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Nonallopatric and parallel origin of local reproductive barriers between two snail ecotypes

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Abstract

Theory suggests that speciation is possible without physical isolation of populations (hereafter, nonallopatric speciation), but recent nonallopatric models need the support of irrefutable empirical examples. We collected snails (*Littorina saxatilis*) from three areas on the NW coast of Spain to investigate the population genetic structure of two ecotypes. Earlier studies suggest that these ecotypes may represent incipient species: a large, thick-shelled 'RB' ecotype living among the barnacles in the upper intertidal zone and a small, thin-shelled 'SU' ecotype living among the mussels in the lower intertidal zone only 10–30 m away. The two ecotypes overlap and hybridize in a midshore zone only 1–3 m wide. Three different types of molecular markers [allozymes, mitochondrial DNA (mtDNA) and microsatellites] consistently indicated partial reproductive isolation between the RB and the SU ecotypes at a particular site. However, each ecotype was related more closely to the other ecotype from the same site than to the same ecotype from another site further along the Galician coast (25–77 km away). These findings supported earlier results based solely on allozyme variation and we could now reject the possibility that selection produced these patterns. The patterns of genetic variation supported a nonallopatric model in which the ecotypes are formed independently at each site by parallel evolution and where the reproductive barriers are a byproduct of divergent selection for body size. We argue that neither our laboratory hybridization experiments nor our molecular data are compatible with a model based on allopatric ecotype formation, secondary overlap and introgression.

Keywords: allozymes, ecological speciation, *Littorina saxatilis*, microsatellites, mtDNA, sympatric divergence

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Introduction

Whether or not reproductive barriers can evolve within a continuous population, that is, in the absence of a physical barrier, is a central issue in the evolutionary debate (Kawecki 1996; Turelli *et al*. 2001; Via 2001; Porter & Johnson 2002). If gene flow is impeded by a physical barrier, stochastic mechanisms such as genetic drift and mutational divergence cause the genomes of the separated populations to become increasingly incompatible (Turelli *et al*. 2001). In sympatric and parapatric situations, stochastic divergence is constrained by gene flow and instead reproductive isolation may result from adaptation to the local ecological conditions by the diverging populations (Schluter 2001). Reproductive isolation may sometimes require sexual selection (Panhuis *et al*. 2001). Classical models of sympatric (reviewed in Johnson & Gullberg 1998) and parapatric speciation (Endler 1977) as well as more recent theoretical models (Doebeli 1996; Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999; Gavrilets & Waxman 2002; Porter & Johnson 2002) suggest various mechanisms of nonallopatric (sympatric

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and parapatric) speciation. A recent model shows that parapatric speciation may be promoted easily by environmental gradients of intermediate steepness (Doebeli & Dieckmann 2003). Several possible examples of nonallopatric speciation have been found in nature (Feder *et al*. 1994; Schliewen *et al*. 1994; Rundle *et al*. 2000; Via 2001; Hendry *et al*. 2002); however, many are disputed because it is usually impossible to reject the alternative explanation of allopatric origin followed by secondary overlap and introgression (but see Berlocher 1998 for biogeographical and phylogenetic patterns that distinguish allopatric and sympatric speciation). Most possible examples also suffer from contradictory or insufficient knowledge about the actual mechanism of divergence (Turelli *et al*. 2001; Jiggins & Bridle 2004).

Strong support for divergence attributable to ecological mechanisms of separation occurs when similar reproductive barriers have evolved independently in separate locations from an ancestral species. A good example is known in stickleback fishes. Large-bodied benthic species prefer mating with a benthic species from another lake rather than with a small-bodied limnetic species from the same lake, even though molecular data show that benthic morphs from different lakes are not related more closely to one another than they are to limnetic morphs of the same lake (Nagel & Schluter 1998; Rundle *et al*. 2000; McKinnon *et al*. 2004). Another example is the walking-stick insect, where populations living on different host plants have diverged in parallel for many morphological and behavioural traits; this suggests that natural selection (host plant adaptation) has played a crucial role in the origin of the reproductive isolation (Nossil *et al*. 2002). These scenarios support ecological models of speciation as it is unlikely that stochastic events will result in exactly the same mechanism of reproductive isolation in different geographical areas (Johannesson 2001). They also suggest that environmental gradients that are consistent over large geographical regions can result in ecotypes that evolve in parallel at different sites. If, furthermore, reproductive isolation between the two ecotypes becomes complete then each may evolve into one polyphyletic species through a process of parallel speciation (Schluter & Nagel 1995; Johannesson 2001; Schluter 2001).

