

Reproductive character displacement by inversion of coiling in clausiliid snails (Gastropoda, Pulmonata)

DENNIS R. UIT DE WEERD^{1*}, DICK S. J. GROENENBERG², MENNO SCHILTHUIZEN³
and EDMUND GITTEBERGER^{1,2}

¹*Institute of Biology, Leiden University, PO Box 9516, 2300 RA Leiden, the Netherlands*

²*National Natural History Museum Naturalis, PO Box 9517, 2300 RA Leiden, the Netherlands*

³*Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Locked Bag 2073, 88999 Kota Kinabalu, Malaysia*

Received 25 July 2004; accepted for publication 4 May 2005

In land snails, a change in the direction of coiling, being associated with a shift in the position of the genital apparatus, may act as a barrier against hybridization between sympatric species. Putative reproductive character displacement by an inversion in chirality has been reported in only a few land snails, based on observations in the field and interbreeding experiments. In this study, we present a new case of possible reproductive character displacement in the direction of coiling, in the clausiliid snail *Isabellaria dextrorsa*. This species is dextral, in contrast with its nearest relatives, including *I. torifera* and *I. lophauchena*, which share plesiomorphic sinistral coiling. Whereas *I. dextrorsa* occurs in sympatry and even syntopically with *I. lophauchena* throughout most of its range, the sinistral species have a mosaic distribution. Phylogenetic analyses of mitochondrial cytochrome c oxidase subunit I (COI) sequences demonstrated that *I. dextrorsa* constitutes a clade with *I. torifera*. In this clade, a shift in coiling direction occurred at least twice, maybe triggered by the presence of a sympatric congeneric sinistral species. The analyses separated the sequences of all *I. dextrorsa* samples from those of sympatric and syntopic *I. lophauchena* samples. The failure to demonstrate gene flow between these species is consistent with the hypothesis of genetic isolation by reproductive character displacement. © 2006 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2006, 88, 155–164.

ADDITIONAL KEYWORDS: chirality – mitochondrial DNA – sympatry.

INTRODUCTION

Reproductive character displacement, the divergence in mating-associated traits that enhances prezygotic reproductive isolation between species in their area of sympatry, is considered one of the most fascinating, yet at the same time controversial, issues in evolutionary biology (for an overview see Howard, 1993). Reproductive character displacement between species can evolve in an area of sympatry when (1) heterospecific mating results in an overall decrease in reproductive success, and (2) divergence in a heritable trait between the species reduces the frequency of such heterospecific mating. A continuing point of criticism has

been the lack of empirical evidence for this hypothesis (Howard, 1993).

One of the best supported (Howard, 1993) and most elegant of all presumed examples of reproductive character displacement concerns the shift in chirality in land snails. Snails can be coiled dextrally (right-handedly) or sinistrally (left-handedly), dependent on two alleles at a single locus. Coiling direction is the classical example of a maternal effect ('delayed inheritance'), in which the genotype of only the female determines the phenotype of all its offspring through the eggs (Boycott *et al.*, 1930; Degner, 1952; Murray & Clarke, 1966). This genetically simple trait can have far-reaching consequences for reproduction in snails. Male and female reproductive organs open asymmetrically on only one side of the body of the generally hermaphroditic pulmonate snails: on the right side in

*Corresponding author. Current address: Academy of Natural Sciences, 1900 Benjamin Franklin Parkway, Philadelphia, PA 19103, USA. E-mail: uitdeweerd@acnatsci.org

dextral animals, and on the left side in sinistral ones. Also in prosobranch snails, which are usually dioecious, the genital organs are situated asymmetrically. The different position of the genital apparatus may prevent or complicate the exchange of gametes between snails of opposite coiling.

So far, two possible cases of character displacement in coiling direction have been reported from land snails: in the prosobranch genus *Diplommatina* (Peake, 1973) and in the pulmonate genus *Partula* (Clarke & Murray, 1969; Murray & Clarke, 1980). The phenomenon was extensively studied in the latter genus only. The initial evidence of reproductive character displacement within *Partula* came from three observations (Clarke & Murray, 1969; Murray & Clarke, 1980): (1) the species of interest are apparently closely related, (2) their ranges touch or overlap, and (3) only in these contact zones do the species have opposite directions of coiling. The potential role of chirality as an isolating mechanism within this genus is demonstrated by the partial reproductive isolation between dextral and sinistral forms of *P. suturalis* (Clarke & Murray, 1969; Lipton & Murray, 1979), caused by attempts at intromission in the wrong place (Lipton & Murray, 1979; Johnson, 1982).

One of the first groups of land snails in which polymorphisms in chirality were studied was the family Clausiliidae (Degner, 1952). These studies focused on the species *Balea biplicata*, and revealed that, unlike the situation in most other snails, the sinistral allele is dominant over the dextral one in this clausiliid species (Degner, 1952). The overwhelming majority of clausiliid species is sinistral (Zilch, 1959: 377), and so sinistral coiling is assumed to be the plesiomorphic condition within the family. The copulation of clausiliid snails can be simultaneously reciprocal (Nordsieck, 1969; Schilthuizen & Lombaerts, 1995) and transfer of spermatophores between individuals of opposite chirality is physically possible (Gittenberger, 1988; Asami, Cowie & Ohbayashi, 1998). Even so, as observations on *Partula* (Johnson, 1982) demonstrate, such copulations may occur less frequently and result in fewer offspring than do copulations between snails with the same direction of coiling.

