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Site-specific genetic divergence in parallel hybrid zones suggests nonallopatric evolution of reproductive barriers

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Abstract

The evolution of reproductive isolation in the presence of gene flow is supported by theoretical models but rarely by data. Empirical support might be gained from studies of parallel hybrid zones between interbreeding taxa. We analysed gene flow over two hybrid zones separating ecotypes of *Littorina saxatilis* to test the expectation that neutral genetic markers will show site-specific differences if barriers have evolved *in situ*. Distinct ecotypes found in contrasting shore habitats are separated by divergent selection and poor dispersal, but hybrid zones appear between them. Swedish islands formed by postglacial uplift 5000 years ago provide opportunities to assess genetic structure in a recently evolved system. Each island houses a discrete population containing subpopulations of different ecotypes. Hybrid zones between ecotypes may be a product of ecological divergence occurring on each island or a consequence of secondary overlap of ecotypes of allopatric origin that have spread among the islands. We used six microsatellite loci to assess gene flow and genetic profiles of hybrid zones on two islands. We found reduced gene flow over both hybrid zones, indicating the presence of local reproductive barriers between ecotypes. Nevertheless, subpopulations of different ecotypes from the same island were genetically more similar to each other than were subpopulations of the same ecotype from different islands. Moreover, neutral genetic traits separating the two ecotypes across hybrid zones were site-specific. This supports a scenario of *in situ* origin of ecotypes by ecological divergence and nonallopatric evolution of reproductive barriers.

Keywords: assortative mating, ecological speciation, *Littorina saxatilis*, microsatellites, morphometrics, parallel speciation

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Introduction

Separating allopatric and nonallopatric isolation using parallel hybrid zones

During the last decade, several studies have presented strong empirical support for ecological mechanisms driving reproductive isolation and speciation (e.g. Mallet 1993; Meyer 1993; Schluter 1996, 2001; Taylor *et al*. 1996; Via 1999; Rieseberg *et al*. 2003). If ecological mechanisms generate reproductive barriers, allopatric isolation is probably no longer necessary during speciation, as shown by various models (Johnson & Gullberg 1998; Tregenza & Butlin 1999; Schluter 2001; Via 2001; Doebeli & Dieckmann 2003). Nevertheless, indisputable examples of nonallopatric (sympatric and parapatric) speciation are few (Coyne & Orr 2004, but see Barluenga *et al*. 2006 and Savolainen *et al*. 2006 for two recent examples).

Studying hybrid zones with partial reproductive barriers is one way to scrutinize speciation mechanisms (Endler 1977; Jiggins & Mallet 2000; Via 2002). Another approach is to compare independent evolution of reproductive barriers within the same species complex (Schluter & Nagel 1995; Rundle *et al*. 2000; McKinnon *et al*. 2004). Indeed, combining the two, by studying parallel hybrid zones between subspecies or ecotypes, seems a particularly fruitful approach (Johannesson 2001).

If, in two or more independent locations, neutral genetic markers show that individuals of different ecotypes are genetically more similar to each other than are individuals of the same ecotype from different geographic locations, this

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suggests local and parallel origins of the different ecotypes. If ecotypes are partly reproductively isolated, these barriers have consequently evolved *in situ* without prior periods of isolation (a nonallopatric scenario) (Johannesson 2001; Via 2001). However, an alternative interpretation is that the geographic clustering of individuals, irrespective of ecotype differences, is the result of secondary overlap and introgression of taxa that were formerly separated during a period of allopatry (e.g. Grahame *et al*. 2006). That is, in this allopatric scenario local hybridization and introgression have caused genetically separate taxa to become secondarily similar in neutral loci by stochastic processes. However, the two scenarios are expected to differ in one important respect; if reproductive barriers have evolved *in situ*, differentiation in neutral markers between ecotypes will be specific to each local hybrid zone. If the hybridizing taxa evolved during previous allopatry, on the other hand, snails of similar ecotype will show eroded signatures of their common allopatric ancestry even when sampled in different populations. Obviously, these two historical scenarios are not mutually exclusive; various degrees of allele frequency differentiation between ecotypes and between island populations are possible, as the relative probabilities of the two scenarios change along a continuum.