We investigate patterns of population genetic structure to examine the extent of genetic divergence at neutral molecular markers between two Spanish ecotypes of a snail species (*Littorina saxatilis*) which shows a microparapatric distribution along the steep environmental gradient from the upper to the lower intertidal zone along the Atlantic coast of Galicia (NW Spain). By microparapatric we mean parapatric over a scale of 10–30 m. Using the definition of sympatric by Futuyma & Mayer (1980), one could possibly argue that the distribution is, rather, sympatric as the dispersal ability of adult snails are such that they can easily move over the complete intertidal zone. The morphological divergence between the two ecotypes is extreme among marine gastropods, and is maintained by strong divergent natural selection most probably on body size and shell thickness, between the upper and lower intertidal habitats (Johannesson *et al*. 1993; Rolán-Alvarez *et al*. 1997). Strong (> 50%) premating reproductive isolation exists when the two ecotypes overlap in a narrow midshore hybrid zone and it has been suggested previously that this is an example of incipient nonallopatric speciation (Johannesson *et al*. 1995a; Rolán-Alvarez *et al*. 1999). However, the earlier use of allozyme markers made alternative explanations possible (due to selected variation in some or all the analysed loci; see, e.g. Johannesson & Tatarenkov 1997). In the present study we added putatively neutral (noncoding) DNA markers to resolve this dilemma. We also used a more complete analysis of the data, for example more detailed partitions of the population genetic structure, and this allowed us to test alternative hypotheses explaining the origin and maintenance of the observed polymorphism. We found the same pattern of variation at three types of molecular markers [seven allozyme loci, one mitochondrial DNA (mtDNA) gene and eight microsatellite loci] which, together with a reanalysis of mating pairs and some laboratory crosses between ecotypes, supported an ecologically driven mechanism for evolution of the reproductive isolation between microparapatric populations. Moreover, our extended analysis showed that this mechanism of isolation has evolved repeatedly and in parallel in different geographical areas.

Materials and methods

L. saxatilis *in Galicia, NW Spain*

The rough periwinkle (*L. saxatilis* (Olivi), Gastropoda) is native to North Atlantic rocky shores (Reid 1996). Sexes are separate and males and females copulate year round to produce juveniles that emerge from the female brood pouch and crawl away to start their life as grazers on microalgae and lichens growing on the rock. Juveniles and adults have a restricted dispersal in the range of a few metres per month, although they occasionally move much longer distances. Populations are typically strongly adapted to local environmental conditions forming ecotypes in different shore habitats (Reid 1996). Wave-exposed shores in NW Spain have two microhabitats for *L. saxatilis*; an upper shore barnacle (*Chthamalus stellatus*) zone and a lower shore blue mussel (*Mytilus galloprovincialis*) zone (Johannesson *et al*. 1993). On these shores *L. saxatilis* occurs in two ecotypes; the upper shore morph is large and robust with a ridged and banded shell (RB ecotype in Fig. 1A) and lives among the barnacles, whereas the lower shore morph is small and fragile with a smooth and unbanded shell (SU ecotype in Fig. 1A) and lives among the mussels (Johannesson *et al*. 1993). The differences between the two ecotypes in shell

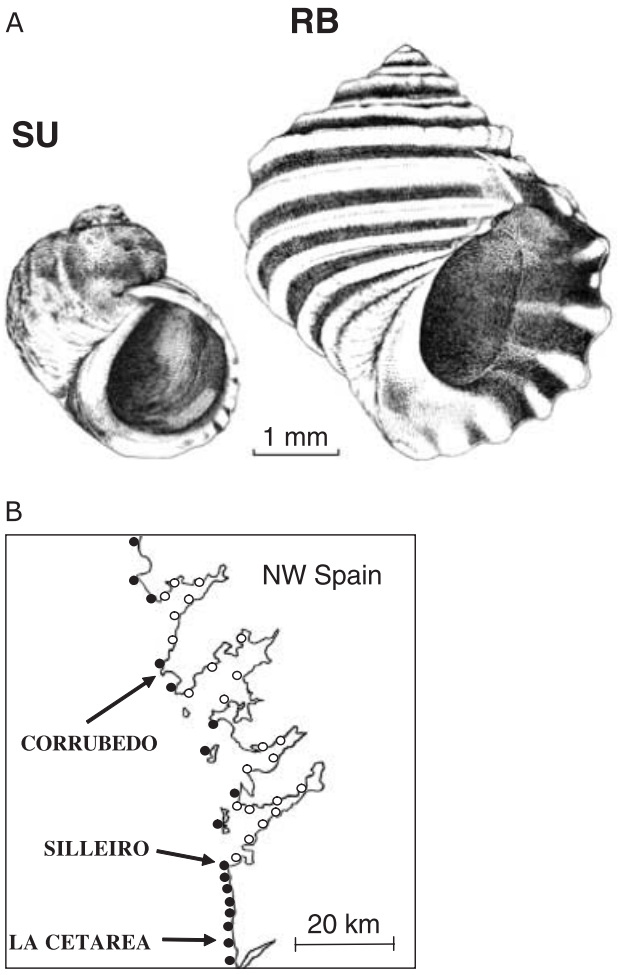

Fig. 1 Distribution of two ecotypes (RB and SU) of *Littorina saxatilis* along rocky shores of Galicia, NW Spain. (A) The RB ecotype is on average twice as large as the SU ecotype and has a shell with ridges and black bands inside grooves; it occupies the barnacle zone of the high intertidal zone on wave-exposed rocky shores. The SU ecotype is relatively small with a smooth, unbanded shell and confined to the mussel belt of the lower intertidal zone of the same shores. Both ecotypes overlap in a metre-wide midshore zone. (B) The ecotypes are confined to the most exposed parts of the coastline resulting in a discontinuous distribution along the coast (filled circles: present; unfilled circles: absent). The arrows show the three localities sampled in the study.