Recent fieldwork in northern Greece, along with molecular analyses, revealed a distribution of dextral and sinistral forms within the clausiliid genus *Isabellaria* reminiscent of that in *Partula*. The genus *Isabellaria* consists of species, dwelling on limestone and marble, which feed on lichens and bryophytes (D.R. Uit de Weerd & E. Gittenberger, pers. observ.). All *Isabellaria* species *sensu* Gittenberger & Uit de Weerd (in press) are sinistral, with the exception of *I. dextrorsa* (Fig. 1). The phylogenetic position of *I. dextrorsa*, nested among sinistral species (Fig. 2), strongly suggests that its dextral coiling evolved secondarily. Par-

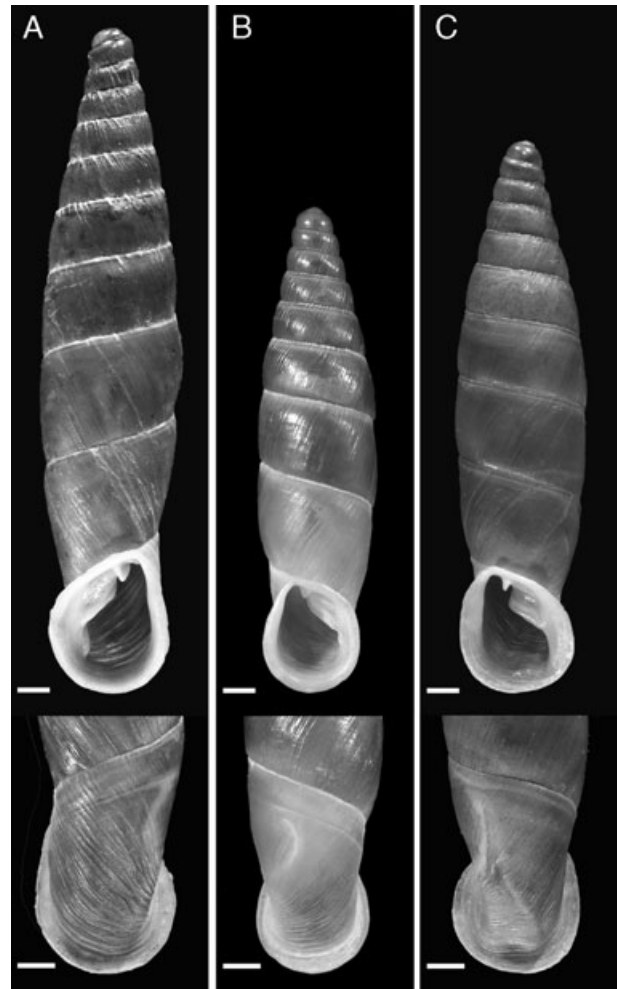


Figure 1. Shells of *Isabellaria dextrorsa* from locality 2 (A), *I. torifera* from locality 10 (B) and *I. lophauchena* from locality 2 (C). Above: ventral aspect; below: cervix, dorsal aspect. Scale bar = 1.0 mm. Locality numbers refer to Figures 3 and 4. Photographs (A) and (C) by A. 't Hooft. Photograph (B) by J. Goud, Leiden.

ticularly striking is the observation that, while among themselves the sinistral *Isabellaria* species have a mosaic distribution (Nordsieck, 1974: 131–132), *I. dextrorsa* is found in sympatry with sinistral *Isabellaria* species throughout its range, which extends from Mount Olympus to the Greek–Macedonian border (Fig. 3). The northern part of this range overlaps broadly with that of *I. lophauchena*, and here both species are often found in syntopy. In the southern part of its range, where *I. lophauchena* is absent, *I. dextrorsa* is found in parapatry with *I. albicosta*. This connection between opposite coiling and syntopic occurrence was the focus of this study.

By providing a genealogical framework, molecular phylogenetic analyses can be an important tool when

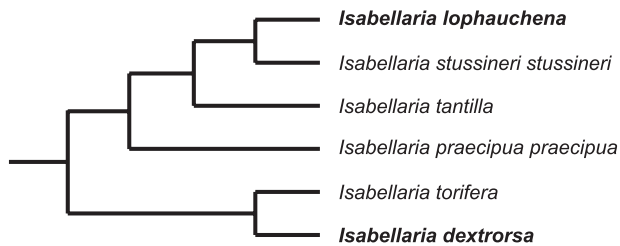


Figure 2. Phylogenetic position of *Isabellaria dextrorsa*, *I. torifera* and *I. lophauchena* among closely related species, as inferred from Bayesian inference (Uit de Weerd, Piel & Gittenberger, 2004). The remaining 25 *Isabellaria* species sampled were placed basal to the clade shown.

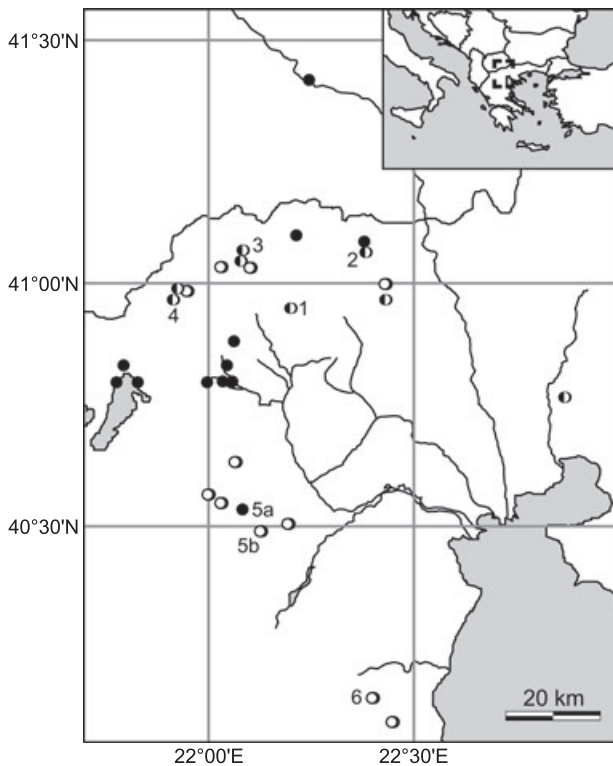


Figure 3. Distribution of *Isabellaria dextrorsa* and *I. lophauchena*, based on the literature and data in the National Museum of Natural History in Leiden. Open circles indicate *I. dextrorsa*, closed circles *I. lophauchena*. Localities where both species were found syntopically are marked with half-filled circles. Numbers refer to the sample sites given in Table 1.

studying inversions in snail chirality. A study of the land snail genus *Euhadra* identified a polyphyletic dextral 'species', consisting of several dextral lineages that had evolved independently from a conchologically similar sinistral 'species' (Ueshima & Asami, 2003). Similarly, *I. dextrorsa* has a 'mirror-image' counterpart: *I. torifera*, a species at *Isabellaria*'s western

fringe. Molecular (Uit de Weerd, Piel & Gittenberger, 2004; Fig. 2) and morphological (Nordsieck, 1972) analyses indicate that *I. torifera* is the closest extant relative of *I. dextrorsa*. Apart from its dextral coiling, *I. dextrorsa* differs from *I. torifera* in its more eastern distribution, completely overlapping with those of other *Isabellaria* species. These differences may have arisen once, after the divergence of lineages leading to *I. dextrorsa* and to *I. torifera*. Alternatively, changes in chirality may have occurred more than once, as in *Euhadra*. This would imply multiple occasions of character displacement, correlating with the presence or absence of syntopic congeneric species.