Using detailed genetic and morphometric data from Swedish hybrid zones of ecologically separate ecotypes of the marine gastropod *Littorina saxatilis*, we tested the predictions of the nonallopatric and allopatric scenarios on the gene flow and genetic profiles of parallel hybrid zones. Although three earlier studies have approached this question using the same model species, two of these (Wilding *et al*. 2001; Grahame *et al*. 2006, UK populations); did not analyse site-specific differences and the results were inconclusive with respect to origin of hybrid zones. The third study (Rolán-Alvarez *et al*. 2004, Spanish populations) did suggest a nonallopatric origin of hybrid zones, but without presenting detailed evidence for this conclusion.

Littorina saxatilis *hybrid zones*

The marine gastropod *Littorina saxatilis* is extremely polymorphic in size, shell shape and behaviour, with traits being habitat-specific and largely inherited, enabling several ecotypes or ecomorphs to be defined (e.g. Johannesson *et al*. 1993; Reid 1996; Johannesson 2003). Where one shore habitat merges into another, hybrids are present, in high frequencies (e.g. in Sweden, Janson & Sundberg 1983) or in lower frequencies (e.g. in Spain, Johannesson *et al*. 1993; Rolán-Alvarez *et al*. 1997, and UK, Hull *et al*. 1996; Grahame *et al*. 2006). Overall, the genetic structure at neutral loci is determined by geographic location rather than by grouping of individuals into distinct ecotypes (Johannesson *et al*. 1993; Wilding *et al*. 2001; Rolán-Alvarez *et al*. 2004); this is a consequence of a restricted dispersal capacity (in the absence of a pelagic larva) combined with either *in situ* origin of ecotypes or with secondary overlap and introgression (as discussed above).

Mate-choice experiments between contrasting ecotypes demonstrate strong positive assortative mating with isolation indexes of 0.9–1 in UK (Hull 1998; Pickles & Grahame 1999), 0.5–0.9 in Spain (Johannesson *et al*. 1995) and 0.6 in Sweden (Hollander, personal communication, and see Hollander *et al*. 2005). (This index varies between 0 indicating random mating and 1 indicating complete isolation, Ribi & Porter 1995.) This suggests an impeded gene flow over hybrid zones, compared to gene flow over similar distances of shore within the distributions of individual ecotypes.

In Sweden, the two dominant ecotypes of *Littorina saxatilis* are the small E-morph with a thin shell and a large aperture that lives on rocky cliffs, and the large S-morph with a thick shell and a small aperture inhabiting boulder beaches (Janson 1982). Morphological differences between ecotypes are likely a result of wave action on cliffs that selects for small, light and large-footed shells, whereas crab predation in boulder areas favours robust shells with a narrow aperture that prevents crushing and peeling (Janson 1983; Johannesson 1986). These ecotypes are found on islands along the Swedish west coast where there are both exposed rocky cliffs and boulder shores, and on a geographic scale their distributions overlap completely. Although we lack detailed records of their complete distribution, they are replaced by other ecotypes somewhere along the Norwegian west coast, probably owing to increased tidal range and additional environmental changes. Their southern limit of distribution is at the entrance of the low-salinity Baltic Sea, and the lack of rocky shores along the Danish coast prevents their further distribution to the southwest. Limited along-shore dispersal results in genetic heterogeneity over distances of 15–150 m (Janson & Ward 1984; Rolán-Alvarez *et al*. 2004). Thus, the minimum distance over which migration is sufficient to prevent isolation by distance is approximately 20 m, which is in the range of the maximum dispersal distance of individual snails over 1–3 months (Janson 1983; Erlandsson *et al*. 1998).

To test the predictions from the allopatric and the nonallopatric scenarios of ecotype formation, we chose two island populations of *Littorina saxatilis* to represent independent, parallel sites, each including one subpopulation of each ecotype (E and S) with an intermediate hybrid zone. We first tested the expectation that populations on separate islands were genetically differentiated as a result of independent evolution. Furthermore, we estimated gene flow within islands to test if assortative mating (Hollander *et al*. 2005) impeded local gene flow between ecotypes. Having shown this, we proceeded to test the predictions from the two scenarios: (i) *in situ* evolution of ecotypes and reproductive barriers in the presence of gene flow (nonallopatry), and (ii) allopatric differentiation of ecotypes followed by secondary overlap and introgression.

