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ORIGINAL ARTICLE

The provenance of the Greek land snail species *Isabellaria pharsalica*: molecular evidence of recent passive long-distance dispersal

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ABSTRACT

Aim The slowly dispersing Greek land snail species *Isabellaria* (*Carinigera*) *pharsalica* (Nordsieck, 1974) can be readily distinguished from related species of the subgenus *Carinigera* on biogeographical grounds, i.e. by a separation of nearly 200 km. Nevertheless, *I. pharsalica* shows overlap in shell morphology with *Isabellaria* (*Carinigera*) *buresi* (A. J. Wagner, 1927). The aim of this paper is to study the interrelationships and phylogeography of *I. pharsalica* and selected *I. buresi* subspecies, in order to reconcile these apparently contradictory observations.

Location Northern Greece, Macedonia, Serbia and southern Bulgaria.

Methods Mitochondrial COI genealogical interrelationships were studied for specimens of *I. pharsalica*, selected *I. buresi* subspecies and other related *Isabellaria* species.

Results COI sequences of *I. pharsalica* constitute a clade nested among sequences of *I. buresi* from north-eastern Greece and adjacent Bulgaria. The genetic divergence among the *I. pharsalica* sequences is remarkably low compared to the divergence among the *I. buresi* sequences.

Main conclusions *I. pharsalica* descended from specimens of *I. buresi* that dispersed into Thessaly, and it should therefore be classified as a subspecies within *I. buresi*. This find is in line with the conchological similarities between *I. buresi pharsalica* and other *I. buresi* subspecies. Given the low vagility of the snails in question and the *c.* 200-km separation by land and sea between the ranges of *I. buresi pharsalica* and the combined range of the other *I. buresi* subspecies, passive dispersal of the *I. pharsalica* ancestors is most likely. Since the inferred area of origin of these hypothetical ancestors of *I. buresi pharsalica* nearly coincides with an ancient marble quarry, human-aided dispersal during marble transport offers a possible explanation for *I. buresi pharsalica*'s erratic distribution.

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Keywords
***Carinigera*, Clausiliidae, cytochrome *c* oxidase I, Greece, human-aided dispersal, land snails, marble transport, mitochondrial DNA, phylogeography.**

INTRODUCTION

In recent years, terrestrial snails have figured in a wide range of biogeographical studies. Their slow mode of dispersal makes them extremely suited to use as a model in vicariance studies (Hausdorf & Hennig, 2004) and in studies on habitat fragmentation (Schweiger *et al.*, 2004), and as indicators in the assessment of spatial patterns of biodiversity (Moritz *et al.*, 2001). According to Solem (1984, p. 4), 'successful long distance dispersal has been an extremely rare event' for snails. Nevertheless, not all land snails are equally passive, and, depending on the group studied, yearly dispersal varies from < 1 to 500 m (for an overview, see Giokas & Mylonas, 2004). Dispersal depends on characteristics such as snail size and homing tendency, as well as on ecological conditions (for an overview, see Baur & Baur, 1995).

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The obligately limestone-dwelling members of the family Clausiliidae belong to the proverbial snails among the snails. Observations on snails placed in the genera *Albinaria* (see Schilthuizen & Lombaerts, 1994; Giokas & Mylonas, 2004), *Cristataria* (see Heller & Dolev, 1994) and *Chondrina* (see Baur & Baur, 1995) yielded yearly dispersal distances that hardly ever exceeded 10 m. At this scale, dispersal is stepping-stone-wise and dictated by the proximity of suitable, i.e. limestone, outcrops (Heller & Dolev, 1994; Schilthuizen & Lombaerts, 1994; Baur & Baur, 1995; Giokas & Mylonas, 2004), on which the snails depend for food (lichens, algae and bryophytes) and shelter (Heller & Dolev, 1994; Giokas & Mylonas, 2002). *Albinaria* and *Cristataria* snails may spend months or longer on a single limestone boulder, not crossing even small distances of unfavourable habitat (Bar, 1977; Schilthuizen & Lombaerts, 1994). Geographical isolation between allopatric 'units' (Nordsieck, 1997), in particular in the north-east Mediterranean region, is thought to have triggered an extreme morphological diversification, reaching its peak within the genus *Albinaria* (see Boettger, 1883; Nordsieck, 1997), which has more than 100 described species, resulting from a largely non-adaptive radiation (Gittenberger, 1991).

Molecular phylogenetic analyses (Uit de Weerd *et al.*, 2004) indicate that geographical isolation has indeed been a dominant factor in the evolution of *Isabellaria*, a monophyletic genus (Gittenberger & Uit de Weerd, 2005) of predominantly Greek species that is closely related to *Albinaria* and *Cristataria* (Uit de Weerd & Gittenberger, 2004; Uit de Weerd *et al.*, 2004). These analyses identified three nearly completely vicariant subclades in *Isabellaria*, which were given the subgeneric names *Carinigera*, *Isabellaria* and *Vallatia* (Gittenberger & Uit de Weerd, 2005).