Molecular phylogenetic analyses can provide an indication of gene flow between two syntopic species, occurring despite having opposite chirality. In this particular case, *I. dextrorsa* and the largely sympatric *I. lophauchena* are closely related, as parts of a single, relatively shallow clade within *Isabellaria* (Uit de Weerd *et al.*, 2004; Fig. 2). Should molecular analyses demonstrate extensive gene flow between syntopic populations of these taxa, then the opposite direction of coil has certainly not been effective as a premating reproductive barrier.

We used part of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) (a) to investigate whether an inversion in chirality evolved only once or more frequently, and (b) to look for gene flow between syntopic *I. dextrorsa* and *I. lophauchena* populations. This genetic marker can be used to reconstruct phylogenetic relationships at both the inter- and the intraspecific level in land snails (e.g. Holland & Hadfield, 2002; Ponder *et al.*, 2003; Gittenberger, Piel & Groenenberg, 2004). In addition, the unlinked non-recombining nature of mtDNA allows for the detection of hybridization even after long time spans (Shimizu & Ueshima, 2000).

MATERIAL AND METHODS

Nearly all samples of *I. dextrorsa* and *I. lophauchena* that were studied came from localities where both species coexist syntopically (Table 1). There were only two exceptions, both from the southern part of their range; in this area, which was less adequately sampled, we did not locate syntopic populations of the species. Therefore, we included *I. lophauchena* and *I. dextrorsa* samples from two localities 6 km apart, localities 5a and 5b, respectively. Two populations from Theodoraki, locality 1, were represented by five individuals each; all other populations were represented by one individual only. An additional *I. dextrorsa* sample (not sympatric with *I. lophauchena*) and an *I. albicosta* sample from adjoining populations on Mount Olympos (locality 6) were also included. Additional ingroup and outgroup

Table 1. Sampling information

Locality	Species	GenBank accession	Coordinates	UTM code
1	<i>Isabellaria dextrorsa</i>	AY438408	40°57'N 22°12'E	FL0034
1	<i>Isabellaria dextrorsa</i>	AY438409	40°57'N 22°12'E	FL0034
1	<i>Isabellaria dextrorsa</i>	AY438410	40°57'N 22°12'E	FL0034
1	<i>Isabellaria dextrorsa</i>	AY438411	40°57'N 22°12'E	FL0034
1	<i>Isabellaria dextrorsa</i>	AY438412	40°57'N 22°12'E	FL0034
2	<i>Isabellaria dextrorsa</i>	AY438413	41°04'N 22°23'E	FL1547
3	<i>Isabellaria dextrorsa</i>	AY438414	41°03'N 22°05'E	EL9144
4	<i>Isabellaria dextrorsa</i> *	AY425585	40°58'N 21°55'E	EL7635
5b	<i>Isabellaria dextrorsa</i>	AY438415	40°29'N 22°08'E	EK9582
6	<i>Isabellaria dextrorsa</i>	AY438416	40°09'N 22°24'E	FK1946
1	<i>Isabellaria lophauchena</i>	AY438400	40°57'N 22°12'E	FL0034
1	<i>Isabellaria lophauchena</i>	AY438401	40°57'N 22°12'E	FL0034
1	<i>Isabellaria lophauchena</i>	AY438402	40°57'N 22°12'E	FL0034
1	<i>Isabellaria lophauchena</i>	AY438403	40°57'N 22°12'E	FL0034
1	<i>Isabellaria lophauchena</i>	AY438404	40°57'N 22°12'E	FL0034
2	<i>Isabellaria lophauchena</i>	AY438405	41°04'N 22°23'E	FL1547
3	<i>Isabellaria lophauchena</i>	AY438406	41°03'N 22°05'E	EL9144
4	<i>Isabellaria lophauchena</i>	AY438407	40°58'N 21°55'E	EL7635
5a	<i>Isabellaria lophauchena</i> *	AY425572	40°32'N 22°05'E	EK9188
7	<i>Isabellaria praecipua praecipua</i> *	AY425574	40°29'N 22°12'E	FK08
8	<i>Isabellaria stussineri stussineri</i> *	AY425591	39°53'N 22°38'E	FK3916
9	<i>Isabellaria tantilla</i> *	AY425592	39°42'N 22°14'E	FJ0596
10	<i>Isabellaria torifera</i> *	AY425593	39°41'N 21°41'E	EJ5891
11	<i>Isabellaria torifera</i>	AY438418	39°41'N 21°35'E	EJ5092
12	<i>Isabellaria septima</i> *	AY425560	41°24'N 22°15'E	FL08
13	<i>Isabellaria thessalonica crassilabra</i> *	AY425579	40°23'N 23°10'E	FK8371
14	<i>Isabellaria albicosta</i> *	AY425583	40°05'N 22°25'E	FK2038
6	<i>Isabellaria albicosta</i>	AY438417	40°09'N 22°24'E	FK1946
–	<i>Albinaria brevicollis brevicollis</i>	AY438419	36°51'N 28°16'E	PA17
–	<i>Albinaria caerulea</i> †	X83390	Unknown	Unknown
–	<i>Albinaria discolor discolor</i>	AY438420	36°44'N 22°54'E	FF6967
–	<i>Albinaria grisea akrocurta</i>	AY438421	36°53'N 22°48'E	FF5983
–	<i>Albinaria puella puella</i> *	AY425550	37°52'N 27°16'E	NB29

*Sequence from Uit de Weerd *et al.* (2004). †Sequence from Hatzoglou *et al.* (1995).

species were selected based on previous phylogenetic inferences (Uit de Weerd *et al.*, 2004). Thus the ingroup consisted of *I. dextrorsa*, *I. lophauchena* and *I. torifera*, with the closest relatives of these species, *I. praecipua*, *I. stussineri* and *I. tantilla* (Fig. 2). For outgroup rooting we used two samples of *I. albicosta*, including the sample from Mount Olympos, *I. thessalonica* and *I. septima*. The additional ingroup and outgroup species surrounded the range of *I. dextrorsa* and *I. lophauchena* (Fig. 4).

