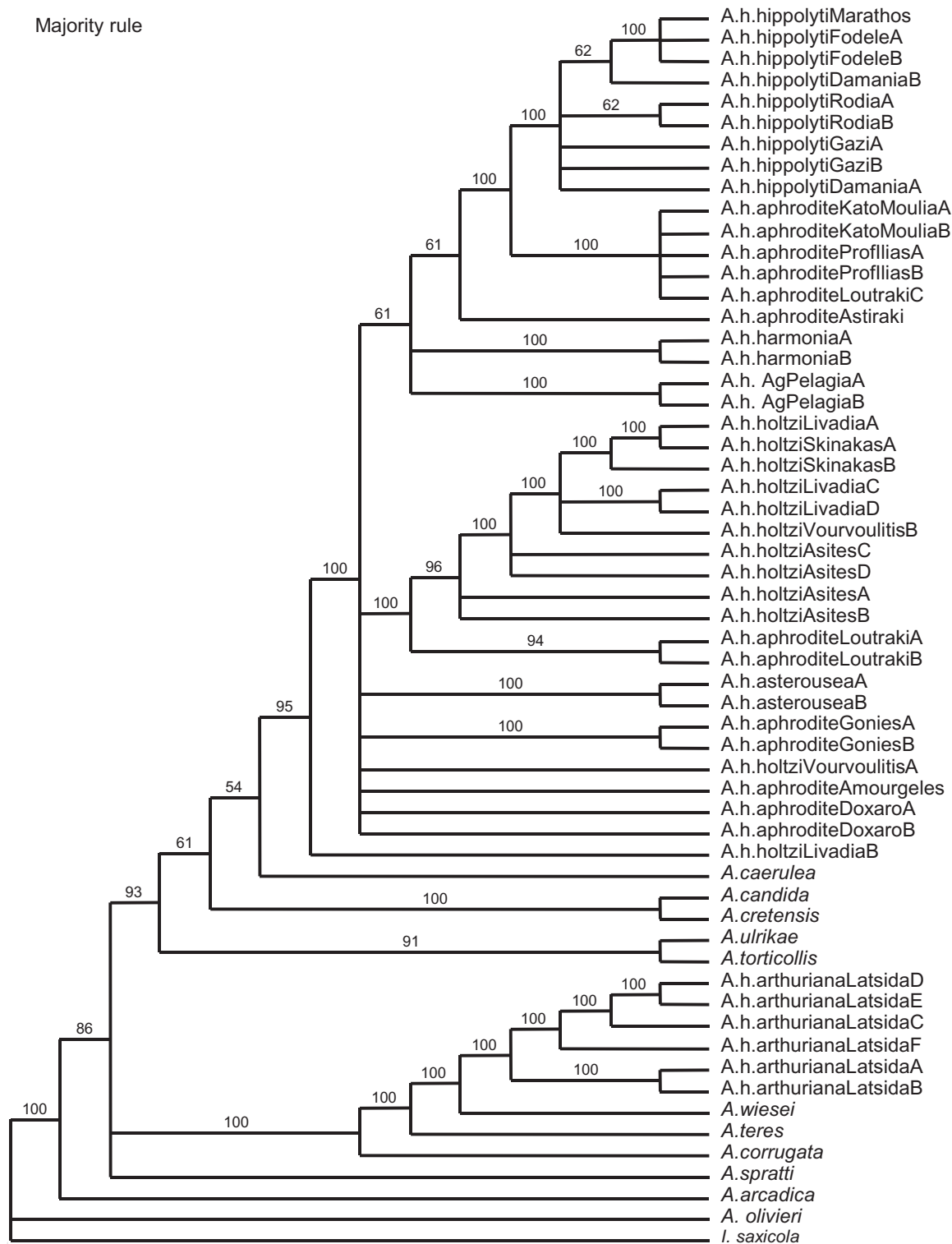


Figure 3. Majority rule consensus tree over 20 000 equally parsimonious trees 22277 in length. The numbers on the branches are consensus indices.

