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Disassociation between weak sexual isolation and genetic divergence in a hermaphroditic land snail and implications about chirality

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Abstract

Examination of the association between reproductive isolation and genetic divergence in a variety of organisms is essential for elucidating the mechanisms causing speciation. However, such studies are lacking for hermaphrodites. We measured premating (sexual) isolation in species pairs of the hermaphroditic land snail *Albinaria* and we compared it with their genetic divergence. We did not find substantial sexual isolation barriers between the species studied. The absence of strong sexual isolation between species implies its minor effect in the evolution of this genus, because distributional, population and life-history characteristics of *Albinaria* make mate-choice possibly redundant. Furthermore, we found disassociation between genetic divergence and sexual isolation, suggesting that they do not form necessarily a cause–effect duet. However, *Albinaria voithii*, the only dextral *Albinaria* species, shows strong sexual isolation against the other sinistral species. We discuss whether change in coiling either has triggered instantaneous speciation, or is an example of character displacement.

Introduction

Even though we can presume the main factors contributing to speciation, their relative importance remains blurred. Reproductive isolation is central for speciation processes, but the relative roles of isolation and selection are controversial (Schilthuizen, 2000), while several questions are still open, for example, whether reproductive isolation arises before or after significant divergence (Berlocher, 1998). Reproductive isolation is the result of premating and/or post-mating barriers. Premating barriers are considered more effective, faster and easier to build up especially in sympatry and in strongly dimorphic taxa (Mendelson, 2003; Coyne & Orr, 2004). Premating isolation includes differences in behaviour, ecology, morphology and reproductive time. Usually, sexual (behavioural) isolation is measured in the laboratory (Rice & Hostert, 1993; Coyne & Orr, 1997), although in a few species it is possible to measure directly in the field (Cruz *et al.*, 2004). The general evidence is that reproductive isolation is positively correlated with genetic divergence (Coyne & Orr, 1989, 1997; Tilley *et al.*, 1990; Sasa *et al.*, 1998; Presgraves, 2002; Christianson *et al.*, 2005), but this is not always the case, especially for sexual isolation (Tilley *et al.*, 1990; Gleason & Ritchie, 1998; Edmands, 2002; Ferguson, 2002; Tregenza, 2002; Moyle *et al.*, 2004). Therefore, similar studies on a variety of organisms with different biological attributes can be useful for clarifying the process of speciation. Furthermore, 'instantaneous' reproductive isolation, that occurs when a single trait becomes fixed in a population, rendering it reproductively isolated from others (Gittenberger, 1988; Orr, 1991), is of particular interest.

Simultaneous hermaphrodites, and particularly land snails, are appealing candidates for studies of reproductive isolation. Their biological properties (e.g. no separate

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sexes, reciprocal sperm transfer, uniparental reproduction) distinguish them from gonochorists, especially for the processes of sexual isolation (for a review see Baur, 1998; Michiels, 1998). For example, as there are no separate sexes, sexual selection has probably a minor role for the evolution of sexual isolation in hermaphrodites (Greeff & Michiels, 1999). In many land snail species partners mount reciprocally each other's shell and crawl in a complicated, yet stereotyped, way before accomplishing copulation (Baur, 1998). This precopulatory behaviour perhaps makes possible the evolution of mate-choice mechanisms and the development of sexual isolation barriers (Schilthuizen, 2003). However, there are relatively few experimental studies on the reproductive isolation between different land snail species (see Schilthuizen & Davison, 2005), possibly because in hermaphrodites it is difficult to get estimates of sexual isolation from mating frequency data, due to the existence of a pooled class of heterotypic pairs instead of the two classes observed in gonochorists and used in sexual isolation estimators (reviewed in Pérez-Figueroa et al., 2005).

Land snails may exhibit mirror-image dimorphism, coiled either clockwise (dextral), or anticlockwise (sinistral). Coiling direction is determined by the maternal genotype at a single locus, with genetic dominance of either the dextral or the sinistral allele (Degner, 1952; Murray & Clarke, 1976; Freeman & Lundelius, 1982). However, most land snail taxa are uniformly coiled, and pervasive stable dimorphism is restricted to very few genera (Rolán-Alvarez & Rolán, 1995; Asami *et al.*, 1998), due to frequency-dependent selection (Lipton & Murray, 1979; Johnson *et al.*, 1990). During mating between individuals with opposite coil, successful copulation is hampered, mainly in snails with globular shells, but also, to a lesser extent, in highly spired ones (Rolán-Alvarez & Rolán, 1995; Asami *et al.*, 1998).