A conspicuous exception to this geographical pattern is the distribution of *Isabellaria* (*Carinigera*) *pharsalica*. This species is part of the subgenus *Carinigera*, which is confined to northern Greece and the adjacent Balkan countries. Occurring near the ancient town of Pharsalos in southern Thessaly, *I.* (*Carinigera*) *pharsalica* is one of the southernmost species within the *Carinigera* clade, and the only one that is more or less surrounded by species belonging to another subgenus. The molecular data nevertheless unite *I.* (*Carinigera*) *pharsalica* with the northernmost species in the subgenus *Carinigera*. These northern species are *Isabellaria* (*Carinigera*) *buresi* (A. J. Wagner, 1927) from north-east Greece and adjacent Bulgaria, *Isabellaria* (*Carinigera*) *eximia* (Moellendorf, 1873) from Serbia, and *Isabellaria* (*Carinigera*) *drenovoensis* (Brandt, 1961), *Isabellaria* (*Carinigera*) *octava* (Brandt, 1962) and *Isabellaria* (*Carinigera*) *septima* (Brandt, 1962), from the Republic of Macedonia (Fig. 1). Of these species, *I.* (*Carinigera*) *buresi* is most closely related to *I.* (*Carinigera*) *pharsalica*, despite the fact that the two species are separated by nearly 200 km of land and sea (Fig. 1). Limestone areas in the intermediate area are widely scattered (see Bornovas & Rondogianni-Tsiambaou, 1983), thus limiting dispersal possibilities for these slowly dispersing limestone-dwelling snails.

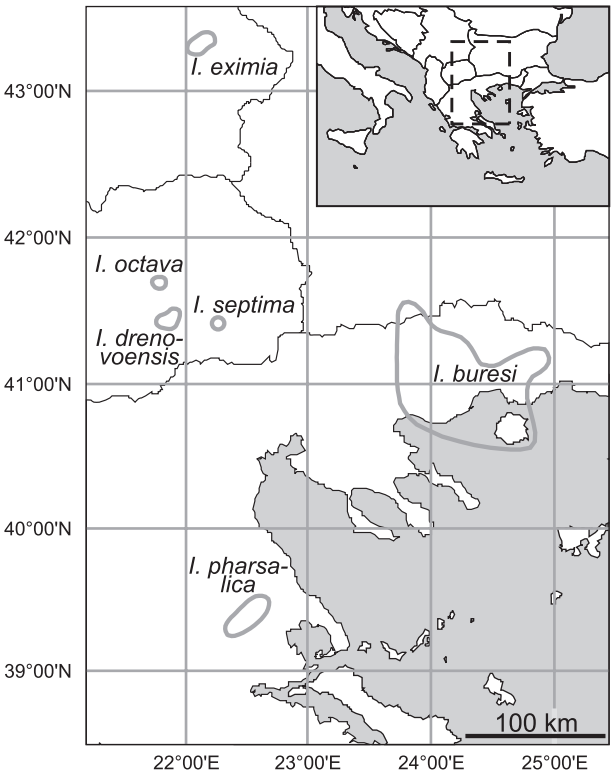

Figure 1 Schematic distribution of species from the north-eastern clade, based on the literature and data in the National Museum of Natural History in Leiden.

Critical for the phylogeography of *I. pharsalica* is its systematic position with respect to the subspecies of the highly diverse *I. buresi*. While *I. pharsalica* does not vary much conchologically and can easily be recognized (Nordsieck, 1974), *I. buresi* is very heterogeneous and lacks a morphological definition. This conchological diversity is reflected in the fact that no less than nine subspecies are recognized within *I. buresi* (Nordsieck, 1977; Gittenberger & Uit de Weerd, 2004): *I. buresi buresi* (A. J. Wagner, 1927), *I. buresi cavallaensis* (Brandt, 1962), *I. buresi conciliatrix* (Fuchs & Käufel, 1936), *I. buresi dramaensis* (Nordsieck, 1977), *I. buresi damjanovi* (Likharev, 1972), *I. buresi insularis* (Urba nski, 1960), *I. buresi militis* (Nordsieck, 1977), *I. buresi nordsiecki* (Gittenberger & Uit de Weerd, 2004) and *I. buresi polimilitis* (Gittenberger & Uit de Weerd, 2004). Several of these taxa were originally described as distinct species (Brandt, 1962, pp. 135–138; Nordsieck, 1972, pp. 9–10). Nordsieck (1974) considered these 'species' to be closely – but not equally closely – related to *I. pharsalica*. More extensive sampling in north-east Greece revealed that intermediate forms indicative of hybridization connect most of these former so-called species. Therefore, they were dealt with as subspecies, while the geographically isolated *I. pharsalica* retained its species status (Nordsieck, 1977).

In this study, we have investigated (1) the relationships between *I. pharsalica* and the northern (sub)species within the subgenus *Carinigera*, in particular the *I. buresi* subspecies, and

(2) the provenance of *I. pharsalica*, taking into account the geographical distribution of the taxa identified as its closest relatives.

MATERIALS AND METHODS

In addition to *I. pharsalica*, the five *Isabellaria* species most closely related to this species (*I. buresi*, *I. eximia*, *I. drenovoensis*, *I. octava*, and *I. septima*) were represented in this study. Seven more distantly related species (*I. albicosta*, *I. dextrorsa*, *I. lophauchena*, *I. praecipua*, *I. schuetti*, *I. superba*, and *I. thessalonica*), representing the other main clades (Uit de Weerd *et al.*, 2004) within the subgenus *Carinigera*, served as an outgroup. In addition, we obtained tissue from six out of nine subspecies of *I. buresi*, three of which were sampled from several localities, i.e. from different populations. When possible, we sampled multiple individuals per population, in order to be able to recognize ancestral polymorphisms or introgression events among populations. Species, subspecies and collection sites are listed in Table 1, and the distribution of sampling sites is shown in Fig. 2. *I. pharsalica* itself is represented by two populations from either end of its range. As an independent marker we used mitochondrial cytochrome *c* oxidase subunit I (COI) nucleotide sequences. Being a mitochondrially encoded gene, COI can be used for phylogeny reconstruction and phylogeography at both the intraspecific (Avise *et al.*, 1987) and the interspecific level.