Nine of the *Isabellaria* COI sequences used in this study had been obtained previously (Table 1); the remaining ones were newly determined. Total genomic DNA was extracted from frozen tissue, collected 1–3 years previously, using the protocol described by Schilthuizen, Gittenberger & Gulyaev

(1995). A 708-bp fragment of the 5' region of the COI gene was PCR-amplified using the forward primer L1490-Alb (5'-ACTCAACGAATCATAAAGATATTGG-3') and the reverse primer H2198-Alb (5'-TATACTT CAGGATGACCAAAAATCA-3') (Gittenberger *et al.*, 2004). The 25 µL PCR reaction mixes (3 mM MgCl₂, 0.2 mM dNTPs, 0.4 µM of each primer, and 1.25 unit *Taq* polymerase) were subjected to 35 cycles of denaturing at 94°C for 60 seconds (first denaturing step for 5 min), annealing at 47°C for 60 seconds, and extension at 72°C for 60 seconds (final extension step for 6 min). PCR fragments were gel-purified using spin columns (Qiaquick Gel Extraction Kit, Qiagen). Sequences of forward and reverse dye-terminator (Big Dye, PE Biosystems) cycle-sequenced PCR products were determined on an ABI 377 automated

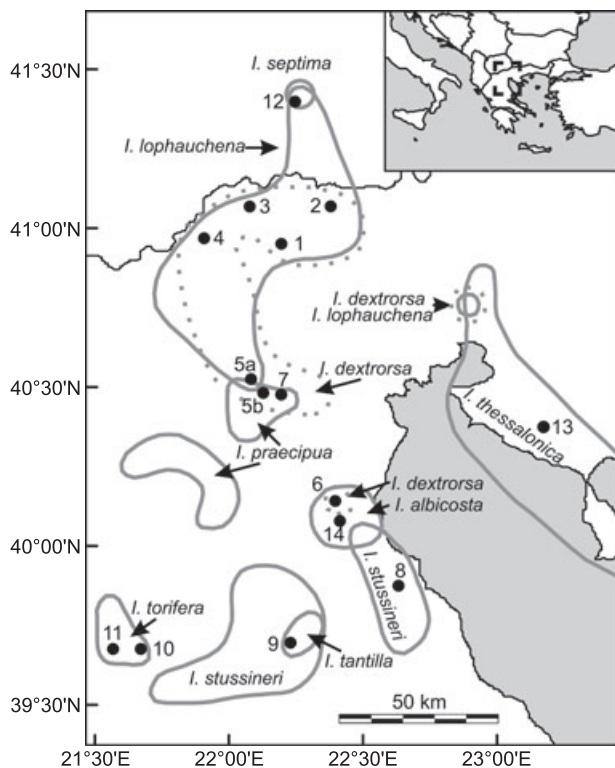


Figure 4. Schematic distribution of the species included in this study, based on the literature and data in the National Museum of Natural History in Leiden. The approximate borders of the range of *I. dextrorsa* are shown by dotted lines, and those of the other, sinistral, species by uninterrupted lines. Numbers refer to the sample sites given in Table 1.

sequencer (PE Biosystems). The forward and reverse sequences were assembled using the program Sequencher (Gene Codes Corp.), and aligned using the Clustal V method implemented in MegAlign 4.03 (DNASTAR Inc., 1999). All sequences were checked for stop codons and missing bases, and were submitted to GenBank.

Phylogenetic inference was based on maximum parsimony (MP) heuristic searches (1000 random addition replicates, TBR and steepest descent) using PAUP* 4.0b10 (Swofford, 2002). Bootstrap support was calculated using 10 000 replicates (one random addition per replicate, TBR and steepest descent). In addition, we performed neighbour-joining (NJ) bootstrap analyses, again based on 10 000 replicates, using the distances calculated on the basis of Kimura's two-parameter model (K2P) and a LogDet model. This was done (1) to test the robustness of the dataset under different phylogenetic approaches, and (2) to provide a bootstrap-supported phylogenetic framework with which to test if K2P and LogDet distances clearly separated *I. dextrorsa* and *I. lophauchena*,

before calculating their inter- and intraspecific DNA divergence (see below).

To estimate the effect of genetic compatibility on gene flow between *I. dextrorsa* and *I. lophauchena* independently from direction of coil, we compared their genetic divergence to that between species of the closely related genus *Albinaria* which have been reported to interbreed (Mylonas *et al.*, 1987). To this end, we also determined COI sequences of four of the purportedly interbreeding *Albinaria* species, *A. brevicollis*, *A. discolor*, *A. grisea* and *A. puella*. The COI sequence of a fifth species used in those studies, *A. caerulea*, was determined by Hatzoglou, Rodakis & Lecanidou (1995) and obtained from GenBank (accession number, X83390). The amount of sequence divergence between *I. dextrorsa* and *I. lophauchena* relative to the sequence divergence within *Albinaria* was determined using the K2P model to correct for multiple hits. This model was chosen to facilitate comparisons with previous studies using K2P corrected divergences between *Albinaria* sequences (Douris *et al.*, 1998; van Moorsel, Schilthuisen & Gittenberger, 2001). In addition, we used a LogDet model to correct for unequal base frequencies (Swofford *et al.*, 1996: 459–461). Only a single sequence from each of the two Theodoraki populations (locality 1) sampled, i.e. AY438400 and AY438408, was used in these calculations, because otherwise this single population might dominate in the analysis. The *Albinaria* sequences, being relatively distantly related to the other sequences, were excluded from the phylogenetic analyses.