Albinaria (Pulmonata, Clausiliidae), a model organism for speciation in terrestrial molluscs (Schilthuizen, 2003), is a land snail genus very common in southern Greece and coastal Asia Minor. It lives usually in high densities on limestone rocks. In nature, specimens mature in 1.5 years, generation time is about 2–2.5 years and life span approximates 7 years (Giokas & Mylonas, 2004). They are active from mid-October with the start of autumn rains, until the beginning of the dry season in late April, when they aestivate for about 5 months. During aestivation, they attach firmly on rock-surfaces and seal their aperture with a thick mucus barrier known as epiphragm (Giokas & Mylonas, 2002). Copulation starts immediately after arousal and eggs are laid approximately after 3 weeks. Shells are sinistral (except for *Albinaria voithii*), slender, spindle-shaped, and high-spired, of about 2 cm height.

There are about 100 *Albinaria* species described (Nordsieck, 1999). Most of them are endemic and have narrow, non-overlapping ranges. Usually there is only a single species at each locality; there are few cases of parapatry, but genuine sympatry is uncommon. The genus exhibits high genetic differentiation and divergence in shell morphology, but both these patterns of divergence are variable (Kemperman & Degenaars, 1992; Ayoutanti *et al.*, 1993; Douris *et al.*, 1995; Schilthuizen & Gittenberger, 1996), and do not seem to be associated with ecological differentiation (Gittenberger, 1991; Schilthuizen, 2003). On the contrary, there is no significant differentiation in genital anatomy among *Albinaria* species (Schilthuizen & Lombaerts, 1995). Additionally, morphological and genetic hybridization and introgression have been reported in some contact zones (e.g. Gittenberger, 1991; Welter-Schultes, 1998; Giokas *et al.*, 2000). This variable disassociation between morphology, ecology and genetic divergence has promoted significant research on *Albinaria*'s evolution during the last two decades.

Reproductive isolation in *Albinaria* has been studied only for a few syntopic species (Schilthuizen, 1994; Giokas *et al.*, 2000) or subspecies (Schilthuizen & Gittenberger, 1994; Schilthuizen, 1995; Schilthuizen & Lombaerts, 1995; Schilthuizen *et al.*, 1999) pairs, by estimation of gene flow and/or hybrid viability. These studies revealed hybrid inferiority and limited gene flow. However, direct estimates of sexual isolation are either lacking or speculative, i.e. the former studies did not provide indices of sexual isolation (Mylonas *et al.*, 1987). Indeed, accurate estimates of sexual isolation are essential to trace factors that are likely to contribute to isolation (i.e. morphology, genetic divergence) and for inferring hypotheses about speciation processes in *Albinaria*.

In the present study, our aim was to examine the contribution of sexual isolation in the evolution of *Albinaria* and to compare its association with genetic divergence and geographic distance. This is the first study in hermaphroditic animals where estimates of sexual isolation and genetic divergence are compared. We measured the degree of sexual isolation among genetically diverged *Albinaria* species, which have been geographically separated for variable time periods. The studied species are unquestionably valid from a phylogenetic perspective, even though their phylogeny reconstructions using mitochondrial and nuclear markers are not always congruent (Douris *et al.*, 1998a, b; Moorsel van *et al.*, 2000; De Weerd & Gittenberger, 2005). For the estimation of sexual isolation we performed a series of mate-choice experiments between several *Albinaria* species. To measure rates of evolution of sexual isolation, we compared, genetic distances (from allozymes and mtDNA) and geographic distances with the magnitude of sexual isolation.