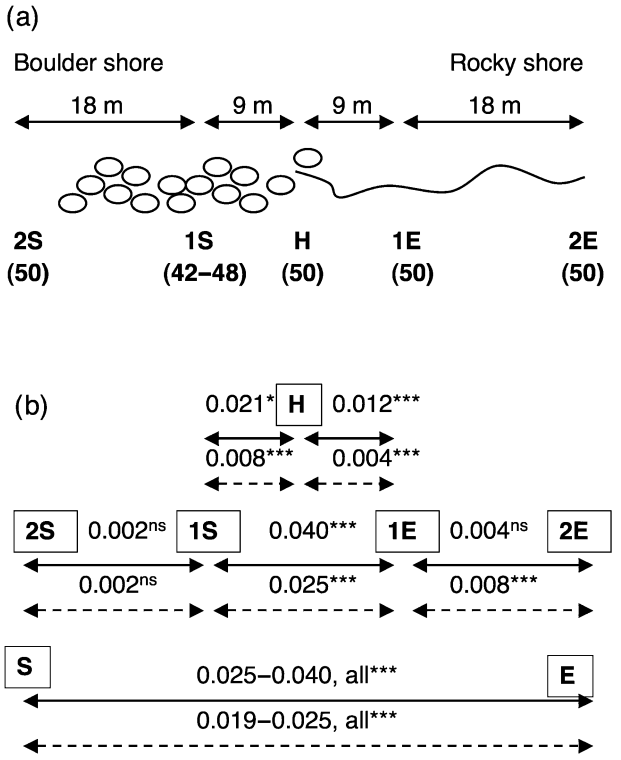

Fig. 1 Subpopulations of *Littorina saxatilis* sampled from two island populations (Ramsö and Ramsholmen). In each population two subpopulations were of E ecotype (1E and 2E) sampled on wave-exposed cliffs, one subpopulation represented a hybrid sample (H) in a zone of intermediate habitat and two subpopulations were of S ecotype (1S and 2S) sampled among boulders. (a) Distances between samples and sample sizes in both populations; (b) pairwise genetic distances ($F_{ST,}$ Weir & Cockerham 1984) between subpopulations on Ramsö (solid lines) and Ramsholmen (dashed lines). Distances are estimated over all loci and accompanied by probabilities derived from log-likelihood genotypic tests with sequential Bonferroni correction: ***$P < 0.0001$, *$P < 0.05$. For four E–S comparisons the range of distances is indicated.

In situ differentiation would imply that an island was colonized by a founder group of any ecotype. It is quite likely that this group would have been small, probably only a single female mated with 7–8 males (Janson 1987; Mäkinen *et al*. submitted). Following establishment, the new population differentiated under selection into separate ecotypes (E and S) that came to occupy different habitats. Initially the ecotypes would differ only at selected loci but, over time, the barrier to gene exchange created by local adaptation (Janson 1983) and assortative mating (Hollander *et al*. 2005) would result in differentiation also at neutral loci. Stochastic *in situ* differentiation would result in unique genetic differences at neutral loci in each island hybrid zone. Allopatric differentiation, on the other hand, assumes that islands were colonized by distinct lineages of E and S ecotypes formed during an earlier period of allopatric isolation. Secondary overlap and introgression would tend to remove differences at neutral loci, but partial reproductive barriers would impede this process. Genetic differentiation remaining at neutral loci between E and S ecotypes on each island would reflect what was left from their earlier allopatric separation, and consequently each local hybrid zone would tend to show more similar profiles of neutral genetic differentiation than do ecotypes that diverged *in situ*.

Materials and methods

Sample collection

Samples of E and S ecotypes of *Littorina saxatilis* were collected on 24April 2003 on two islands (Ramsö 58°50′N, 11°04′E, and Ramsholmen 58°51′N, 11°03′E) close to Tjärnö Marine Biological Laboratory. The distance between the islands is 2 km. The islands are representative of the archipelago of the area, with boulder shores and rocky cliffs inhabited by snails of *L. saxatilis*. On each island we sampled along a horizontal transect including a boulder beach, an exposed rocky cliff and the intermediate habitat in between. Sampling sites were chosen so that on each island we had two samples from each habitat at equal distances (18 m) and an additional sample from the middle of the intermediate zone (Fig. 1a). Distance between samples was comparable to the maximum dispersal range of snails found in earlier experiments (Janson 1983; Panova, unpublished). In each site 42–50 snails were collected over 1–2 m^2 areas with snail densities of 10^2–10^3 per m^2. Snails from the same island are referred to as a population, while snails from the same habitat within an island are referred to as a subpopulation (thus we sampled E, S and H = hybrid subpopulations).