Apart from the outgroup sequences and sequences of six of the ingroup species (see Table 1) that had been previously obtained, all COI sequences were newly determined. Tissue of the specimens sampled either was kept frozen at −80 °C after collection or had been stored in 70% ethanol (see Table 1). DNA from fresh tissue was extracted using the protocol described by Schilthuizen *et al.* (1995), whereas a modified protocol was used in order to reduce loss of DNA from older ethanol-stored tissue (Uit de Weerd & Gittenberger, 2004). Extracted DNA was amplified in a PCR reaction consisting of 35 cycles with an annealing temperature of 47 °C using the forward primer (ACT CAA CGA ATC ATA AAG ATA TTG G) and the reverse primer (TAT ACT TCA GGA TGA CCA AAA AAT CA) (Gittenberger *et al.*, 2004). PCR product was gel-purified using spin columns (Qiaquick® Gel Extraction Kit; Qiagen, Hilden, Germany) and dye-terminator cycle-sequenced in both directions (Big Dye™; PE Biosystems, Foster City, California, USA). The cycle-sequenced samples were subsequently run on an ABI 377 automated sequencer (PE Biosystems®). Sequences thus obtained were assembled and edited in Sequencher (Gene Codes Corp., Ann Arbor, Michigan, USA). All sequences were aligned in MegAlign 4.03© (DNASTAR, Inc., Madison, Wisconsin, USA), compared with the complete COI nucleotide sequence of *Albinaria caerulea* (Hatzoglou *et al.*, 1995; GenBank acc. no. X83390), and checked for stop codons and gaps.

Tests for data quality and phylogenetic analyses were performed in PAUP* 4.0b10 (Swofford, 2002). We checked for deviations in base frequencies for each codon position separately using a χ^2-test. The presence of a phylogenetic signal was tested using a 1000-replicate permutation test with 10 random addition replicates, TBR, and steepest descent, within each replicate (Archie, 1989; Faith & Cranston, 1991). In addition, a g_1-value was calculated from the distribution of 10,000 random trees (Hillis & Huelsenbeck, 1992). Phylogenetic analyses were based on both maximum parsimony (MP) and maximum likelihood (ML) approaches. Maximum parsimony heuristic searches were performed using 1000 random addition replicates, TBR and steepest descent. Maximum parsimony bootstrap analyses consisted of 10,000 bootstrap replicates (1 addition per bootstrap replicate, TBR and steepest descent). The ML search, with 10 random addition replicates (TBR, without steepest descent), was carried out using the GTR + I + Γ model, selected by MrModeltest 1.1b (Nylander, 2002) after running the standard modelblock in PAUP* 4.0b10. Apart from the base frequencies, for which empirical values were used, all other parameters were estimated during the ML analysis. Identical parameter settings were subsequently used in a ML bootstrap analysis (GTR + I + Γ) consisting of 100 bootstrap replicates (1 random addition per bootstrap replicate, TBR, without steepest descent). For computational efficiency, all populations identified as monophyletic in the ML analysis were constrained to be monophyletic in the ML bootstrap analysis, as was the ingroup as a whole. In order to speed up the analysis further, we restricted the maximum number of rearrangements per bootstrap replicate to 250.

RESULTS

We were able to determine the bases of 633–657 positions of COI for the samples included in this study. The data set contained neither gaps nor stop codons. For none of the codon positions were significant deviations in base frequencies detected ($P > 0.95$). Both the permutation test ($P = 0.001$) and the g1 value ($P < 0.01$) indicated a highly significant phylogenetic signal within the data set. The total data set contained 243 variable positions, 218 of which were potentially parsimony-informative. Within the ingroup, 228 positions were variable, the vast majority (97.8%) containing synonymous variation. Only five positions harboured non-synonymous variation. The number of potentially parsimony-informative positions within the ingroup was 203.

The six most parsimonious trees (score: 977; CI: 0.432; rescaled CI: 0.293, see Fig. 3a for strict consensus) and the ML tree (GTR + I + Γ; −lnL 4900.31308; proportion of invariable sites: 0.58649587 and α: 0.967911, see Fig. 3b) agreed with respect to the position of the *I. pharsalica* sequences as a clade nested among *I. buresi* sequences. In fact, the MP strict consensus and the ML tree are identical with respect to the phylogenetic relationships among the *I. pharsalica* and the most closely related *I. buresi* sequences in clade A. Four successive branches with *I. buresi* subspecies separate *I. pharsalica* from the other *Isabellaria* taxa sampled, according to the MP trees. In addition to these, an additional most basal