Materials and methods

Selection and collection of species

We selected seven *Albinaria* species for which there were available genetic divergence estimates from previous studies (Ayoutanti *et al.*, 1993; Ayoutanti, 1994; Douris *et al.*, 1995, 1998a, b; Douris, 1997) (Appendix S1). We chose species that are valid on morphological and molecular grounds (Douris *et al.*, 1998a, b; Nordsieck, 1999; Moorsel van *et al.*, 2000; De Weerd & Gittenberger, 2005). Furthermore, we used representative populations of species exhibiting a wide spectrum of genetic and morphological divergence, geographic proximity, and palaeogeographic isolation, and whose geographic ranges do not currently overlap, except for the parapatric occurrence of *A. schuchii* and *A. voithii* at a single locality (see Appendix S1 for the list of sampling localities). Adult individuals were collected within a small sampling area (100 × 100 cm) from each locality during aestivation, and each individual was kept separately in dry conditions (wrapped with soft paper) to prevent accidental early arousal and copulation, before the start of the experiments.

Mate-choice experiments

We performed 21 multiple mate-choice experiments in two subsequent years during the normal copulation period for *Albinaria* (mid October–early November). Two types of experiments were performed: 'Single design' using one pair of species (11 experiments), and 'Pooled design' (10 experiments) with more than one species pair (Table 1 and Appendices S1–S3). We used various combinations of species and populations. Experiments 1 and 3, 8 and 9, 14 and 23, 20 and 28 were identical. In each experiment (except experiment 4), all populations had an equal number of specimens (varying from 10 to 40 among experiments). In order to emulate the natural mating conditions of these species, marked specimens were sprayed with water to emerge from aestivation, and placed in glass 'arenas' (40 × 60 × 40 cm) with soil, stones and food. We kept wet conditions during the experiments. We recorded the number of successful copulations, i.e. when penises were fully inserted in the genital openings of each partner, and we kept notes about mating behaviour, during at least 12 h.

Estimation of sexual isolation

We used the number of observed mating pairs to estimate sexual (premating) isolation as in multiple-choice experiments (*sensu* Knoppien, 1985), where for two particular species (A and B) four different mating pairs are expected: male A–female A (AA), male A–female B (AB), male B–female A (BA), and male B female B (BB). However, *Albinaria* is hermaphrodite and so only three classes of pairs were produced: AA, AB (including both AB and BA types that cannot be distinguished) and BB types. Such difference, however, does not represent any theoretical difference for the estimation procedure, as any estimate of sexual isolation is, in fact, a measure of the relative importance of homotypic pairs (AA and BB types) to the heterotypic ones (AB pairs; Gilbert & Starmer, 1985; Knoppien, 1985; Rolán-Alvarez & Caballero, 2000). In most land snails the two individuals from every mating pair are usually reciprocally fertilized (Baur, 1998; This study). Therefore, to obtain realistic sexual isolation estimates the observed heterotypic mating pairs were equally allocated in AB and BA pair types (for example in experiment 7 from Appendix S3; the seven observed heterotypic pairs are allocated in 3.5 AB and 3.5 BA pairs).

We used the I_{PSI} sexual isolation estimator in our data set corrected as suggested above. This index is the joint isolation (*I*) index calculated on the PSI coefficients instead of observed numbers of matings (see Rolán-Alvarez & Caballero, 2000), and it represents the best-known alternative to estimate sexual isolation at biological sample sizes (Pérez-Figueroa *et al.*, 2005). Thus,

$$I_{\mathrm{PSI}} = \frac{(\mathrm{PSI}_{\mathrm{AA}} + \mathrm{PSI}_{\mathrm{BB}}) - (\mathrm{PSI}_{\mathrm{AB}} + \mathrm{PSI}_{\mathrm{BA}})}{\mathrm{PSI}_{\mathrm{AA}} + \mathrm{PSI}_{\mathrm{AB}} + \mathrm{PSI}_{\mathrm{BA}} + \mathrm{PSI}_{\mathrm{BB}}}$$

with $\mathrm{PSI}_{\mathrm{AA}}$, $\mathrm{PSI}_{\mathrm{AB}}$, $\mathrm{PSI}_{\mathrm{BA}}$ and $\mathrm{PSI}_{\mathrm{BB}}$ being the PSI coefficients for the respective mating pair combinations (Rolán-Alvarez & Caballero, 2000). The PSI coefficients are defined, for every mating pair combination, as the number of observed pair types divided by the expected pair types from mates assuming random mating (see detailed algorithms in Rolán-Alvarez & Caballero, 2000).The PSI coefficients have been proved to be the least-biased estimators of mate-choice in the presence of mate propensity (Rolán-Alvarez & Caballero, 2000). I_{PSI} varies from −1 to +1, with −1 representing maximum disassortative mating, 0 random mating, and +1 maximum assortative mating (complete sexual isolation). The estimates were exclusively calculated when at least two of the three classes of mating types (AA, AB or BB) were present, to avoid no sense sexual isolation estimates due to low sample sizes.