form and size are strongly inherited (Johannesson *et al*. 1993, 1997; Carballo *et al*. 2001) and ecological studies have shown that divergent selection maintains the polymorphism between the two microhabitats (Rolán-Alvarez *et al*. 1997). The main agent of selection in the low-shore mussel zone is heavy wave-action, and small snails escape dislodgement by hiding in cracks and crevices. Snails with a proportionately larger shell aperture and foot have a better grip on the substrate. Thus a small shell size with a relatively large aperture is typical of the lower shore SU ecotype (Johannesson *et al*. 1993). In the upper shore, the agents of selection are predation by the crab *Pachygrapsus marmoratus* and more severe physical stress from heat, desiccation and rainfall. Here, RB snails that have a large and strong shell with a relatively small aperture are likely to be more resistant to crab attacks (Johannesson 1986), and the smaller aperture probably also reduces the rate of water loss.

The two ecotypes are thus distributed along a micro-environmental vertical gradient within each shore. The dispersal rate of individual snails is large enough to allow adult snails to move between shore levels several times during a life-time (1–8 m/month by mark–recapture experiments), although they show preferences for living at their particular shore level (Erlandsson *et al*. 1998). However, similar proportions of the two snail ecotypes are present in true sympatry at the midshore, where barnacles and mussels overlap in a patchy zone about 1 m wide. A variable percentage of snails with intermediate shell characters ('hybrids': 5–37%, data from 10 localities, Rolán-Alvarez *et al*. 1999) having either ribs but not bands, or bands but not ribs are also present (Johannesson *et al*. 1993). Mating between the RB and SU ecotypes is strongly assortative in the zone of overlap, with an average isolation index of 0.77, where 1 is complete assortative mating and 0 is random mating (data from Rolán-Alvarez *et al*. 1999; reanalysed using the I_{PSI} statistic, Rolán-Alvarez & Caballero 2000).

The population genetic structure of this hybrid zone was analysed in 1989 using five allozyme loci, and it was found that the average genetic differentiation between ecotypes was larger at microgeographical scales (< 100 m) than at geographical scales (> 10 km), indicating the presence of local reproductive barriers because of the assortative mating between ecotypes (Johannesson *et al*. 1993, 1995a). However, in other European populations of this species the allele frequencies of some loci (e.g. *Pgm*-2) varied gradually with microhabitat, possibly because of selection on these loci or at a locus tightly linked to the marker locus (Johannesson *et al*. 1995b; Johannesson & Tatarenkov 1997). The possibility of selection at some allozyme loci confounded our previous interpretation (Johannesson *et al*. 1993; Johannesson & Tatarenkov 1997). In the present study we added putatively neutral genetic markers (synonymous mtDNA substitutions and microsatellites) to estimate more effectively micro- and macrogeographical patterns of genetic differentiation.

Sampling scheme

We scored allozyme, microsatellite and mitochondrial polymorphisms for snails collected in 1999 and allozyme polymorphisms for snails collected in 2003 from the same three localities (25–77 km apart; Fig. 1B) sampled in 1989 (Johannesson *et al*. 1993). In each locality, the three shore levels were sampled along two replicate transects that were 10–27 m long and 15–45 m apart (Fig. 2). Barnacles