Table 1 Samples included in the analyses and their locality information

Species, with sample codes	GenBank acc. no.	Loc.	Coordinates	UTM code	Initial state
Ingroup					
Isabellaria (*Carinigera*) *buresi cavallaensis* 191	AY438377	1	40°56′ N 24°24′ E	KF8234	Frozen
Isabellaria (*Carinigera*) *buresi conciliatrix* 343	AY438378	2	41°04′ N 24°17′ E	KF7149	Frozen
Isabellaria (*Carinigera*) *buresi conciliatrix* 344	AY438379	2	41°04′ N 24°17′ E	KF7149	Frozen
Isabellaria (*Carinigera*) *buresi conciliatrix* 1126	AY438381	2	41°04′ N 24°17′ E	KF7149	Frozen
Isabellaria (*Carinigera*) *buresi conciliatrix* 1115	AY438380	3	41°02′ N 24°20′ E	KF74	70% ethanol
Isabellaria (*Carinigera*) *buresi damjanovi* 1172	AY438382	4	41°31′ N 23°48′ E	GM2706	70% ethanol
Isabellaria (*Carinigera*) *buresi polimilitis* 1138	AY438390	5	41°02′ N 24°38′ E	LF0145	70% ethanol
Isabellaria (*Carinigera*) *buresi polimilitis* 1140	AY438391	5	41°02′ N 24°38′ E	LF0145	70% ethanol
Isabellaria (*Carinigera*) *buresi dramaensis* 188	AY438383	6	41°13′ N 23°54′ E	GL4266	Frozen
Isabellaria (*Carinigera*) *buresi dramaensis* 194	AY438385	6	41°13′ N 23°54′ E	GL4266	Frozen
Isabellaria (*Carinigera*) *buresi dramaensis* 192	AY438384	7	41°13′ N 23°59′ E	GL4967	Frozen
Isabellaria (*Carinigera*) *buresi dramaensis* 978	AY438386	7	41°13′ N 23°59′ E	GL4967	Frozen
Isabellaria (*Carinigera*) *buresi nordsiecki* 337*	AY425552	8	40°54′ N 24°14′ E	KF6731	Frozen
Isabellaria (*Carinigera*) *buresi nordsiecki* 934	AY438389	8	40°54′ N 24°14′ E	KF6731	Frozen
Isabellaria (*Carinigera*) *buresi nordsiecki* 330	AY438387	9	40°42′ N 24°05′ E	KF5312	Frozen
Isabellaria (*Carinigera*) *buresi nordsiecki* 345	AY438388	9	40°42′ N 24°05′ E	KF5312	Frozen
Isabellaria (*Carinigera*) *drenovoensis* 1134*	AY425553	10	41°25′ N 21°54′ E	EL78	70% ethanol
Isabellaria (*Carinigera*) *drenovoensis* 1163	AY438392	10	41°25′ N 21°54′ E	EL78	70% ethanol
Isabellaria (*Carinigera*) *eximia* 1104*	AY425554	11	43°19′ N 22°08′ E	EN99	70% ethanol
Isabellaria (*Carinigera*) *eximia* 1114	AY438393	11	43°19′ N 22°08′ E	EN99	70% ethanol
Isabellaria (*Carinigera*) *octava* 1103	AY438394	12	41°42′ N 21°48′ E	EM61	70% ethanol
Isabellaria (*Carinigera*) *octava* 1113*	AY425557	12	41°42′ N 21°48′ E	EM61	70% ethanol
Isabellaria (*Carinigera*) *pharsalica* 195*	AY425558	13	39°29′ N 22°37′ E	FJ3871	Frozen
Isabellaria (*Carinigera*) *pharsalica* 933	AY438395	13	39°29′ N 22°37′ E	FJ3871	Frozen
Isabellaria (*Carinigera*) *pharsalica* 1125	AY438396	13	39°29′ N 22°37′ E	FJ3871	Frozen
Isabellaria (*Carinigera*) *pharsalica* 1136	AY438397	14	39°20′ N 22°25′ E	FJ25	70% ethanol
Isabellaria (*Carinigera*) *pharsalica* 1141	AY438398	14	39°20′ N 22°25′ E	FJ25	70% ethanol
Isabellaria (*Carinigera*) *septima* 1137*	AY425560	15	41°24′ N 22°15′ E	FL08	70% ethanol
Isabellaria (*Carinigera*) *septima* 1142	AY438399	15	41°24′ N 22°15′ E	FL08	70% ethanol
Outgroup					
Isabellaria (*Carinigera*) *albicosta* 307*	AY425583	21	40°05′ N 22°25′ E	FK2038	Frozen
Isabellaria (*Carinigera*) *dextrorsa* 760*	AY425585	22	40°58′ N 21°55′ E	EL7635	Frozen
Isabellaria (*Carinigera*) *lophauchena* 268*	AY425572	18	40°32′ N 22°05′ E	EK9188	Frozen
Isabellaria (*Carinigera*) *praecipua praec.* 844*	AY425574	19	40°29′ N 22°12′ E	FK08	Frozen
Isabellaria (*Carinigera*) *schuetti* 1235*	AY425559	16	41°07′ N 23°38′ E	GL2554	Frozen
Isabellaria (*Carinigera*) *superba* 193*	AY425561	17	41°13′ N 23°54′ E	GL4266	Frozen
Isabellaria (*Car.*) *thessalonica crassilabra* 266*	AY425579	20	40°23′ N 23°10′ E	FK8371	Frozen

*From Uit de Weerd *et al.* (2004).

I. buresi branch is identified in the ML analysis. In particular, a sister-group relationship between *I. pharsalica* and the *I. buresi conciliatrix* population 2 is highly supported (MP bootstrap 94%, ML bootstrap 91%). In contrast, there is low support for the monophyly of *I. buresi* as a whole plus *I. pharsalica*. The monophyly of this group as found in the ML analysis is supported by only two of the six most parsimonious trees; the four other trees group the *I. buresi* populations 3, 4 and 9 with *I. octava*. The ML bootstrap supports the monophyly of *I. buresi* plus *I. pharsalica* at a level of only 6%. All populations included are found to be monophyletic in the MP analysis, and all except one, namely number 7, in the ML analysis. The sequences of population 7 are paraphyletic with respect to population 6. However, the short terminal branches leading to these two sequences from locality 7 and the short internal branch separating them suggest that their paraphyly may have been spuriously inferred, a conclusion supported by the observation that 53% of the ML bootstrap replicates retrieved population 7 as monophyletic.