Morphological analysis

The technique of landmark-based morphometrics (Bookstein 1991; Rohlf & Marcus 1993) was applied to describe shell shape and size of each snail that was later genotyped. This method captures the geometry of morphological structures and creates a number of size-independent variables, analogous to principal components that can be used in standard multivariate statistical procedures (Adams *et al*. 2004). The size of the snails is estimated from the distances between landmarks and the centroid (Bookstein 1991; for details see Hollander *et al*. 2005). The analysis was performed on live snails and thereafter they were individually labelled and stored at –70° until DNA extraction.

Microsatellite genotyping

DNA was extracted from small pieces of foot tissue (approximately 1 mm^3) with DNeasy Plant Mini Kit (QIAGEN).

All snails from the morphological analysis were typed for six microsatellite loci: Lsub62, Lsub32, Lsub16 and Lsub8 developed for *Littorina subrotundata* (Tie *et al*. 2000) and Lsax6 and Lx23 developed for *L. saxatilis* (Sokolov *et al*. 2002). All loci consist of trinucleotide repeats. In addition, Lx23 includes several poly T regions that produce alleles with one or two nucleotide differences in size. Polymerase chain reactions (PCR) were performed in 12-μL reaction volume with 2 μL of DNA extract, 0.6 U of *Taq* polymerase (Fermentas or TaKaRa Bio Inc), 1× supplied Tris-HCl buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.5 μM or 0.125 μM of fluorescence-labelled forward primer and reverse primer (for Cy5-labelled primers from CyberGene AB and D2, D3 and D4-labelled primers from Proligo). The reactions were conducted in Eppendorf thermal cyclers with cycling conditions following those described by the authors of the primers, except a decrease in annealing temperature for Lsub62 (54 °C instead of 64 °C) and Lsub32 (55 °C instead of 62 °C).

To screen the Ramsö population we used Cy5-labelled forward primers and ran the products on ALFexpress II automated sequencer (Amersham Pharmacia Biotech) according to the manufacturer's recommendations. Analysing the Ramsholmen population we performed the poolplexing of the products labelled with three dyes: 1 μL D2-Lsub62 + 1 μL D3-Lsub16 + 2 μL D4-Lsub32 or 1 μL D2-Lx23 + 1 μL D3-Lsub8 + 2 μL D4-Lsax6 with 36 μL Sample Loading Solution and 0.55 μL DNA Size Standard Kit-400 (CEQ™). Poolplexed samples were run on CEQ 8000 Genetic Analysis System and analysed by CEQ FRAGMENT ANALYSIS software. A set of individuals from both populations representing different alleles was run on both automated sequencers to assure consistency of allele sizes.

Statistical analysis

Gene diversity (expected heterozygosity corrected for sample size) was calculated in MICROSATELLITE ANALYSER (MSA, Dieringer & Schlötterer 2003). GENEPOP on the Web, version 3.3 (Raymond & Rousset 1995), was used to calculate the inbreeding coefficient F_{IS} (Weir & Cockerham 1984) and to perform exact tests (Guo & Thompson 1992) for Hardy–Weinberg equilibrium at each locus. The Hardy–Weinberg tests were performed for each subpopulation separately, to eliminate the risk of Wahlund effects owing to isolation by distance or impeded gene flow between ecotypes. Linkage disequilibrium between loci was tested in each subpopulation by Fisher's exact test in GENEPOP. Whenever we did multiple tests, we used sequential Bonferroni correction of probabilities (Sokal & Rohlf 1995).

Four of the six genotyped loci showed significant heterozygote deficiency, and possible genotyping errors (null alleles, stuttering and large allele dropout) were tested by comparing the observed and expected frequencies of all heterozygote classes using the software MICRO-CHECKER (van Oosterhout *et al*. 2004). MICRO-CHECKER suggested null alleles as the most probable cause of heterozygote deficiency (that is, the deficiency was equally distributed among all heterozygote classes). Indeed, for some populations and loci the number of failed amplifications corresponded to the estimated number of null-allele homozygotes. At the same time, a low number of failed amplifications was observed even in the two loci that were in Hardy–Weinberg equilibrium, suggesting additional genotyping problems, for example random dropout of one allele in heterozygotes due to poor template quality. Anyway, for loci that showed significant heterozygote deficiency we corrected genotype frequencies for putative null alleles by randomly replacing excessive homozygotes with null-allele heterozygotes in MICRO-CHECKER. Corrected genotype data were used in the analyses of population structure based on the assumption of Hardy–Weinberg equilibrium (AMOVA and pairwise genetic distances between subpopulations).