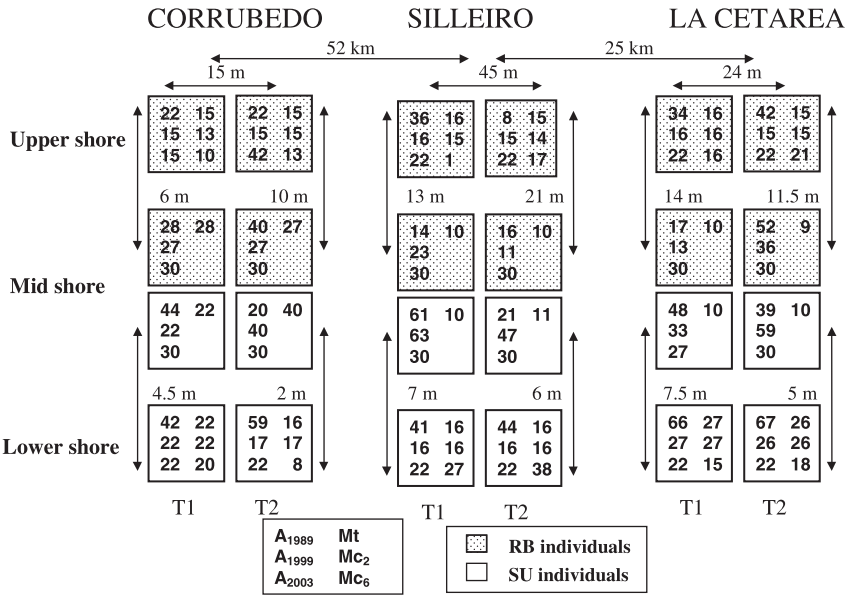

Fig. 2 Scheme showing the sampling design and sample size. Upper-, mid- and low-shore samples were taken at two replicate transects at each of three localities. While upper and lower samples consisted of RB and SU, respectively, the midshore samples contained both ecotypes as well as a minor proportion of hybrids. Hybrids were not, however, included in this study. Mean sample sizes used for the different genetic markers are provided following this code: for allozymes in 1989 (A1989), 1999 (A1999) and 2003 (A2003), for the mitochondrial polymorphic region (Mt) and for the two (Mc_2) and six (Mc_6) microsatellite data sets.

Table 1 Genetic differentiation at a macrogeographical scale (three localities at 25–77 km distance): F_E, between ecotypes (RB and SU) with samples of the same ecotype from different localities grouped, and F_L, among localities with samples of ecotypes from each locality grouped. Genetic differentiation at a microgeographical scale (samples within localities at 10.5–45 m distance): $F_{E(TL)}$, between ecotypes within localities, and $F_{T(EL)}$, among replicate samples of the same ecotype within localities. Allozyme 1989 data are reanalysed from Johannesson *et al*. (1993). Eight microsatellite loci, five allozyme loci and one mtDNA locus were scored. Upper–lower estimates include only data from upper shore (RB ecotype) and lower shore (SU ecotype) samples. Midshore estimates compare differentiation between RB and SU ecotypes in the midshore zone of overlapping distribution, hybrids were not included

Data	Shore level	*N*	F_E	F_L	$F_{E(TL)}$	$F_{T(EL)}$
Microsatellites 1999 (two loci)	Upper–lower	215	−0.002 NS	0.057**	0.045***	0.026***
Microsatellites 1999 (six loci)	Upper–lower	251	−0.001 NS	0.013***	0.058***	0.039***
mtDNA 1999	Upper–lower	215	−0.071 NS	0.477**	0.143***	0.079***
	Midshore	197	−0.044 NS	0.267**	0.073***	0.032***
Allozymes 1989	Upper–lower	499	−0.007 NS	0.077**	0.028 NS	0.001*
	Midshore	381	−0.010 NS	0.070**	0.022***	0.023***
Allozymes 1999	Upper–lower	217	−0.014 NS	0.069**	0.068***	0.037***
	Midshore	417	0.003 NS	0.055*	0.039 NS	−0.029 NS
Allozymes 2003	Upper–lower	264	−0.005 NS	0.062**	0.059***	0.015***
	Midshore	360	0.003 NS	0.053**	0.046***	0.013***

NS: nonsignificant, $^{**}P < 0.01$, $^{***}P < 0.001$.

dominated the upper shore and these samples contained almost exclusively RB ecotype snails. The barnacles and mussels overlap in the middle shore level and here a mixture of the RB ecotype, the SU ecotype and hybrids between them was present. The lower shore zone was in the mussel belt and was dominated by the SU ecotype. Snails were collected from 1 m² plots at each combination of localities, shore levels and transects, resulting in a total of 24 samples (midshore samples were subdivided into RB and SU specimens) in both 1999 and 2003.

Allozyme polymorphism

Three sets of allozyme data, each using snails collected during a different year and comprising an overlapping but nonidentical group of loci, were analysed (Table 1; see below). The 1989 samples were reanalysed from Johannesson *et al*. (1993). This included 880 specimens, 48 per sample on average (range 4–100), for five polymorphic loci: phosphoglucose isomerase (*Pgi*, EC 5.3.1.9), phosphoglucose mutase (*Pgm-1*, EC 5.3.1.9), mannose phosphate isomerase

(*Mpi*, EC 5.3.1.8), aspartate aminotransferase (*Aat-1*, EC 2.6.1.1) and purine nucleoside phosphorilase (*Pnp*, EC 2.4.2.1). The second data set was obtained in 1999, including a total of 634 individuals, 26.4 per sample on average (range 15–67) for five polymorphic loci: *Pgi*, *Aat-1*, *Pgm-2*, leucine aminopeptidase (*Lap*, EC 3.4.1.1) and arginine kinase (*Ark*, EC 2.7.3.3). The third allozymic data set was obtained in 2003, including a total number of 624 individuals, 26 per sample on average (range 22–30) for six polymorphic loci *Pgi*, *Pgm-2*, *Aat-1*, *Lap*, *Ark* and *Pnp*. Snails from both the second and the third data set were stored at –80 °C, and the soft parts used to obtain the homogenate for scoring the loci.

The locus *Pgm-2* was a posteriori excluded from the analysis, as during both 1999 and 2003 it showed evidence of differential selection between upper and lower shore habitats (E. Rolán-Alvarez, unpublished results). Exclusion of this locus can be justified because in Swedish *L. saxatilis* populations different alleles of *Pgm-2* are being selected on upper and lower shores (Johannesson & Tatarenkov 1997).