The significance of the I_{PSI} coefficients in 'single' multiple-choice experiments was calculated by resampling 10 000 times the observed frequencies of mating pairs using the program JMATING available on the web (http://webs.uvigo.es/genxb2/; see A. Carvajal-Rodríguez and E. Rolán-Alvarez, unpublished data) using 0.5 instead of zero values in cells (a conservative procedure which allow statistical inference when sexual isolation is maximum). The significance of estimates across 'pooled' multiple-choice experiments was calculated by the *t*-test using averaged I_{PSI} estimates obtained independently from the pseudo-replicated pooled experiments (Sokal & Rohlf, 1996).

Genetic and geographic distances and their association with sexual isolation

For the estimation of genetic divergence we used allozyme and 16S mtDNA data from previous studies (Ayoutanti *et al.*, 1993; Ayoutanti, 1994; Douris *et al.*,

Table 1 Sexual isolation estimates (I_{PSI} *sensu* Rolán-Alvarez & Caballero, 2000) from experiments with two (single) or more than two (pooled) species using always a multiple choice design.

	A. caerulea		*A. grisea*		*A. schuchii*		*A. voithii*		*A. discolor*		*A. turrita*		*A. puella*	
	Single	Pooled	Single	Pooled	Single	Pooled	Single	Pooled	Single	Pooled	Single	Pooled	Single	Pooled
A. caerulea														
Mean (SD)	0.03 (0.187)	0.14 (0.431)		0.20 (0.374)		−0.10 (0.654)	0.91 (0.094)**	0.99 (0.035)*	−0.60 (0.422)	0.26 (0.442)	$0.35^{1/2}$ (0.502)	0.20 (0.418)		0.37 (0.358)
N	3	30		9		6	1	7	1	12	2	18		12
A. grisea														
Mean (SD)						1	1 (0.192)*	0.81 (0.160)		0.56 (0.294)		0.18 (0.377)		0.34 (0.362)
N						1	1	3		4		4		3
A. schuchii														
Mean (SD)								0.72		0.29 (1.004)		−0.39 (0.564)		0.58
N								1		2		3		1
A. voithii														
Mean (SD)									0.90 (0.094)***	0.96 (0.056)*	1 (0.220)*	0.97 (0.067)*		0.88 (0.202)*
N									1	6	1	5		3
A. discolor														
Mean (SD)											−0.54 (0.410)	0.02 (0.549)		0.49 (0.172)
N											1	6		4
A. turrita														
Mean (SD)														0.49 (0.310)
N														5

N is the number of choice experiments used to calculate the mean values. The significance of the Single test was obtained by resampling 10 000 times the observed frequencies of pairs (see text), while the significance of the Pooled test was calculated by a *t*-test on the averaged estimates from pseudo-replicated choice experiments.

$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, '1/2' represent one significant ($P < 0.05$) case from two independent tests.

1995, 1998a, b; Douris, 1997) and from GenBank. We chose these two genetic markers because typically they show different levels of introgression between different species (Chan & Levin, 2005), are considered neutral and are widely used (especially allozymes) in similar studies (Edmands, 2002; Avise, 2004). Allozyme data were available for all the species and mtDNA sequences were available for all the species except for *A. schuchii* (Appendix S1). We used two types of genetic divergence estimates: Nei's (1972) distances based on allozyme frequencies for 27 loci, and Kimura's (1980) two-parameter distances for the 16S mtDNA sequences (Table 2). Genetic distances were calculated with PHYLIP 3.6 (Felsenstein, 2004).

We used the geographic distances between the studied populations (Appendix S1) to calculate the average and the minimum geographic distances among the studied species (Table 2).

We examined the association between genetic distances and sexual isolation for pairs of species comparing their respective matrices as in Coyne & Orr (1989, 1997). We used the matrices of (i) sexual isolation estimates (using preferentially the pooled estimates from Table 1), (ii) Nei genetic distances for allozyme loci, (iii) Kimura two-parameter distances for mtDNA sequences, (iv) average geographic distances and (v) minimum geographic distances. We performed standard correlation analysis between the former matrices, but due to the possible absence of independence in different replicates, we employed also the Mantel test to determine their significance (Mantel, 1967; Smouse *et al.*, 1986), using NTSYSpc (Rohlf, 2002).