Genetic variation among populations and among subpopulations within populations was analysed using AMOVA (Excoffier *et al*. 1992) as implemented in ARLEQUIN 2.000 (Schneider *et al*. 2000) using 1000 permutations. To estimate differentiation between subpopulations the pairwise F_{ST} values (Weir & Cockerham 1984) were calculated in GENEPOP and their significance was assessed by the log-likelihood exact *G*-test on genotypes (Goudet *et al*. 1996). We chose to use Cavalli-Svorza & Edwards's (1967) chord distance to estimate genetic distances because it models short-term divergence due to drift alone (Nei 1987) and is thus most suitable for comparing closely related intraspecific groups. Cavalli-Svorza's distance statistics were calculated from the data corrected for null alleles in GENDIST and neighbour-joining clustering of distances was done in the NEIGHBOUR program of the PHYLIP package (Felsenstein 1989). Statistical support for the neighbour-joining tree was provided by bootstrap, resampling data 100 times and making a consensus tree using the programs SEQBOOT and CONSENSE of the PHYLIP package.

An additional analysis of population structure, the multivariate ordination of subpopulations by principal component analysis (PCA) of allele frequencies, was conducted in PCAGEN software (J. Goudet, unpublished; www2.unil.ch/popgen/softwares/pcagen.htm). This analysis is completely independent of the assumption of Hardy–Weinberg equilibrium. We used noncorrected data for this analysis, to control for possible bias that might be introduced by the null-allele correction. We also used PCA to estimate the impact of individual microsatellite alleles on differentiation between subsamples of ecotypes over the hybrid zones and thus to discriminate between site-specific (nonallopatric) and allopatric origin of divergence.

To analyse genetic relationships between hybrids and pure ecotypes an assignment test was performed using the GENECLASS 2 software (Piry *et al*. 2004). The E and S

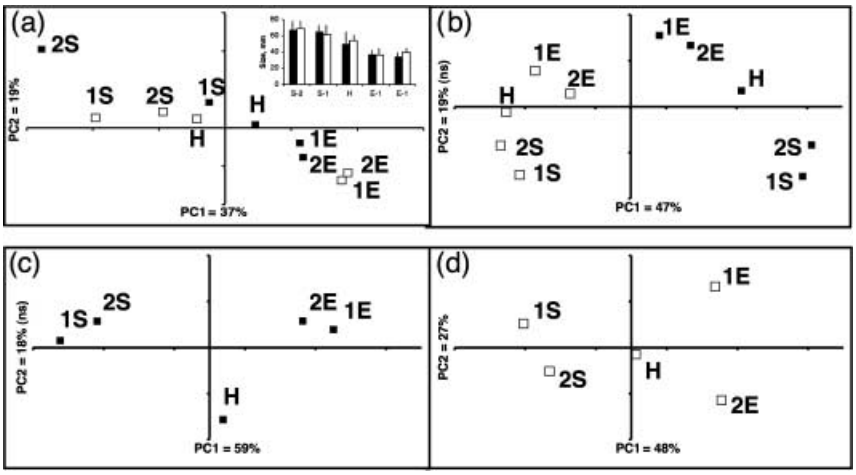

Fig. 2 Principal components analyses of shell shape and microsatellite allele frequencies in *Littorina saxatilis* subpopulations from cliff, boulder and intermediate habitats. Populations: filled squares and columns — Ramsö; empty squares and columns — Ramsholmen; subpopulation references as in Fig. 1. Percentage of explained variation per axis is indicated along axes. (a) PCA of shell shape in two populations with small diagram showing means and standard deviations of shell size. (b) PCA of microsatellite allele frequencies in two populations. (c) PCA of microsatellite allele frequencies in Ramsö population. Ten alleles with maximum impacts on the first axis in descending order are Lsub32-6, Lsax6-10, Lsub16-6, Lsub8-10, Lsub16-5, Lsub32-5, Lsub8-7, Lsub8-15, Lsax6-8 and Lsax6-11. (d) PCA of microsatellite allele frequencies in Ramsholmen population. Ten alleles with maximum impacts on the first axis in descending order are Lsub62-7, Lsub16-3, Lsub62-1, Lsub16-2, Lsub8-5, Lsub16-12, Lsax6-6, Lsub16-8, Lsax6-12 and Lsub16-16.

subpopulations nearest to each hybrid subpopulation were used as alternative source populations. We used two different methods of assignment implemented in this software: Rannala and Mountain's Bayesian probability based on allele frequencies in samples and interindividual Cavalli-Sforza and Edwards' chord distances. When genetic differentiation is low the first method performs better (Cornuet *et al*. 1999), but the probabilities are calculated under the assumption of Hardy–Weinberg equilibrium. For this reason, we used this method only with the two loci in equilibrium and we adopted the second method, which does not assume Hardy–Weinberg equilibrium, when analysing all six loci. The performance of each method was estimated as the proportion of correct self-assignments of the two source populations.