RESULTS

Of the amplified region, 651 bases could be identified for all samples. Base frequencies at codon positions 1 and 2 were not significantly heterogeneous across the total set of taxa ($P = 1.00$), whereas significant heterogeneity was detected at codon position 3 ($P < 0.001$). These differences appeared to correlate with the phylogenetic tree inferred from position 3. Thus, the sequences with the highest percentage of adenosine, those of the *I. praecipua*, *I. torifera* and *I. dextrorsa* samples, all clustered within a single clade. We therefore tested whether this compositional bias at position 3 had introduced a systematic error into the phylogenetic inferences. To this end, the phylogenetic signal inferred from position 3 was compared in a partition homogeneity test (1000 replicates, heuristic search, five random additions per replicate, TBR and steepest descent) with that of the unbiased positions 1 and 2 combined, excluding all uninformative positions. We found no evidence for any significantly contradicting phylogenetic signal between position 3 and positions 1 and 2 combined ($P = 0.12$). We also com-

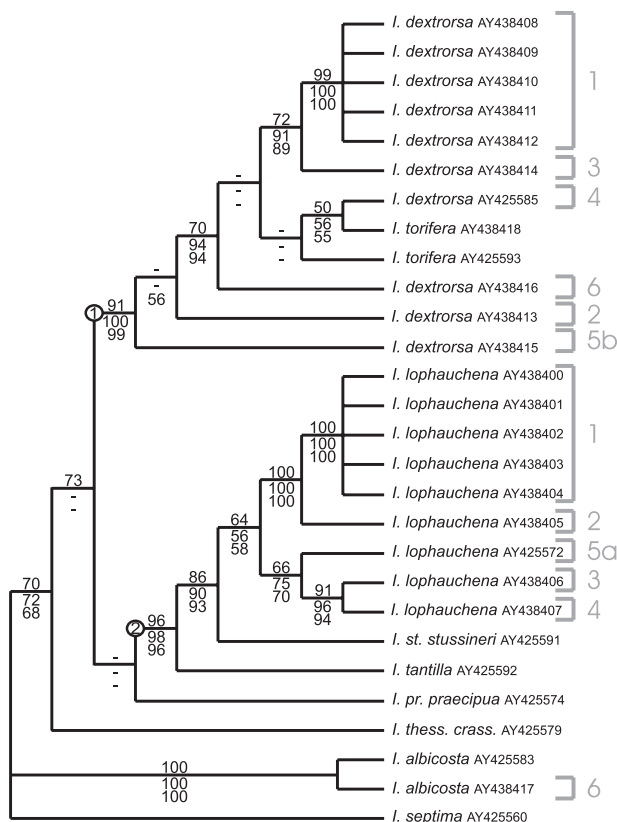


Figure 5. Strict consensus cladogram of the four most parsimonious trees. Numbers above the branches represent maximum parsimony bootstrap values, based on 10 000 replicates. Numbers below the branches are from the neighbour-joining bootstrap analyses using Kimura's two-parameter distances (upper number) and LogDet distances (lower number), respectively, based on 10 000 replicates. Encircled numbers below branches indicate clades 1 and 2, as mentioned in the text. Numbers to the far right refer to collection sites. The GenBank accession numbers of the sequences used are given next to the species names.

pared, for the total dataset, the parsimony scores of MP trees with those of the NJ tree based on LogDet distances, which are considered robust to base composition bias (Swofford *et al.*, 1996: 459–461). Four most parsimonious trees were found, with a score of 680. In spite of differences between the two methods with respect to the type of data used and the tree-building process (Page & Holmes, 1998: 178–193), the LogDet NJ tree required only two additional transformations, which is a non-significant difference (one-tailed Wilcoxon signed-ranks test: $0.25 < P$). Consequently, we based our phylogenetic inferences on the four MP trees, and we included the third codon positions in all subsequent analyses.

The four most parsimonious trees differed only in the inferred relationships between the sequences from

each population from locality 1 (Theodoraki). The strict consensus of these trees and the bootstrap support for its constituent clades are shown in Figure 5. Nearly all clades supported by MP bootstrap values exceeding 50% received comparable or higher bootstrap support from the two NJ bootstrap analyses. In addition, the K2P and LogDet NJ bootstrap analyses supported a grouping of *I. praecipua*, *I. torifera* and *I. dextrorsa* by 85% and 80%, respectively (not shown).

Both the MP and the NJ analyses grouped *I. dextrorsa* and *I. torifera* in a well-supported clade (clade 1), but refuted the monophyly of *I. dextrorsa*. In the MP tree (Fig. 5), the *I. dextrorsa* sequences were paraphyletic with respect to *I. torifera*, a topology supported by only moderate (70%) MP bootstrap support. The 64 most parsimonious trees indicating a monophyletic *I. dextrorsa* had a score of 689. All of these were significantly less parsimonious than were each of the MP trees, but some of them only just so (one-tailed Wilcoxon signed-ranks test: $P \leq 0.05$). The paraphyly of *I. dextrorsa* with respect to *I. torifera* was far better supported by NJ bootstrap (both K2P and LogDet) analyses (94%).

In addition, the MP and NJ analyses clearly separated the *I. dextrorsa*–*torifera* and the *I. lophauchena* sequences, placing these in different highly supported clades, clades 1 and 2, respectively. Both these clades had been identified previously using an independent, nuclear marker, ITS1 and 2 sequences (Uit de Weerd *et al.*, 2004). Those analyses moreover identified a topology within clade 2 identical to that found in this study. For the *I. lophauchena* sequences, a monophyletic origin was inferred. The *I. dextrorsa* and *I. lophauchena* sequences from locality 1 (Theodoraki) constituted two monophyletic groups.

The average pairwise divergence (Table 2) between sequences of *I. dextrorsa* and *I. lophauchena* was slightly lower (K2P: 0.168584; LogDet: 0.176944) than was the average estimated divergence between the *Albinaria* sequences (K2P: 0.175832; LogDet: 0.183873). These roughly similar divergences could not be attributed to saturation effects, as the K2P and LogDet pairwise divergences between *I. dextrorsa* and *I. lophauchena* populations were lower on average than were those of either species to *Albinaria* (Table 2), indicating that a maximum divergence level has not yet been reached between *I. dextrorsa* and *I. lophauchena*. Genetic divergence within *I. dextrorsa* was significantly lower than was that within *I. lophauchena* (two-tailed Mann–Whitney *U*-test: $P < 0.001$). This difference could not be attributed to sampling, since a comparison of an equal number of *I. lophauchena* samples and adjoining *I. dextrorsa* samples, those of localities 1–5, likewise revealed significant differences (two-tailed Mann–Whitney *U*-test: $P < 0.005$).