These three data sets were subdivided further into upper/lower shore samples, which include differentiation between RB from upper shore and SU from lower shore, and midshore samples, that include differentiation between RB and SU from the midshore, allowing the use of six pseudoreplicates to study allozymic population differentiation (the last six rows from Table 1). The repeated analysis of allozyme variation also allowed us to assess whether the pattern of allozyme variation was stable over time.

Mitochondrial polymorphism

The same homogenate in a subset of the individuals of the 1999 sample (N = 412; Table 1) was used to extract its DNA using a chelex method (Method 2 in Estoup *et al*. 1996). We used polymerase chain reaction (PCR) to amplify a 232 base pairs (bp) mtDNA region fragment that included the 3′ end of the COI gene and the 5′ end of the COII gene. Primers were designed based on the partial mtDNA sequence described by Wilding *et al*. (1999). Individual snails were analysed with SSCP (single-stranded conformation polymorphism) by running them on a denaturing acrylamide gel then using silver-staining to visualize the DNA fragments. We observed two different fragment patterns and sequenced nine random replicates per SSCP pattern. The sequence analysis confirmed that only two mtDNA haplotypes were present and these differed by two synonymous substitutions (positions 39 and 69 of the COI gene; GenBank Accession nos: AF416614 and AF416615).

Microsatellite polymorphism

We also analysed 215 of the individuals sampled in the upper- and lower-shore samples used in our mtDNA study, using PCR primers for two microsatellite loci (Sokolov *et al*. 2002). However, at Vigo we scored a faster and brighter locus 'Lx-12 fast' that had alleles ranging in size from 118 to 155 bp rather than the dimmer 'Lx-12' locus of 151–220 bp that was described originally for this primer pair (Sokolov *et al*. 2002). Lx-12 fast and Lx-20 were genotyped at Vigo by running them on a denaturing acrylamide gel and visualizing the DNA fragments using silver-staining. An additional 251 upper- and lower-shore specimens from the same sampling period had their DNA extracted at Vigo (using proteinase-K digestion; see method in Tie *et al*. 2000) but were genotyped at Tjärnö Marine Biological Laboratory for six microsatellite loci (Lsub6, Lsub8 and Lsub62 from Tie *et al*. 2000; Lx-12, Lx-18 and Lx-23, from Sokolov *et al*. 2002). All genotyping at Tjärnö was performed on an ALF II DNA analyser (Amersham-Pharmacia Biotech) using fluorescently labelled forward primers and the manufacturer's recommended internal and external size standards.

Analysis of molecular variation

We used F_{ST} statistics for the three different types of molecular markers to assess genetic differentiation within and among localities and ecotypes by using a two-level hierarchical partition (groups and populations within groups) of population genetic differentiation by AMOVA (Excoffier *et al*. 1992). The data were rearranged in different ways to analyse different pieces of information (see Fig. 2). At a macrogeographical level, F_E estimates the genetic differentiation between ecotypes (after grouping samples from all localities and transects within ecotypes) and F_L estimates the genetic differentiation between localities (after grouping samples from transects and ecotypes within localities). At a microgeographical level the groups were all combinations of transects (or ecotypes) with localities, while populations within groups represented the two ecotypes (for the $F_{E(TL)}$ analysis) or the two transects (for the $F_{T(EL)}$ analysis). Therefore, $F_{E(TL)}$ is the mean estimate of genetic differentiation between ecotypes within transect and locality (averaged across all the comparisons; two transects × three localities), and $F_{T(EL)}$ estimates the mean genetic differentiation between transects within ecotype and locality (averaged across all the comparisons; two ecotypes × three localities). We calculated the variance ratios F_{ct} (differentiation between groups) for F_E and F_L, and F_{sc} (differentiation between populations within groups) for $F_{E(TL)}$ and $F_{T(EL)}$ using the program ARLEQUIN, which estimates probability values by permutation of populations within groups (F_{ct}) and by permutation of genotypes among populations but within groups (F_{sc}) (Weir & Cockerham 1984; Excoffier *et al*. 1992). Different population differentiation statistics (F_E, F_L, $F_{E(TL)}$, $F_{T(EL)}$; from Table 1) could be compared across pseudoreplicates (from different genetic markers and data sets) using a randomization ANOVA programmed in BASIC (available at

http://webs.uvigo.es/c03/webc03/XENETICA/XB2/anova.zip), and by a classical sign test (Sokal & Rohlf 1995).

Prezygotic and postzygotic isolation

We knew from earlier field data that mating is nonrandom in the midshore zone where the two ecotypes overlap (e.g. Rolán-Alvarez *et al*. 1999). To trace the mechanism behind the nonrandom mate choice, we reanalysed data on mating pairs collected from wild populations by Cruz *et al*. (2004). They took 28 shell measurements for both members of 154 mating pairs that had been sampled from the midshore region of two localities. For each of the shell measurements, we estimated the male–female correlation across pairs in order to find the most probable trait responsible for the mate choice.