Hybrid composition was assessed by correlating individual genotypes and morphology using individual scores from genetic and morphological PCAs and performing Mantel's test (Sokal & Rohlf 1995) on two matrices of interindividual Euclidean distances. Finally, we calculated the correlation between individual assignment scores based on genotype (the probabilities to be assigned to either ecotype subpopulation, divided by the sum of those probabilities) and individual morphology using a simple correlation coefficient.

Results

Shell morphology

The first two principal components, explaining 56% of total shape variation, separated snails from boulder and cliff habitats in a consistent way on both islands. Furthermore, the PCA showed snails from the hybrid zones to be morphologically intermediate between those from cliffs and boulders (Fig. 2a). In both populations, snails from boulders were much larger than snails from cliffs, and snails from hybrid zones were of intermediate size (Fig. 2a).

Microsatellite genotypes

All six loci were highly variable, with many alleles and high gene diversity (Table 1). One to nine private alleles were found at each locus in each population (hypervariable Lx23 excluded), but none of these was found to be ecotype-specific. Significant linkage disequilibrium ($P < 0.05$ after Bonferroni correction) was found between the loci Lsub16 and Lsub32 in one S ecotype subpopulation from Ramsö and one S subpopulation from Ramsholmen. The other loci were in linkage equilibrium in all subpopulations.

Out of six studied loci, two (Lsub62 and Lsub32) were in Hardy–Weinberg equilibrium, but four (Lsub16, Lsub8, Lsax6, Lx23) showed significant heterozygote deficiency in all or nearly all subpopulations (Table 1). Since nonrandom mating, inbreeding, etc., should affect all loci uniformly, we believe that the observed heterozygote deficiency was likely a technical artefact. Most probably, this was caused by null alleles as suggested by the analyses of genotype frequencies in MICRO-CHECKER (Table 1). (Indeed this suggestion is strongly supported by preliminary data from an ongoing study of microsatellite inheritance using half-sibs, Panova, André & Mäkinen, unpublished data.) In addition,

Table 1 Characteristics of six microsatellite loci screened in Ramsö and Ramsholmen populations of *Littorina saxatilis*. N_A, total number of alleles in both populations; H_{Exp}, total gene diversity; F_{IS}, inbreeding coefficient (Weir & Cockerham 1984) range over 10 subpopulations; *N* (exact tests), number of subpopulations with significant deviation from Hardy–Weinberg equilibrium (HWE) estimated by Fisher's exact test with sequential Bonferroni correction at 5% probability level. Genotype distribution based on exact tests and the results of MICRO-CHECKER are indicated for loci out of HWE; *correction for putative null allele was made in later analyses

Locus	N_A	H_{Exp}	F_{IS}	*N* (exact tests)	Genotype distribution
Lsub62	13	0.705	(–0.105; +0.080)	0/10	HWE
Lsub32	13	0.755	(–0.092; +0.202)	1/10	HWE
Lsub16	25	0.860	(+0.375; +0.665)	10/10	Heterozygote deficiency due to null alleles *
Lsub8	24	0.894	(+0.153; +0.465)	9/10	Heterozygote deficiency due to null alleles *
Lsax6	15	0.855	(+0.283; +0.764)	10/10	Heterozygote deficiency due to null alleles* and stutters
Lx23	41	0.926	(+0.132; +0.410)	7/10	Heterozygote deficiency due to null alleles* and stutters

Table 2 Summary of analysis of molecular variance (AMOVA) over six microsatellite loci in *Littorina saxatilis*. Two populations, Ramsö and Ramsholmen, contained five subpopulations each: two of E ecotype, two of S ecotype and one hybrid population. Allele frequencies were corrected for putative null alleles at four loci

Source of variation	d.f.	*P*	Percentage of variation	Fixation indices
Among populations	1	0.004	2.4	0.024
Among subpopulations within populations	8	0.000	1.5	0.015
Within subpopulations	970	0.000	96.1	0.039
Total	980		100	

random allele dropout in heterozygotes, as well as stuttering (erroneously scoring weak one-repeat shorter band as 'noise' instead of a true allele in Lsax6 and Lx23) might be a problem, although much less severe than that of null alleles. Therefore for those statistics that assumed Hardy–Weinberg equilibrium (AMOVA, F_{ST} and Cavalli-Sforza's chord distance) we corrected genotype frequencies for putative null alleles at these four loci. Principal component analysis did not assume Hardy–Weinberg equilibrium and was performed using the original allele frequencies.