In order to compare amounts of sequence divergence within *I. pharsalica* and related *I. buresi* populations, we first checked for rate constancy in clade A. Both MP and ML analyses provide fairly high bootstrap values for this clade, and identify a highly similar and thus robust topology, with the only differences residing within the *I. buresi conciliatrix* population (no. 2). Using a likelihood ratio test, we compared the likelihood values of clade A under GTR + I + Γ, with and without the assumption of rate constancy (Felsenstein, 1988).

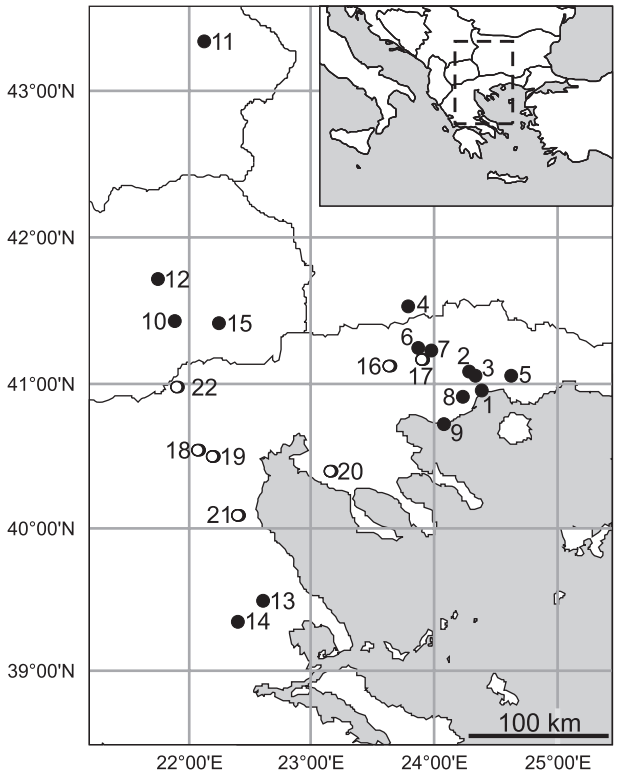

Figure 2 Distribution of sampling sites. Black dots refer to the localities of the ingroup samples; open circles, to those of outgroup samples.

Under the molecular clock constraint, the two *I. buresi nordsiecki* samples were used as a monophyletic outgroup to the remaining sequences within this clade A, in line with the inferred topology within this clade. The test did not refute rate constancy within this clade, for either the six MP topologies or the ML topology (d.f. = 11: $0.30 < P < 0.60$).

In view of the rate constancy within clade A, the genetic divergence within *I. pharsalica* is remarkably low compared with that within *I. buresi* from this clade. Taking into account all *I. pharsalica* sequences, seven sites are variable and no more than four nucleotides differ between any pair of sequences. Given this low number of sites that are variable between the *I. pharsalica* sequences and the clustering of these sequences in the tree, multiple hits are highly unlikely. Hence we estimated the amount of divergence by calculating uncorrected distances only. The sequences of the two individuals from the southernmost *I. pharsalica* population (no. 14) are identical, whereas intrapopulational divergence ranges from 0.31% to 0.62% in the three sequences from the northern population (no. 13). The divergence between the two *I. pharsalica* populations is 0.62%. This interpopulational divergence within *I. pharsalica* lies within the range of 0.15–0.77% observed within the *I. buresi* populations of clade A. The maximum uncorrected distance within *I. buresi* as a whole amounts to 14.84%, and is found between the samples from populations 1 and 3.

DISCUSSION AND CONCLUSIONS

Our analyses unambiguously nest the *I. pharsalica* sequences among the sequences of *I. buresi*, a position supported not only by high bootstrap values but also by the extremely small divergence among the *I. pharsalica* sequences as compared with those of *I. buresi*.

From gene tree to species tree

If the COI sequences of *I. pharsalica* as a clade are nested among those of the more northerly species *I. buresi*, our tree is discordant with geography (Fig. 1). The observed paraphyly of the *I. buresi* sequences can be explained in three ways: (1) as arising from ancestral polymorphisms, (2) as caused by introgression events, or (3) as a result of the paraphyly of the 'species' itself. We will first consider the first two explanations, in particular since they appear to apply to the mitochondrial genealogy of other land snail species (e.g. Douris *et al.*, 1998; Goodacre & Wade, 2001; van Moorsel *et al.*, 2001).

The nested phylogenetic position of *I. pharsalica* sequences with respect to those of *I. buresi* can hardly be attributed to retention of polymorphisms present in their common ancestor. Because the *I. pharsalica* mitochondrial lineage is grouped with *I. buresi* lineages in at least four successively nested clades (Fig. 3), this explanation would imply: (1) four mitochondrial lineages in their common ancestor, (2) retention of these lineages within *I. buresi*, and (3) loss of three lineages in *I. pharsalica*. Generally, lineage sorting takes place quite rapidly, with the expected fixation time, measured as the number of generations, of a mitochondrial lineage in a hermaphroditic population equalling the effective population size (Avise *et al.*, 1984). The monophyly of all populations sampled in this study, as well as that of *I. pharsalica*, suggests that lineage sorting has indeed been fairly effective at erasing mitochondrial lineages, even within *I. buresi*. Such a completely population-wise clustering of mitochondrial variants is highly exceptional in land snails (cf. Ross, 1999; Davison & Clarke, 2000; Watanabe & Chiba, 2001; Goodacre, 2002; Holland & Hadfield, 2002; Hugall *et al.*, 2002; Pfenninger & Posada, 2002). Theoretically, ancestral polymorphisms could persist over a far longer time span within a species as a whole than they do in populations or demes (Thomaz *et al.*, 1996; Edwards & Beerli, 2000), if populations or demes conform to a stepping-stone model such as observed in *Albinaria* (Schilthuizen & Lombaerts, 1994). Nevertheless, analyses (Uit de Weerd *et al.*, 2004) using the nuclear marker ITS and incorporating a subset of our ingroup specimens (i.e. one for each species) yielded a topology nearly completely congruent with that found here. Taking all observations together, we consider retention of ancestral polymorphisms as a too far-fetched hypothesis to explain the patterns observed here.