To assess the amount of postzygotic isolation between ecotypes, we raised immature RB juveniles (< 3 mm shell height) in the laboratory (September 1999) until maturation at 8 months. The specimens were sexed every week and incipient males were kept separated from virgin females. Mature virgin RB females were paired with either RB males or with SU males using eight females and eight males in each aquarium. There were 16 aquaria: eight had RB-females/RB-males and eight had RB-females/SU-males. We replaced males every month, as their sexual activity decreased with time. After 8 months we dissected all females, counted the number of embryos in the brood pouches of pregnant females and determined the frequency of aborted embryos following Janson (1985).

Results

Patterns of genetic differentiation

Levels of variation (expected heterozygosity and number of alleles) in allozymes for the second, 1999 ($H_E = 0.397$, $n_a = 4.2$) and third, 2003 ($H_E = 0.350$, $n_a = 3$) data sets were similar to those obtained for the first, 1989, data set ($H_E = 0.374$, $n_a = 3.4$; see Johannesson *et al*. 1993). The two haplotypes observed in the mitochondrial region analysed showed similar levels of variation (the frequency of one of the two haplotypes was 0.234), but the microsatellites were more variable both for the two-loci ($H_E = 0.785$, $n_a = 10$) and six-loci ($H_E = 0.815$, $n_a = 9.9$) data sets.

Populations from different localities were genetically distinct in all markers because of isolation by distance (F_L significantly larger than zero in all cases; see Table 1). However, none of the genetic markers showed an overall genetic difference between the two ecotypes at a macrogeographical scale (F_E was not significantly different from zero in any case). Thus, the two ecotypes did not cluster on a macrogeographical scale, despite their considerable morphological divergence. Most interesting was our finding that the two ecotypes were genetically distinct within their particular geographical localities ($F_{E(TL)}$ was typically significantly different from zero). This was not a consequence of isolation by distance over microgeographical distances within sites, as the genetic differentiation between snails of different ecotype within localities ($F_{E(TL)}$; separated by 10–27 m) was significantly greater than the differentiation between snails of the same ecotype over similar distances at the same shore level ($F_{T(EL)}$; separated by 15–45 m; *F*-test by a randomization ANOVA = 6.03, d.f.$_1$ = 1, d.f.$_2$ = 9, P = 0.0256; and $P_{\text{sign test}} = 0.021$ for all 10 comparisons). Both the macro- and the microgeographical patterns of genetic variation were robust for all three marker types (mtDNA, microsatellites and allozymes) and over time (for allozymes only: 1989, 1999 and 2003).

Reproductive barrier mechanisms

We looked for evidence of postzygotic reproductive isolation but crosses between RB females and RB and SU males showed equal percentages of successful fertilization (with RB males: 7.8%; with SU males: 9.4%; $\chi^2 = 0.099$, $P > 0.05$), equal numbers of embryos born per replicate cross (RB: 7.00 ± 9.81; SU: 8.87 ± 9.36; Mann–Whitney *U*-test = 28, $P = 0.660$) and equal proportions of abortive embryos in pregnant females (RB: 8.1%; SU: 2.3%; $\chi^2 = 1.458$, $P > 0.05$). Thus, the results point towards no postzygotic barriers to gene flow, although we were unable to obtain male SU–female SU crosses and male RB–female SU crosses.

The analysis of mating pairs collected from the wild showed that only a minor proportion of the pairs were RB-male/SU-female (3.9%) and SU-male/RB-female (8.4%), showing that prezygotic reproductive isolation was strong but not complete (0.63 ± 0.055). To identify which morphological traits were important to mate choice, we estimated the correlation between 28 male and female shell traits. Male and female shell height showed the highest positive correlation (Fig. 3; $\tau_b = 0.428$, $n = 154$, $P < 0.001$) and this correlation remained significant when it was estimated within ecotype (RB/RB: $\tau_b = 0.357$, $n = 19$, $P = 0.033$; SU/SU: $\tau_b = 0.313$, $n = 80$, $P < 0.001$). The RB ecotype is on average twice the size of the SU ecotype and therefore the positive correlation between male and female sizes of a mating pair resulted in nonrandom mating of the two ecotypes.

Discussion

The population genetic data show two important results: first, ecotypes were not significantly genetically differentiated at a macrogeographical scale ($F_E = 0$); second, ecotypes were significantly genetically differentiated at a microgeographical scale ($F_{E(TL)} > 0$) within each of the three localities, suggesting that local barriers to gene flow between the two ecotypes were present. It seems, at first