Table 2. Kimura's 2-parameter corrected and LogDet genetic distances between samples

Samples	Genetic distance	
	K2P	LogDet
<i>Albinaria</i> samples		
<i>A. discolor discolor</i> – <i>A. brevicollis brevicollis</i>	0.19094	0.196559
<i>A. puella puella</i> – <i>A. b. brevicollis</i>	0.154556	0.163869
<i>A. p. puella</i> – <i>A. d. discolor</i>	0.177611	0.189392
<i>A. caerulea</i> – <i>A. b. brevicollis</i>	0.154831	0.165477
<i>A. caerulea</i> – <i>A. d. discolor</i>	0.189712	0.198261
<i>A. caerulea</i> – <i>A. p. puella</i>	0.154556	0.16828
<i>A. grisea akrocurta</i> – <i>A. b. brevicollis</i>	0.178775	0.181407
<i>A. g. akrocurta</i> – <i>A. d. discolor</i>	0.188528	0.194067
<i>A. g. akrocurta</i> – <i>A. p. puella</i>	0.179249	0.185701
<i>A. g. akrocurta</i> – <i>A. caerulea</i>	0.189559	0.195716
Average within <i>Albinaria</i>	0.175832	0.183873
Averages for <i>I. dextrorsa</i> and <i>I. lophauchena</i> samples		
Within <i>I. dextrorsa</i> population 1	0.001232	0.001413
Between <i>I. dextrorsa</i> populations	0.055241	0.062214
Within <i>I. lophauchena</i> population 1	0.001232	0.001425
Between <i>I. lophauchena</i> populations	0.100326	0.107319
<i>I. dextrorsa</i> – <i>I. lophauchena</i>	0.168584	0.176944
<i>I. dextrorsa</i> – <i>Albinaria</i> species	0.187705	0.189568
<i>I. lophauchena</i> – <i>Albinaria</i> species	0.216102	0.220428

DISCUSSION

The nested position of the *I. torifera* sequences among *I. dextrorsa* sequences in clade 1 points to character displacement. Unless we are being misled by extensive sorting of ancestral mitochondrial lineages about the interrelationships between the specimens sampled (e.g. Goodacre & Wade, 2001; but see Uit de Weerd, Schneider & Gittenberger, 2005), inversions in coiling direction must have occurred more than once in this clade, eventually leading to a situation with dextral forms in the presence of sympatric sinistral *Isabel-laria* species, and sinistral ones in their absence. Thus, either (1) dextral lineages, assigned to *I. dextrorsa*, evolved independently five times, or (2) dextrality evolved only once and a reversal to the sinistral '*I. torifera*' occurred twice. The latter alternative requires fewer chirality changes (three instead of five). Comparable reversals in chirality have also been inferred in the land snail genus *Euhadra* (Ueshima & Asami, 2003). It is also the more plausible alternative on theoretical grounds, because the sinistral allele, if it is indeed dominant as in *B. biplicata*, can be expected to reach fixation more easily (Orr, 1991; van Batenburg & Gittenberger, 1996). It should be noted, however, that many of the decisive branches within the *I. dextrorsa*–*torifera* clade were poorly bootstrap-supported and that most of its subclades lacked bio-

geographical coherence. When all branches supported by less than a threshold bootstrap value of 70% (Hillis & Bull, 1993) were collapsed, both scenarios implied two transformations.

Our results suggest strongly that *I. dextrorsa* and *I. lophauchena* are reproductively isolated. No evidence of gene flow between the two species was found, which is consistent with the absence of morphologically identifiable hybrids between syntopic *I. dextrorsa* and *I. lophauchena*. In spite of their apparent reproductive isolation, the genetic distance between *I. dextrorsa* and *I. lophauchena* was found to be of the same order of magnitude as that within *Albinaria* species reported to interbreed in the laboratory (Mylonas *et al.*, 1987). Nearly all these distances fall within the range of mtDNA genetic distances (11–18%) expected for congeneric species (Douris *et al.*, 1998). Assuming, on the basis of their genetic divergence (Edmands, 2002), that *I. lophauchena* and *I. dextrorsa* are genetically as compatible as are the interbreeding *Albinaria* species, we conclude that their opposite coiling direction may have contributed to their reproductive isolation. Hybrid mortality, such as has been inferred in *Albinaria* (Giokas, Mylonas & Sotiropoulos, 2000; Schilthuizen & Lombaerts, 1995; Schilthuizen, 1995), may have driven the divergence in coiling direction. Even so, actual hybridization is not a necessary con-

dition for reproductive character displacement to evolve, since heterospecific mating can have other adverse effects (Noor, 1999; Servedio, 2001; Servedio & Noor, 2003). Even if no resources are allocated to the production of offspring, mating in snails still involves other costs (Baur, 1998: 282, 283).

The combination of an opposite direction of coiling and reproductive isolation in areas of sympatry of *I. dextrorsa* and *I. lophauchena* is suggestive of reproductive character displacement. Niche differentiation in sympatry offers an unlikely alternative for which we have no arguments. Admittedly, niche differentiation between related sympatric or syntopic species has been reported for other land snails (Murray, Johnson & Clarke, 1982; Ledergerber *et al.*, 1997; Chiba, 1999, 2002), and has been inferred for syntopic *Albinaria* species displaying differences in radula structure and in substrate usage (Kemperman, 1992: 117–119). Syntopic rock-dwelling clausiliid snails can even feed on different lichen species (Baur, Baur & Fröberg, 1994). Also feeding on lichens and bryophytes, *I. dextrorsa* and *I. lophauchena* could have developed a similar fine-patterned food partitioning that could be detected only by detailed studies. Feeding-related differentiation into two mirror images has been observed in cichlid fish (Hori, 1993) and in crossbills (Benkman, 1988, 1996). However, these groups are asymmetrical in their feeding apparatus, which is not the case in clausiliid snails (Kemperman, 1992: 85). It is hard to imagine a way in which differences in chirality would facilitate a partitioning of their food resources. Similar studies on mirror types within *Partula* were likewise unable to relate these to ecology (Clarke & Murray, 1969). Although we cannot exclude some extent of niche differentiation between *I. dextrorsa* and *I. lophauchena*, this is unlikely to be the driving force behind the evolution of the mirror types.