Introgression of mitochondrial DNA from *I. buresi* to *I. pharsalica* provides an equally unlikely explanation for the nested position of *I. pharsalica* sequences among those of *I. buresi*. True enough, such interspecific introgression has

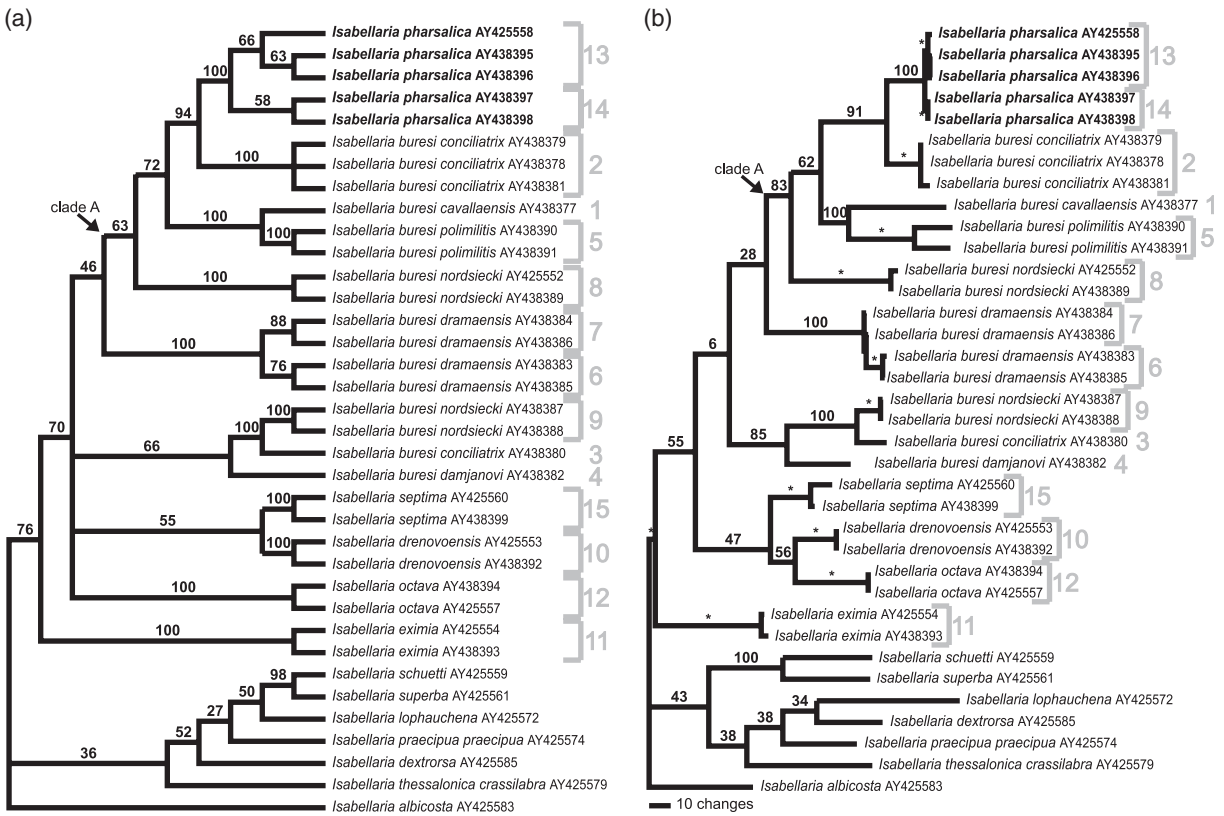

Figure 3 Phylogenetic trees of the COI sequences, with the localities listed in Table 1 given in grey numbers. (a) Strict consensus of the six most parsimonious cladograms. Numbers above branches represent bootstrap values derived from 10,000 bootstrap replicates. (b) maximum likelihood (ML) tree, based on a general time-reversible (GTR) model with gamma distribution and a proportion of invariable sites (see text). Numbers above branches represent ML bootstrap values derived from 100 bootstrap replicates with an upper limit of 250 rearrangements. Asterisks indicate clades constrained in the ML bootstrap analyses.

been reported from *Albinaria* (Douris *et al.*, 1998; van Moorsel *et al.*, 2001), a genus closely related to the *Isabellaria* species of this study. However, those observations concern species that are known to hybridize in the field. To our knowledge, only one possible case has been described for land snails in which past hybridization between two presently widely disjunct species has left its imprint in the distribution of mtDNA lineages (Shimizu & Ueshima, 2000). Those species are not as widely separated as the ones considered here, and occur in a geologically much more dynamic region, which is thought to have induced local extinctions and migrations (see also Hayashi & Chiba, 2000). The wide geographical separation between *I. pharsalica* and *I. buresi* makes past secondary contact between *I. pharsalica* and *I. buresi* highly unlikely. We therefore also dismiss interspecific introgression of DNA as an explanation of the inferred position of the *I. pharsalica* sequences, and conclude that *I. pharsalica* shares a common ancestor with a subdivision of *I. buresi*.