Genetic structure of populations

We included two hierarchical levels: populations (islands) and subpopulations (individuals of same ecotype from the same island) in an analysis of molecular variation (AMOVA). Most of the variation, 96.1%, was allocated within subpopulations, which is typical for microsatellite data. Nevertheless, differentiation between populations and between subpopulations within populations was significant and explained 2.4% and 1.5% of the genetic variation, respectively (Table 2). Pairwise, between-island comparisons of subpopulations showed significant levels of differentiation (F_{ST} = 0.02–0.07; all significant after Bonferroni correction), while within-island comparisons revealed less differentiation between subpopulations (F_{ST} = 0–0.04; all, except of three comparisons within ecotypes, were significant after Bonferroni correction) (Fig. 1b).

Based on Cavalli-Sforza's chord distances, subpopulations clustered primarily by island, but within islands they clustered by ecotype (Fig. 3). Within populations, there was no significant differentiation between subpopulations of the same ecotype, with the exception of the two E subpopulations from Ramsholmen. In contrast, we found significant genetic differentiation over both hybrid zones (Fig. 1b). Indeed, differentiation was observed both between subpopulations of pure ecotypes on either side of the hybrid zone, and between hybrids and adjacent subpopulations of E or S on either side, despite these distances being only half the others (Fig. 1b). These results indicated that gene flow over the hybrid zones was impeded. The overall level of genetic differentiation over the hybrid zone on Ramsö was higher than on Ramsholmen (Fig. 1b).

Allele frequencies separated the two island populations along the first axis of a principal component analysis (PCAGEN); this axis contributed significant variation explaining 47% of total variation among subpopulations (Fig. 2b). The second axis separating the ecotypes was nonsignificant. However, when each population was analysed separately the first axis separated the ecotypes, being significant and explaining 59% and 48% of the variation in the two populations (Fig. 2c, d). This result indicated that genetic differentiation between ecotypes was specific to each island population and became insignificant when summarized in one analysis. Noticeably, the separation of the two morphs was caused by completely different changes in allele

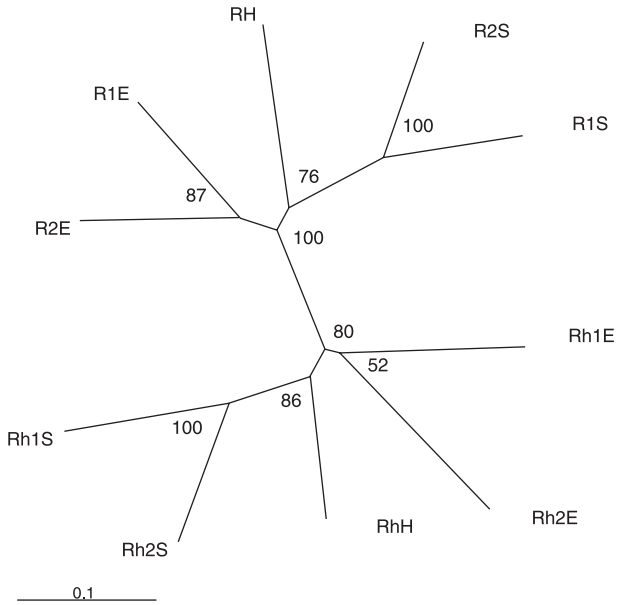

Fig. 3 Consensus neighbour-joining tree based on pairwise Cavalli-Svorza's chord distances between *Littorina saxatilis* subpopulations from the two islands Ramsö (R) and Ramsholmen (Rh) (subpopulation references as in Fig. 1). Bootstrap support from 100 resamplings is indicated next to nodes.

frequencies in Ramsö and Ramsholmen. The 10 alleles that contributed the most to ecotype separation on one island were all different from the 10 alleles most important to separation of ecotypes on the other island (Fig. 2c, d); furthermore, the impacts of each allele on the PC axes separating the morphs on one island and those on the other island were not correlated ($r = 0.18$; $P = 0.12$; calculated for 75 alleles that were found on both islands). This is very strong support for *in situ* evolution of ecotypes and hybrid zones on each island. The alternative, the merging of formerly allopatrically separated ecotypes, would have required heavy introgression to erase all earlier differences in neutral loci and the subsequent evolution of new (local) stochastic differences. Finally, the results of the principal component analyses were in good agreement with the results obtained from AMOVA and pairwise genetic distances, suggesting that the correction for null alleles did not introduce significant bias in the data.