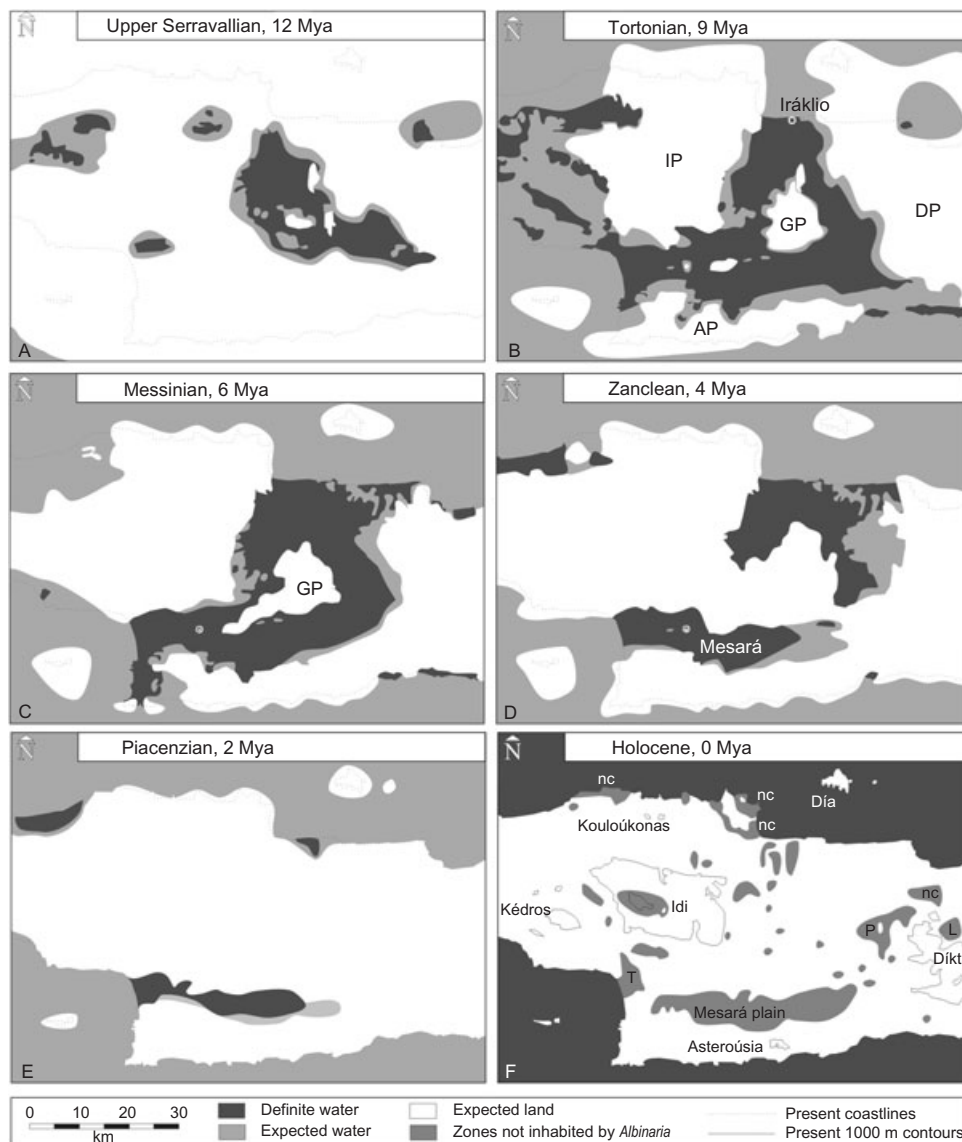


Figure 4. Presumed land/water distributions in central Crete during six periods over the past 12 Myr. In 4B, the location of the present-day capital, Iráklion, is indicated. Other details of palaeogeography include the four palaeoislands (DP, Díkti-Palaeoisland; IP, Idi-Palaeoisland; GP, Gioúchtas-Palaeoisland; AP, Asterouússia-Palaeoisland), and the Mesará-bay (4D). In 4F, non-limestone areas (uninhabited by *Albinaria*) have been indicated with ‘nc’.

The *ITS-1* data, however, do not support such a scenario. Although the mutation rate in *ITS-1* is unknown for these snails, the spacer is generally considered to be a fast-evolving region; for example, a rate of divergence of 2.4% per million years has been calculated for *Drosophila* (Schlötterer *et al.*, 1994). Nevertheless, several populations on the Gioúchtas-Palaeoisland, which should have been isolated for 12 Myr (e.g. *A. h. hippolyti* f. ‘Damánia’), branched off very high in the tree, and differed from their consub-specific counterparts on the Idi-Palaeoisland by less than 1% (Kimura’s (1980) 2-parameter distance). In

fact, *A. h. hippolyti* f. ‘Damánia’ had *ITS-1* sequences virtually identical to those of the populations of *A. h. hippolyti* f. ‘Gázi’, which, in contrast, occupies a region that belonged to the Idi-Palaeoisland. This suggests that the populations east of the Idi-Palaeoisland are recent in origin, having dispersed there over the newly dried-up plains in the Pleistocene, and subsequently suffered a range reduction (possibly due to climatic change or competition with the widely distributed *A. corrugata* and *A. praeclara*). In some cases, such long-distance dispersal may even have happened in historical times. Welter-Schultes (1998b) pointed

out that the shells of the isolated *A. h. aphrodite* population of Káto Mouília are identical to those of an ancient quarry at Profítis Iliás, 13–14 km to the north. He suggests that this isolated population was founded by human movement of limestone blocks from the quarry. The *ITS-1* data support this hypothesis, as sequences from both places are virtually identical.

The *ITS-1* tree does, however, support a relatively ancient origin for *A. h. asterousea*, which branched off basally to most *A. h. holtzi* sequences. The Kimura's 2-parameter distance between these two subspecies was *c.* 3%, which would suggest a divergence time of roughly 1 Mya. This is supported by a 16S (mtDNA) distance of *c.* 6% (Douris *et al.*, 1998; D. Thomaz, pers. comm.). Nevertheless, this still falls short of the divergence time (11.8 Myr) expected on the basis of geology.

In summary, the phylogeographical patterns in *A. h. hippolyti* as revealed by *ITS-1* sequences are not consistent with a scenario that relies on major vicariance events in the Miocene and Pliocene. It appears that most of the evolutionary history of the species has taken place in the Pleistocene, when no large transgressions and regressions occurred in this part of Crete. It is as yet not clear what bearing this has on the possible modes of speciation. The fact that geological vicariance has apparently not played a significant role does not rule out allopatric speciation. Land snails with limited dispersal abilities may become isolated by less severe geological or environmental barriers.

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