Congruence between the molecular and conchological data

The inferred descent of *I. pharsalica* from *I. buresi* is supported by the congruence of patterns revealed by our DNA analysis with conchological data. First, our analyses show that the morphological diversity within *I. buresi* (see Nordsieck, 1977) is matched by a comparably high amount of genetic divergence, whereas COI sequences for the two populations of the conchologically far less variable *I. pharsalica* are highly similar. Second, both the morphological (Nordsieck, 1974) and our molecular characters question the reciprocal monophyly of *I. pharsalica* and *I. buresi*, suggesting instead that *I. pharsalica* should be combined with a subgroup of *I. buresi*. The mitochondrial lineages do not coincide with the morphologically defined subspecies of *I. buresi*. This is hardly surprising, given the many intermediate forms between most of these subspecies (Nordsieck, 1977), suggesting extensive hybridization with many opportunities for introgression of mtDNA. In accordance with the evidence now available, we propose classifying *I. pharsalica* as a subspecies of *I. buresi*, and thus extending the earlier revision of *I. buresi* (Nordsieck, 1977). We will refer to it as *I. buresi pharsalica* from now on.

Dispersal into Thessaly

The geographical distribution of *I. buresi pharsalica* is clearly discordant with its phylogenetic position. Our data strongly suggest that *I. buresi pharsalica* originates from the eastern part

of the Greek province Macedonia or from Thracia, since it is nested in a *I. buresi* clade distributed in that region. The failure of morphological surveys to identify existing *I. buresi pharsalica* populations in Macedonia or Thracia could be the result of any of the following scenarios: (1) inadequate sampling, (2) loss of the combination of characters typical of *I. buresi pharsalica* because of gene flow with other forms of *I. buresi*, (3) extinction of the subspecies in its area of origin. Alternatively, the combination of shell features typical of *I. buresi pharsalica* could have evolved after its dispersal to Thessaly. Given the rather extensive sampling of Macedonian and Thracian *C. buresi* populations (see Gittenberger & Uit de Weerd, 2004; see also Fig. 4), it is unlikely that ancestral *I. buresi pharsalica* populations have been overlooked. Apart from this, we can only speculate about the cause of the absence of *I. buresi pharsalica* in Macedonia and Thracia.

The presence of *I. buresi pharsalica* in Thessaly can be interpreted as resulting from long-distance dispersal only, given the wide geographical separation from its closest relatives within *I. buresi*. Such long-distance dispersal in land snails is generally accomplished through passive transport (Dörge *et al.*, 1999). Long-distance dispersal events in other Clausiliidae have thus been attributed to (1) rafting (Douris *et al.*, 1998), (2) transport by birds (Preece & Gittenberger, 2003), and (3) transport by humans (Boettger, 1891, p. 62; Liebegott, 1986, p. 18; Welter-Schultes, 1998). Below we will consider these possibilities for *I. buresi pharsalica*.

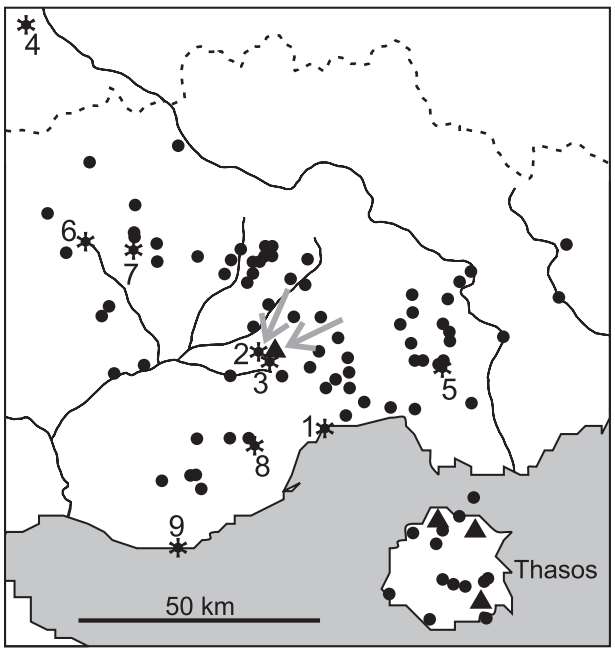

Figure 4 Coincidence of ancient marble quarries (triangles) and known localities of *I. buresi* in north-east Greece and adjacent Bulgaria. Asterisks refer to *I. buresi* populations sampled in this study. The localities of other *I. buresi* populations (dots) are from Gittenberger & Uit de Weerd (2004). Locations of the marble quarries are based on Koželj (1988); Waelkens *et al.* (1988a) and Dworakowska (1975). The arrows mark the sister group of *I. buresi pharsalica* and the Philippi quarries.

Dispersal by sea is unlikely in this particular case. The areas of distribution of *I. buresi pharsalica* and the other subspecies of *I. buresi* are separated by a Neogene mountain chain (Higgins & Higgins, 1996, p. 91), consisting of the Olympos, Ossa, Mavrovouni and Pilio mountains, which precludes a direct connection by sea in the recent past. Moreover, the possibility of long-distance rafting has recently been questioned (Welter-Schultes, 2000, p. 78).

Aerial dispersal by birds could, in theory, explain the occurrence of *I. buresi pharsalica* in Thessaly. Over 100 species of birds migrate across Greece each year, either as winter visitors or as passage migrants (Handrinos & Akriotis, 1997). Birds may be responsible for an extreme case of long-distance dispersal in the clausiliid genus *Balea*, with snails crossing thousands of kilometres of ocean waters (Preece & Gittenberger, 2003). However, whereas *Balea* is known for its 'exceptionally sticky mucus' (Rees, 1965, p. 275), we do not know of any observations in favour of dispersal by birds in *Isabellaria*, nor in any of its closest relatives. At this point, there is insufficient information about bird migratory routes to test this hypothesis.