Our data provide very few clues into the interactions between the two species in the field. Mating behaviour between dextral and sinistral Clausiliidae has been studied only in the clausiliid genera *Balea* and *Alopi*a, and these observations were made under artificial conditions. Crosses between dextral and sinistral individuals of *B. biplicata* are possible and result in offspring (Degner, 1952), but these crosses were forced in the sense that each pair was kept in isolation. The study on *Alopi*a (Nordsieck, 1978) consisted of two experiments, each with different *Alopi*a species, and each with 15 individuals of a dextral and 15 of a sinistral species. Summed over the two experiments, three out of 'about 20' copulations observed were between mirror-image species, the remaining being between conspecific individuals. These observations suggest that copulation between *I. dextrorsa* and *I. lophauchena* might also be possible physically. Indeed, molecular surveys of

coil-polymorphic *Partula* species suggest that a difference in direction of coil per se does not impose a barrier to gene flow (Johnson, Murray & Clarke, 1987; Goodacre, 2002).

At this point, observations on the interactions between syntopic individuals of *I. dextrorsa* and *I. lophauchena*, preferably from the field, are needed to identify mechanisms responsible for their genetic and morphological distinctness. Where both species occur in syntopy, individuals of *I. dextrorsa* and *I. lophauchena* are often found in small (approximately 5–50 individuals) clusters of conspecifics (D.R. Uit de Weerd & E. Gittenberger, pers. observ.), which may reduce the chances of heterospecific mating. Increased assortative mating in sympatry has been observed in species of the pulmonate genus *Lymnaea* (Wullschleger, Wiehn & Jokela, 2002). Future studies should focus on the questions of (a) whether heterospecific mating between *I. dextrorsa* and *I. lophauchena* does occur, (b) whether this results in exchange of gametes, and (c) whether viable and fertile hybrids are produced.

ACKNOWLEDGEMENTS

We thank T. Backeljau, W. H. Piel, and two anonymous reviewers for their constructive comments.

REFERENCES

- Asami T, Cowie RH, Ohbayashi K. 1998. Evolution of mirror images by sexually asymmetric mating behavior in hermaphroditic snails. *American Naturalist* **152**: 225–236.
- van Batenburg FHD, Gittenberger E. 1996. Ease of fixation of a change in coiling: computer experiments on chirality in snails. *Heredity* **76**: 278–286.
- Baur B. 1998. Sperm competition in molluscs. In: Birkhead TR, Møller AP, eds. *Sperm competition and sexual selection*. London: Academic Press, 255–305.
- Baur A, Baur B, Fröberg L. 1994. Herbivory on calcicolous lichens: different food preferences and growth rates in two co-existing land snails. *Oecologia* **98**: 313–319.
- Benkman CW. 1988. A 3 : 1 ratio of mandible crossing direction in white-winged crossbills. *Auk* **105**: 578–579.
- Benkman CW. 1996. Are the ratios of bill crossing morphs in crossbills the result of frequency-dependent selection? *Evolutionary Ecology* **10**: 119–126.
- Boycott AE, Diver C, Garstang SL, Turner FM. 1930. The inheritance of sinistrality in *Limnea peregra* (Mollusca, Pulmonata). *Philosophical Transactions of the Royal Society of London, Series B* **219**: 51–131.
- Chiba S. 1999. Character displacement, frequency-dependent selection, and divergence of shell colour in land snails *Mandarina* (Pulmonata). *Biological Journal of the Linnean Society* **66**: 465–479.