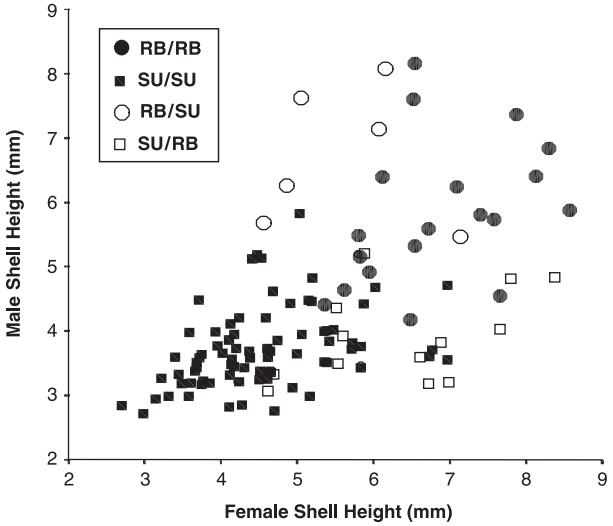

Fig. 3 Correlation of male and female sizes within copulating pairs of *Littorina saxatilis* sampled from natural populations. Four different pair combinations are shown: RB/RB (male/female): black circles; RB/SU: open circles; SU/RB: open squares; and SU/SU: black squares. Correlations were significant within RB/RB and within SU/SU pairs (see text).

glance, a paradox that the differentiation between ecotypes is larger on a microgeographical scale than on a macrogeographical scale ($F_{E(TL)} > F_E$), but this is actually what is expected if the reproductive barrier evolves independently at each of the local sites. Our interpretation of the data is that the reproductive barriers between these two ecotypes of *L. saxatilis* have evolved independently in each locality by parallel evolution without a period of physical isolation. In this scenario, a local origin of the ecotypes renders a situation in which genetic differences between ecotypes are created at a microgeographical scale but ecotypes remain undifferentiated overall. Local reproductive barriers, arising as a byproduct of ecological divergence between ecotypes, will generate neutral genetic differences between ecotypes at random. That is, the difference between ecotypes in the frequency of an allele at a particular marker, will deviate from zero in one direction or the other simply by chance. However, when differences between ecotypes are summed up over geographically isolated localities, the mean differences in frequency between ecotypes averaged over many localities would be expected to be centred around zero, producing a lack of overall differentiation between ecotypes.

An alternative scenario assumes that an allopatric origin of the two ecotypes is followed by secondary overlap and hybridization which, to some extent, eliminates the neutral genetic differences between ecotypes. However, this scenario fails to explain the patterns of variation we observed. If the two ecotypes originated in allopatry then they could have acquired different initial allele frequencies, which might be maintained during the phase of secondary overlap in their distribution. Such differences in allele frequencies could be removed if partial or complete hybridization between ecotypes followed the secondary contact. However, no allopatric scenario seems likely in which the original genetic differences between ecotypes are maintained at each locality while the overall differences between ecotypes summed over all localities disappear because of hybridization.

A second alternative scenario in which the two ecotypes originate in allopatry but where dispersal allows favourable mutations to spread from the site where they appeared to populations in other localities could, in theory, explain the observed genetic structure. However, our 'localities' have a discontinuous distribution along the Galician coast (Fig. 1B), therefore high along-shore dispersal of favourable mutations that contribute to the within–locality barriers seems unlikely. Additionally, in order to fit our observation that the ecotypes were not genetically differentiated at a macrogeographical scale, this scenario would require that our neutral markers were unlinked to the loci containing the favourable mutations for each ecotype. It is possible that very rare dispersal events among localities do spread favourable mutations in this manner but they would have to be rare enough not to prevent genetic differentiation among localities at neutral loci.

A third alternative scenario would be that the ecotypes evolved in allopatry independently at each locality (microallopatry). However, we know of no geological or oceanographic mechanism that could have kept the two ecotypes physically separated (at the scale of a few meters) within each locality over long enough time to accumulate the genetic differentiation obtained. In addition, we do not expect similar mechanisms of premating reproductive isolation to evolve repeatedly if only stochastic mechanisms are operating (i.e. genetic drift in physically isolated populations).

Finally, a fourth alternative scenario would be that the ecotypes have a nonallopatric and nonparallel origin. That is, the new ecotype became reproductively isolated from the parent population at one site and thereafter spread along to other sites (keeping the same mechanism of isolation). With such a scenario, however, we would predict that populations group by ecotype at a macrogeographical scale, owing to a common origin of populations of the same ecotype, and this was not observed.

There are further arguments favouring the hypothesis of nonallopatric and parallel evolution of reproductive isolation. Recent reproductive barriers that have formed nonallopatrically are expected to be prezygotic rather than postzygotic, as the former are observed to form first (Coyne & Orr 1997; Berlocher 1998). Crossing the ecotypes of *L. saxatilis* in the laboratory gave no indications of any postzygotic barrier. Furthermore, previous results show

that hybrid fitness is similar to the fitness of parental ecotypes in the midshore, both with respect to survival and to male and female fecundity of natural populations (Rolán-Alvarez *et al*. 1997; Johannesson *et al*. 2000; Cruz *et al*. 2001). Hybrid fitness is low on average, not because of low fitness in the midshore region but because of low fitness in both the upper and the lower shore habitats (Rolán-Alvarez *et al*. 1997). Simulation results on this hybrid zone suggest that low hybrid fitness in the midshore is not necessary for the maintenance of this particular polymorphism (Pérez-Figueroa *et al*. 2004). These simulations support the non-allopatric and parallel evolution scenario, as observed levels of assortative mating, disruptive selection and gene flow between ecotypes within a locality (around four to nine migrants per generation; Rolán-Alvarez *et al*. 1996) are able to maintain the observed allele frequency differences between ecotypes.