Results

Mating behaviour and sexual isolation

Immediately after emerging, individuals started looking for partners (not for food, even if it were available). Partners mounted reciprocally each other's shell and they were crawling over the shell surface of its mate with the shells aligned in parallel before accomplishing copulation. In general, we did not observe any prior preference for mates but several (not counted) mounting attempts were repeatedly unsuccessful, between and within different species. These failed mating attempts were abandoned before the protrusion of penises. This precopulatory behaviour lasted for about 15 min. Afterward, in cases of successful copulations, penises were protruded and fully inserted in the genital openings of each partner. Partners remained joined, on average for 20 min, transferring spermatophores. Mating success, i.e. the percentage of successfully copulating individuals in each experiment, varied from 18.5% to 73.8% (mean = 55.2, SD = 16.1) (Appendix S2). Differences in mating success were not systematic, i.e. they were not correlated with the number of populations and individuals used in each experiment (results not shown), and perhaps can be attributed to stochastic differential response to experimental conditions. In any case, our index of sexual

Table 2 Genetic and geographic distances between the studied *Albinaria* species. Genetic distances (Nei distances for allozymes and Kimura distances for mtDNA) are shown above the diagonal, and geographic distances (average and minimum, in km) are shown below the diagonal.

	A. caerulea		*A. grisea*		*A. schuchii*		*A. voithii*		*A. discolor*		*A. turrita*		*A. puella*	
	Nei	Kimura	Nei	Kimura	Nei	Kimura	Nei	Kimura	Nei	Kimura	Nei	Kimura	Nei	Kimura
A. caerulea														
Average			0.937	0.0994	0.479	–	0.628	0.1232	0.574	0.1090	0.554	0.1135	0.328	0.1194
Minimum														
A. grisea														
Average	197.9				0.537	–	0.706	0.0985	0.782	0.0948	0.905	0.1053	1.000	0.1084
Minimum	23													
A. schuchii														
Average	289.2		200				0.400	–	0.356	–	0.443	–	0.550	–
Minimum	207		200											
A. voithii														
Average	289.8		179		33				0.448	0.1019	0.571	0.1243	0.879	0.1153
Minimum	171		158		0									
A. discolor														
Average	242.5		120.3		83.3		80.5				0.443	0.1136	0.761	0.1118
Minimum	51		37		31		31							
A. turrita														
Average	130.4		97		210.5		204.3		153				0.656	0.1138
Minimum	41		60		207		189		81					
A. puella														
Average	158.9		228		382.3		374.8		317		183.8			
Minimum	25		208		361		353		243		159			

isolation is not affected by these differences (Rolán-Alvarez & Caballero, 2000).

We found weak and no significant sexual isolation between most of the species studied, although *A. puella* showed apparently high (but nonsignificant) sexual isolation with the others (Table 1). However, there was a notable exception; *A. voithii*, the only dextral species, exhibited significant sexual isolation when compared with the sinistral species. There was also an apparently case of sexual isolation between *A. schuchii* and *A. grisea* (but no test available as it belonged to a pooled design without replication). The estimates of sexual isolation were similar in 'single' and 'pooled' designs, especially for *A. voithii*.

Sexual isolation, geographic distance and genetic divergence

We found no significant association between sexual isolation and genetic divergence (Fig. 1) ($I_{\rm PSI}$ vs. Nei distances: $r = 0.073$, $P = 0.752$, $N = 21$; $I_{\rm PSI}$ vs. Kimura distances: $r = 0.195$, $P = 0.486$, $N = 15$). Mantel test on sexual isolation and genetic divergence matrices yielded the same outcome ($I_{\rm PSI}$ vs. Nei distances: $P = 0.385$; $I_{\rm PSI}$ vs. Kimura distances: $P = 0.414$). Similarly, lack of association was found between allozymic and mtDNA divergence (Nei vs. Kimura distances: $r = -0.375$, $P = 0.116$, $N = 15$; Mantel test, Nei vs. Kimura distances: $P = 0.173$). We did not find any significant association even when we excluded *A. voithii* from our analyses, in order to test if this reverse-coiled species had a special influence on the results (results not shown).