- Chiba S. 2002.** Ecological diversity and speciation in land snails of the genus *Mandarina* from the Bonin Islands. *Population Ecology* **44**: 179–187.
- Clarke B, Murray J. 1969.** Ecological genetics and speciation in land snails of the genus *Partula*. *Biological Journal of the Linnean Society* **1**: 31–42.
- Degner E. 1952.** Der Erbgang der Inversion bei *Laciniaria biplicata* MTG. (Gastr. Pulm.). *Mitteilungen aus dem Hamburgischen Museum und Institut* **51**: 3–61.
- Douris V, Cameron RAD, Rodakis GC, Lecanidou R. 1998.** Mitochondrial phylogeography of the land snail *Albinaria* in Crete: long-term geological and short-term vicariance effects. *Evolution* **52**: 116–125.
- Edmands S. 2002.** Does parental divergence predict reproductive compatibility? *Trends in Ecology and Evolution* **17**: 520–527.
- Giokas S, Mylonas M, Sotiropoulos K. 2000.** Gene flow and differential mortality in a contact zone between two *Albinaria* species (Gastropoda; Clausiliidae). *Biological Journal of the Linnean Society* **71**: 755–770.
- Gittenberger E. 1988.** Sympatric speciation in snails: a largely neglected model. *Evolution* **42**: 826–828.
- Gittenberger E, Piel WH, Groenenberg D. 2004.** The Pleistocene glaciations and the evolutionary history of the polytypic snail species *Arianta arbustorum* (Gastropoda, Helicidae). *Molecular Phylogenetics and Evolution* **30**: 64–73.
- Gittenberger E, Uit de Weerd DR. 2006.** Reconsidering the generic position of the species once classified in *Carinigera*, *Isabellaria* and *Sericata*. *Basteria*, in press.
- Goodacre SL. 2002.** Population structure, history and gene flow in a group of closely related land snails: genetic variation in *Partula* from the Society Islands of the Pacific. *Molecular Ecology* **11**: 55–68.
- Goodacre SL, Wade CM. 2001.** Patterns of genetic variation in Pacific island land snails: the distribution of cytochrome *b* lineages among Society Island *Partula*. *Biological Journal of the Linnean Society* **73**: 131–138.
- Hatzoglou E, Rodakis GC, Lecanidou R. 1995.** Complete sequence and gene organization of the mitochondrial genome of the land snail *Albinaria coerulea*. *Genetics* **140**: 1353–1366.
- Hillis DM, Bull JJ. 1993.** An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182–192.
- Holland BS, Hadfield MG. 2002.** Islands within an island: phylogeography and conservation genetics of the endangered Hawaiian tree snail *Achatinella mustelina*. *Molecular Ecology* **11**: 365–375.
- Hori M. 1993.** Frequency-dependent natural selection in the handedness of scale-eating cichlid fish. *Science* **260**: 216–219.
- Howard DL. 1993.** Reinforcement: Origin, dynamics, and fate of an evolutionary hypothesis. In: Harrison RG, ed. *Hybrid zones and the evolutionary process*. Oxford: Oxford University Press, 46–69.
- Johnson MS. 1982.** Polymorphism for direction of coil in *Partula suturalis*: behavioural isolation and positive frequency dependent selection. *Heredity* **49**: 145–151.
- Johnson MS, Murray J, Clarke B. 1987.** Independence of genetic subdivision and variation for coil in *Partula suturalis*. *Heredity* **58**: 307–313.
- Kemperman TCM. 1992.** Systematics and evolutionary history of the *Albinaria* species from the Ionian islands of Kephallinia and Ithaka (Gastropoda Pulmonata: Clausiliidae). DPhil Thesis, Leiden University. Univ. Book. Serv., Leiden.
- Ledergerber S, Baminger H, Bisenberger A, Kleewein D, Sattmann H, Baur B. 1997.** Differences in resting-site preference in two coexisting land snails, *Arianta arbustorum* and *Arianta chamaeleon* (Helicidae), on alpine slopes. *Journal of Molluscan Studies* **63**: 1–8.
- Lipton CS, Murray J. 1979.** Courtship of land snails of the genus *Partula*. *Malacologia* **19**: 129–146.
- van Moorsel CHM, Schilthuizen M, Gittenberger E. 2001.** Phylogeny reconstruction in *Albinaria*: incompatible results, causes and solutions. In: *Molecular phylogenetics of a speciose group: Albinaria and the search for homology*. Unpublished DPhil Thesis. Leiden University, 87–103.
- Murray J, Clarke B. 1980.** The genus *Partula* on Moorea: speciation in progress. *Proceedings of the Royal Society of London, Series B* **211**: 83–117.
- Murray J, Clarke B. 1966.** The inheritance of polymorphic shell characters in *Partula* (Gastropoda). *Genetics* **54**: 1261–1277.
- Murray J, Johnson MS, Clarke B. 1982.** Microhabitat differences among genetically similar species of *Partula*. *Evolution* **36**: 316–325.
- Mylonas M, Krimbas C, Tsakas S, Ayoutanti A. 1987.** The genus *Albinaria* VEST. (Clausiliidae, Gastropoda). Is there any true species? *Biologia Gallo-Hellenica* **13**: 161–164.
- Noor MAF. 1999.** Reinforcement and other consequences of sympatry. *Heredity* **83**: 503–508.
- Nordsieck H. 1969.** Zur Anatomie und Systematik der Clausilien, VI. Genitalsystem und Systematik der Clausiliidae, besonders der Unterfamilie Alopiinae. *Archiv für Molluskenkunde* **99**: 247–265.
- Nordsieck H. 1972.** Zur Anatomie und Systematik der Clausilien, XI. Neue Formen und taxonomische Revision einiger Gruppen der Alopiinae. *Archiv für Molluskenkunde* **102**: 1–51.
- Nordsieck H. 1974.** Zur Anatomie und Systematik der Clausilien, XV. Neue Clausilien der Balkan-Halbinsel (mit taxonomischer Revision einiger Gruppen der Alopiinae und Baleinae). *Archiv für Molluskenkunde* **104**: 123–170.
- Nordsieck H. 1978.** Beobachtungen bei der Haltung von Aloprien. *Mitteilungen der deutschen malakozoologischen Gesellschaft* **3**: 371–373.
- Orr HA. 1991.** Is single-gene speciation possible? *Evolution* **45**: 764–769.
- Page RDM, Holmes EC. 1998.** *Molecular evolution. A phylogenetic approach*. Oxford: Blackwell Science.
- Peake JF. 1973.** Species isolation in sympatric populations of the genus *Diplommatina* (Gastropoda, Prosobranchia, Cyclophoridae, Diplommatininae). *Malacologia* **14**: 303–312.
- Ponder WF, Colgan DJ, Gleeson DM, Sherley GH. 2003.** Relationships of *Placostylus* from Lord Howe Island: an

- investigation using the mitochondrial cytochrome *c* oxidase gene. *Molluscan Research* **23**: 159–178.
- Schilthuizen M. 1995.** A comparative study of hybrid zones in the polytypic land snail *Albinaria hippolyti*. *Netherlands Journal of Zoology* **45**: 261–290.
- Schilthuizen M, Gittenberger E, Gultyaev AP. 1995.** Phylogenetic relationships inferred from the sequence and secondary structure of ITS1 rRNA in *Albinaria* and putative *Isabellaria* species (Gastropoda, Pulmonata, Clausiliidae). *Molecular Phylogenetics and Evolution* **4**: 457–462.
- Schilthuizen M, Lombaerts M. 1995.** Life on the edge: a hybrid zone in *Albinaria hippolyti* (Gastropoda: Clausiliidae) from Crete. *Biological Journal of the Linnean Society* **54**: 111–138.
- Servedio MR. 2001.** Beyond reinforcement: the evolution of premating isolation by direct selection on preferences and postmating, prezygotic incompatibilities. *Evolution* **55**: 1909–1920.
- Servedio MR, Noor MAF. 2003.** The role of reinforcement in speciation: theory and data. *Annual Review of Ecology, Evolution and Systematics* **34**: 339–364.
- Shimizu Y, Ueshima R. 2000.** Historical biogeography and interspecific mtDNA introgression in *Euhadra peliomphala* (the Japanese land snail). *Heredity* **85**: 84–96.
- Swofford DL. 2002.** *PAUP*: phylogenetic analysis using parsimony (* and other methods)*, Version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.
- Swofford DL, Olsen GJ, Waddell PJ, Hillis DM. 1996.** Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular systematics*. Sunderland, Massachusetts: Sinauer Associates, 407–514.
- Ueshima R, Asami T. 2003.** Single-gene speciation by left-right reversal. *Nature* **425**: 679.
- Uit de Weerd DR, Piel WH, Gittenberger E. 2004.** Widespread polyphyly among Aloiinae snail genera: when phylogeny mirrors biogeography more closely than morphology. *Molecular Phylogenetics and Evolution* **33**: 533–548.
- Uit de Weerd DR, Schneider D, Gittenberger E. 2005.** The provenance of the Greek land snail species *Isabellaria pharsalica*: molecular evidence of recent passive long-distance dispersal. *Journal of Biogeography* **32**: 1571–1581.
- Wullschleger EB, Wiehn J, Jokela J. 2002.** Reproductive character displacement between the closely related freshwater snails *Lymnaea peregra* and *L. ovata*. *Evolutionary Ecology Research* **4**: 247–257.
- Zilch A. 1959.** Euthyneura 2. In: Schindewolf OH, ed. *Handbuch der Paläozoologie* 6. *Gastropoda* 2. Berlin: Borntraeger, 201–400.