The polymorphism seems to be maintained instead by a prezygotic barrier between the two ecotypes consisting of assortative mating within the midshore zone. This assortative mating occurs in part due to a nonrandom mate choice and in part due to non–random associations with particular microhabitats (Johannesson *et al*. 1995a; Rolán-Alvarez *et al*. 1999; Cruz *et al*. 2004). The significant size correlation between mates of mating pairs observed in the present study (Fig. 3), as well as the pattern of mating observed within and between ecotypes (Cruz *et al*. 2004), supports size-based mate choice, which has also been observed in fishes and lizards (e.g. Foote & Larkin 1988; Nagel & Schluter 1998; Richmond & Reeder 2002). The size difference between these snail ecotypes is largely heritable (Johannesson *et al*. 1997; Cruz *et al*. 2001) and most probably maintained by directional selection favouring large snails on the upper shore and small snails on the lower shore (Rolán-Alvarez *et al*. 1997). This supports the hypothesis that the local reproductive barriers we observe between the Spanish ecotypes have evolved as a secondary consequence (byproduct) of natural selection for divergent sizes in two adjacent microhabitats.

There is additional support for the parallel evolution of reproductive barriers in this species. The species *L. saxatilis* is known to produce ecotypes adapted to the different ecological conditions existing in different habitats and geographical areas (reviewed in Reid 1996). In fact, both Swedish and British populations have multiple ecotypes adapted to particular shore microhabitats in a similar way to that observed in Spain, although the type and distribution of microhabitats as well as the corresponding snail phenotypes are different.

Swedish populations of *L. saxatilis* show evidence of assortative mating between a small ecotype confined to wave-exposed microhabitats and a large and robust ecotype present in heavy predated and moderately exposed boulder microhabitats (Hollander *et al*. in prep.). Similarly, Swedish populations show a pattern of increased isolation by distance at allozyme loci which is independent of snail ecotype (Janson & Ward 1984; Janson 1987).

In Britain, phenotypic divergence has also led to partial reproductive barriers between the H and M ecotypes of *L. saxatilis* (Pickles & Grahame 1999) but, unlike in Galicia, there is evidence for decreased fertility and higher embryo abortion rates in hybrids (Hull *et al*. 1996) and this might instead suggest secondary contact after divergence in allopatry (Berlocher 1998). Wilding *et al*. (2001) found that about 5% of the AFLP markers studied in populations of the H and M ecotypes could partially explain the ecotype differences at a local scale, although the data could not distinguish between a sympatric and an allopatric (followed by secondary contact) origin of the ecotypes.

It is essential that conclusions about the phylogeny of these populations are based on molecular variation that is neutral, at least with respect to the two microhabitats considered. The allozyme data set we used in a previous study (Johannesson *et al*. 1993) was confounded partially by selection which made it impossible to infer correctly the relationships among ecotypes from different localities. Indeed, a macrogeographical genetic difference between ecotypes (F_E) is expected if natural selection favours particular alleles in each microhabitat. For example, if the allozyme locus *Pgm*-2 is included in the analysis, F_E becomes significantly different from zero in all 3 years (F_E = 0.041** in 1989, 0.083** in 1999 and 0.057** in 2003), but this is due probably to direct or indirect effects of natural selection on this enzyme. In fact, about 60% of the among-sample variation in *Pgm*-2 was explained by the microhabitat in Swedish populations of *L. saxatilis* (Johannesson & Tatarenkov 1997).

Several examples of parallel ecological speciation are known (reviewed in Johannesson 2001; Schluter 2001), but very few examples have unambiguous support for a non-allopatric origin of the reproductive barriers between the incipient species. Although there is evidence for parallel evolution of reproductive barriers, in a Pacific salmon (Taylor *et al*. 1996), in some African cichlids (Galis & Metz 1998), in some sticklebacks (Nagel & Schluter 1998; Rundle *et al*. 2000) and in some walking-stick insects (Nossil *et al*. 2002), either the mechanism driving the speciation process is insufficiently known, or the speciation process has already been completed, making it difficult to test whether the process had an allopatric or nonallopatric origin. Indeed, if speciation had already been completed in these Spanish *L. saxatilis*, it is likely that gene flow within and the lack of gene flow between the two derived species (RB and SU) would, over time, result in an apparently allopatric pattern of differentiation and made this system less useful.

We do not yet know whether the incomplete reproductive isolation between these two Spanish ecotypes of *L. saxatilis* will ever become complete. Computer simulations

predict that the population genetic differentiation and the phenotypic polymorphism we observe could be maintained given the estimated levels of migration, assortative mating and divergent selection (Pérez-Figueroa *et al*. 2004). Meanwhile, this model system exemplifies how consistent divergent selection on ecologically important traits might result in parallel evolution of reproductive barriers in the presence of gene flow.

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