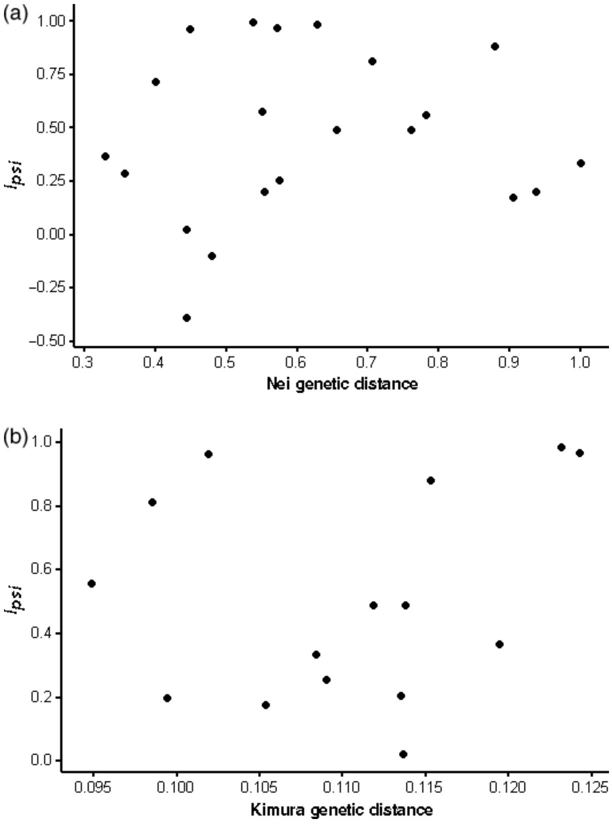

Fig. 1 Scatterplots showing the relationships between sexual isolation (from pooled data) and Nei genetic (allozymic) distance (a), and Kimura genetic (mtDNA) distance (b).

Similarly, we found lack of association between average geographic distance and $I_{\rm PSI}$, Nei and Kimura distances ($I_{\rm PSI}$ vs. average geographic distances: $r = 0.069$, $P = 0.768$, $N = 21$; Nei vs. average geographic distances: $r = 0.287$, $P = 0.207$, $N = 21$; Kimura vs. average geographic distances: $r = 0.425$, $P = 0.114$, $N = 15$). Mantel test confirmed these results ($I_{\rm PSI}$ vs. average geographic distances: $P = 0.624$; Nei vs. average geographic distances: $P = 0.886$; Kimura vs. average geographic distances: $P = 0.936$). The same result was observed when we used the minimum geographic distances (results not shown).

Discussion

Our study showed that the *Albinaria* species studied, except the dextral *A. voithii*, have not evolved substantial sexual isolation barriers. The absence of strong sexual isolation implies that it has played a minor role in the evolution of this group. Our results could be biased by the particular characteristics of the species studied, i.e. they are hermaphrodites and even though copulation is reciprocal they occasionally may behave preferentially like male or female (Baur, 1998). However, results for *A. voithii* were unambiguous and repeatable, suggesting that any possible bias is overwhelmed when high sexual isolation exists.

Several factors may account for the weak sexual isolation observed. One might be allopatry. Many *Albinaria* species, especially on islands, have evolved and remain in geographical isolation for long (2–9 Myr; Douris *et al.*, 1995, 1998a, b), and as elevated sexual isolation is usually correlated with sympatry (Coyne & Orr, 1989, 1997) perhaps they were not forced to develop strong sexual isolation. This is also implied by the absence of genital differentiation (Schilthuizen & Lombaerts, 1995). Although post-mating isolation may exist in the form of hybrid inferiority (Schilthuizen, 1994; Giokas *et al.*, 2000), the lack of secondary contact between most of these species, makes it unlikely that reinforcement plays a major role on the development of sexual isolation (Servedio, 2004). Another factor might be that for *Albinaria* individuals it is easy to find a partner, given that it is a hermaphroditic organism and forms large and dense populations. Thus, it seems that sperm waste is not a major constraint. Furthermore, because the copulatory period is short (Giokas & Mylonas, 2002) they have to mate in a short time with the first partner available. In our experiments, individuals started to search for partners and to copulate immediately after arousal. The short copulatory period of several land snails is related to unpredictable adverse conditions, dehydration, locomotion and energy cost, and predation pressure during copulation (Baur, 1998). Additionally, populations of *Albinaria* are strongly structured, i.e. they consist of demes, with very little migration between neighbouring demes (Schilthuizen & Lombaerts, 1994; Giokas & Mylonas, 2004). This can result in mating between relatives and may increase inbreeding and its potential deleterious effects. Consequently, favouring migrant individuals could be advantageous.