Human-aided dispersal of *I. buresi*, on the other hand, is supported by several observations. Such dispersal of eastern Mediterranean land snails is indicated by archaeological finds of land snail shells among the cargo of a 3300-year-old shipwreck (Welter-Schultes, 2001), and may have had an impact on their present-day distribution patterns (Mylonas, 1984; Glaubrecht, 1990, unpubl. MSc thesis). The snails studied here dwell on marble and limestone rocks, which have often been used as building or sculpturing blocks in the past. Marble in particular was transported across large distances in the eastern Mediterranean area (e.g. Tykot & Ramage, 2002). Quarried marble blocks were often transported in a raw form and then finished at the site of destination (Waelkens *et al.*, 1988a; Déroche *et al.*, 1989; Herrmann & Barbin, 1993). Consequently, snails may have been transported along with the rocks. Such a mode of dispersal has been inferred to explain distributional patterns within the related genus *Albinaria* (Pfeiffer, 1955, 1956; Liebegott, 1986, p. 18; Welter-Schultes, 1998). For instance, morphological (Welter-Schultes, 1998) and molecular (Schilthuizen *et al.*, 2004) surveys of *A. hippolyti* consistently place an isolated population of this species with a population from an ancient quarry, despite a 13–14 km separation. The hypothesis that *I. buresi* has been artificially dispersed was previously advanced by Frank (1988). She reports specimens of *I. buresi cavallaensis* from the ancient temple of Zeus in Athens (Frank, 1987, 1988), a find that has never, however, been confirmed.

We know of two regions in north-east Greece within the home range of *I. buresi* that contain well-documented marble quarries dating from antiquity, namely an area near Philippi (Waelkens *et al.*, 1988b; Mentzos *et al.*, 2002) and the island of Thasos (Dworakowska, 1975; Koželj, 1988). At least some of the Philippi quarries were situated above the town of Lidia

(Waelkens *et al.*, 1988b, note 93), which places them within 4 km of collection site 2, where the sister group of *I. buresi pharsalica* is found (see Fig. 4). Thus the ancestors of *I. buresi pharsalica* may have been transported from one of these quarries to Thessaly. The island of Thasos is inhabited by *I. buresi insularis*, for which no sequences could be obtained. Thassian marble has been studied in far greater detail than marble from Philippi (Mentzos *et al.*, 2002), and it has been identified from archaeological sites throughout the eastern and central Mediterranean area (e.g. Maniatis *et al.*, 1988; Herrmann, 1992; Tykot *et al.*, 2002). It was transported as far as ancient Delphi, situated beyond the present range of *I. buresi pharsalica*, as early as the sixth century BC (Déroche *et al.*, 1989), and has been found *c.* 30 km south-east of the range of *I. buresi pharsalica* in Thessaly (Mentzos *et al.*, 2003). Large blocks of marble were transported far more easily across water than across land (Tykot & Ramage, 2002), and long-distance transport of marble from the Philippi quarries, which are located 10–15 km farther inland than those of Thasos, may have been more problematic. Nevertheless, several devices for marble transport across land, such as four-wheeled carts, are known from antiquity (Wurch-Kozelj, 1988).

In order to date the dispersal event it is necessary to estimate the divergence time. It should be noted, however, that the estimate of this quantity rests on uncorroborated assumptions and may therefore be flawed. We can arrive at only a crude estimate of the minimal time span from the deepest divergences within the *I. buresi pharsalica* clade, based on the range of previously reported mitochondrial divergence rates, each of unknown precision (see Graur & Martin, 2004). The highest mitochondrial sequence divergence so far reported within land snails, calculated from mitochondrial lrDNA and srDNA, amounted to 10% pair-wise divergence per million years (Chiba, 1999). This is markedly higher than the estimations in *Albinaria*, which range from 1–1.2% (Douris *et al.*, 1998) to 5% (Douris *et al.*, 1995). Considering the maximum pair-wise difference between *I. buresi pharsalica* sequences of 0.62%, the uppermost estimate of 10% would imply that the two populations shared a common ancestor at the very least *c.* 62,000 yr BP. This minimum estimate outdates marble quarrying in Macedonia and Thracia by more than a factor of 10. As such, it provides a wider time margin for changes to erase a morphological connection between *I buresi pharsalica* in Thessaly and particular populations from its area of provenance.

We note, however, three caveats. First, all inferred mitochondrial divergence rates within land snails have so far been based on ribosomal DNA, not on protein-coding genes harbouring synonymous variation. Second, the low maximum number of four sites that are variable between *I. buresi pharsalica* sequence pairs, may introduce a large stochastic error. Finally, the divergence of the mitochondrial lineages present within *I. buresi pharsalica* may predate the dispersal into Thessaly. In fact, under the assumption of passive dispersal through marble transport, it is highly likely that numerous snails would be transported at once. Provided that complete lineage sorting has not been accomplished since then, several of these ancestral lineages may have been sampled. This possibility should not be ignored, in view of the fact that the amount of DNA divergence within *I. buresi pharsalica* is of the same order of magnitude as that within single *I. buresi* populations from north-east Greece.

Our estimates thus lack the precision needed to reject the hypothesis of transport of the ancestors of *I. buresi pharsalica* with Philippi marble to Thessaly. A denser sampling of *I. buresi* populations from north-east Greece is needed in order to locate the provenance of the ancestors of *I. buresi pharsalica* with greater confidence. Further studies on the genetic variation within *I. buresi pharsalica* may help to pinpoint its site of introduction in Thessaly. These results could then be compared with archaeological data.

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