The lack of correlation between genetic divergence and sexual isolation implies that in *Albinaria* genetic divergence is a weak predictor of sexual isolation. This outcome is unusual, but not unique, for animals (Tilley *et al.*, 1990; Gleason & Ritchie, 1998; Tregenza & Butlin, 1999; Ferguson, 2002; Coyne & Orr, 2004). Moyle *et al.* (2004) report a similar disassociation between sexual isolation and genetic divergence for two angiosperm genera (hermaphroditic plants). The erratic evolution of sexual isolation might be related to the strength of selection and the lability of behaviour (Edmands, 2002). Furthermore, a high positive correlation between sexual isolation and genetic divergence is predicted when sympatry prevails (Coyne & Orr, 1989, 1997) and for strongly sexual dimorphic taxa where there is a more important role for sexual selection (Mendelson, 2003). Although, sexual selection is a potential cause of speciation in land snails (Charnov, 1979; Schilthuizen, 2003; Parmakelis *et al.*, 2005), its significance for hermaphrodites is disputed (Greeff & Michiels, 1999), and empirical evidence is still lacking. Therefore, more studies on hermaphroditic organisms are necessary to confirm whether they are exceptional for the evolution of sexual isolation.

An alternative explanation might be that we missed isolation because we looked across a long or a short evolutionary spectrum, or we did not examine enough species. However, our sample of species and populations is representative, as it includes both phylogenetically close and distant species with variable geographical and historical isolation (Douris *et al.*, 1995, 1998a, b; Moorsel van *et al.*, 2000; De Weerd & Gittenberger, 2005). Therefore, the disassociation between sexual isolation and genetic divergence is not due to insufficient time to accumulate reproductive isolation or to a nonrepresentative sample of species. Perhaps, the absence of a clock-like evolution of sexual isolation can be attributed to the act of different evolutionary forces on isolation processes in different lineages (Reed & Frankham, 2001; Edmands, 2002).

The lack of correlation between the two estimates of genetic divergence might reflect different selective pressures on different lineages for different markers (Tregenza & Butlin, 1999; Fitzpatrick, 2002) or different levels of introgression between species (Chan & Levin, 2005). Such disassociation has been found in many organisms (Chan & Levin, 2005), and particularly in snails (e.g. Pinceel *et al.*, 2004). This disassociation possibly implies departures from neutral evolution for one or both markers. In principle, both markers are considered as neutral (Avise, 2004) but there is accumulated evidence that they can be affected by selection (Rand, 2001; Shapiro & Von Sternberg, 2005). This evidence questions their utility for examining the effect of selection on reproductive isolation. However, in many cases they still provide our only means of estimating evolutionary timing (Edmands, 2002). Even though allozymes are more subject to the action of natural selection than mtDNA, recent meta-analyses (Fitzpatrick, 2002) suggest that allozymes are more tightly correlated with the degree of reproductive isolation than silent DNA differences, and perhaps are more useful for studying reproductive isolation and the effect of selection.

The absence of correlation between the current geographic distances and sexual isolation and genetic divergence is expected as the area of distribution of *Albinaria*, and particularly the Aegean archipelago, has a complex palaeogeographic history (Meulenkamp, 1985), where erratic vicariant events prevail and not isolation by distance processes. Several studies (Douris *et al.*, 1995, 1998a; Beerli *et al.*, 1996; Chatzimanolis *et al.*, 2003; Poulakakis *et al.*, 2003, 2005; Kasapidis *et al.*, 2005; Parmakelis *et al.*, 2005), suggest that the present, often mosaic, distribution of most of the terrestrial Aegean taxa, is mainly shaped through vicariant phenomena resulting from the complex geological history of the east Mediterranean and especially of the Aegean archipelago. Furthermore, long-distance dispersal over sea is also involved (Douris *et al.*, 1998a; Dennis *et al.*, 2000; Kasapidis *et al.*, 2005).

Although we observed several mating attempts between snails of opposite coiling, the strong sexual isolation of the dextral *A. voithii* against all the other sinistral species contrasts previous reports of successful copulations between individuals of opposite chirality in clausiliids (Degner, 1952; Nordsieck, 1978). What is, therefore, the effect of shell coiling in the evolution of reproductive isolation in land snails and especially in this genus? Both experimental evidence and computer simulations have yielded contradictory results (for a review see Schilthuizen & Davison, 2005). Some researchers suggest that chirality can be an effective reproductive barrier because snails differing in coiling mate less frequently than those with the same coiling (Lipton & Murray, 1979). Ueshima & Asami (2003) proposed that left–right reversal in reciprocally mating snails could accomplish sexual isolation at a single locus, overcoming the majority rule of chirality. Furthermore, theoretical studies suggest that this kind of single-gene speciation, although very rare, is possible, under certain conditions such as small, isolated populations, with few invaders and low mating success (Orr, 1991; van Batenburg & Gittenberger, 1996; Stone & Björklund, 2002).

On the contrary, others assert that because of the physical difficulty for copulation between snails having opposite coiling, frequency-dependent selection reduces the chiral minority and prohibits chiral speciation

(Johnson, 1982; Johnson *et al.*, 1990; Asami *et al.*, 1998). Studies on *Partula* (Clarke *et al.*, 1996; Goodacre, 2002) and *Achatinella* (Holland & Hadfield, 2002) suggest that different coiling does not impose a long-term barrier to gene flow, and is unlikely to have been important in speciation because of its unusual pattern of inheritance. Model simulations resulted in a small likelihood that coil reversal can initiate single-gene speciation in land snails (Davison *et al.*, 2005), as the delayed inheritance makes such reproductive barriers ineffective, and hence speciation by chiral reversal is possible only in the context of a complex biogeographical process. Reverse-coiled species are more likely to show reinforcement or reproductive character displacement that can promote the evolution of new chiral morphs, tending to counteract the positive frequency-dependent selection that would otherwise drive the more common chiral morph to fixation (De Weerd *et al.*, 2005).

Lacking more data it is difficult to conclude if coil reversal in *A. voithii* represents an example of character displacement or initiated single-gene speciation. The complete sexual isolation of *A. voithii* and the strong structure of *Albinaria* populations may account for the fixation of opposite chirality. An intermediate alternative would be if the change of coiling direction can contribute to the speciation process, but without being necessarily the principal factor. The populations of *A. voithii* are geographically surrounded by four sinistral *Albinaria* species, and perhaps in the past, possible contacts and production of inferior hybrids may have reinforced reproductive isolation by fixing different chiral forms in the incipient ecological species. Such a possibility could explain the above apparently contradictory results from simulations. Therefore, to choose the most probable scenario we need estimates of gene flow between sinistral and dextral species in the current contact zones.

Even though we found that sexual isolation has a small contribution to the evolution of *Albinaria* species, it is still unclear which factors trigger and accomplish speciation. The role of natural selection is obscure as, until now, research on *Albinaria*'s ecology has not revealed any clear association between ecological differentiation and species divergence (Gittenberger, 1991; Schilthuizen, 2003). Furthermore, in the absence of sexual isolation, we can consider other isolating barriers, such as hybrid inferiority and sperm competition (Baur, 1998). Therefore, much more work in needed to uncover how divergence and subsequent speciation relate to environmental conditions and adaptation. Studies on contact zones and more elaborate mate-choice and breeding experiments (Schilthuizen, 2003) may clarify questions about the evolutionary processes in these land snails.

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Supplementary Material

The following supplementary material is available for this article online:

Appendix S1. List of species and populations, sampling localities (with abbreviations), number of experiments (pooled or single) for each population, and availability of genetic divergence estimates for each population.

Appendix S2. List of the experiments, showing for each experiment the number of the populations used, the number of individuals per population, the percentage of mated individuals, and the observed number of homotypic and heterotypic matings.

Appendix S3. The observed number of mating pairs in each experiment (in matrix format). For abbreviations of populations, see Appendix S1.

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