Hybrid composition

Shell shape of hybrid snail subpopulations overlapped substantially with shell shape variation of parental subpopulations (Fig. 4a, b). There was, however, no correlation between individual morphology and individual multilocus microsatellite genotype (Mantel test: $P = 0.45$ and 0.52 in the two populations). Furthermore, no linkage disequilibria were detected for any of the loci in the hybrid subpopulations. These results both support the hybrid subpopulations being homogeneous rather than being a mixture of two overlapping populations.

We assigned hybrids to the nearest E and S subpopulations in each population, based on microsatellite genotype. We used the allele frequency method, including only the

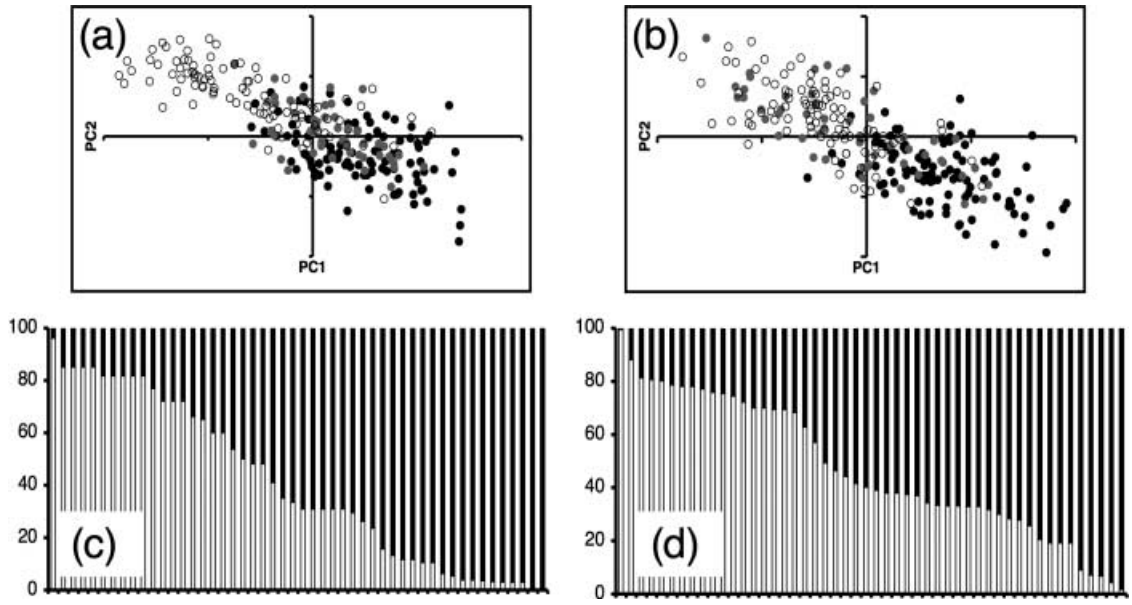

Fig. 4 Hybrid composition in *Littorina saxatilis* populations. (a, b): Individual shell shape of snails in Ramsö and Ramsholmen populations, respectively; empty circles — S snails; grey circles — hybrids; and black circles — E snails. (c,d): Ranked distribution of assignment scores to neighbouring E and S morph subpopulations in hybrids from Ramsö and Ramsholmen, respectively. Each column represents a hybrid individual and its probability of assignment to the two alternative source populations: S morph (white part) and E morph (black part).

two loci that were in Hardy–Weinberg equilibrium (because Hardy–Weinberg equilibrium is assumed by this method) and the chord distance method including all six loci. Both methods performed satisfactorily (66–77% were correctly self-assigned by the allele frequency method and 78–83% by the chord distance method), showing that most of the hybrids had introgressed genotypes; consequently smooth distributions of ranked assignment scores were produced in both populations [Fig. 4 (c, d) shows the result of the allele frequency method; the result of the chord distance method was almost identical]. Values of individual assignment scores of hybrids correlated weakly with individual shell shape ($r = 0.33$, $P = 0.02$) but not with shell size on Ramsholmen, and with shell size ($r = 0.23$; $P = 0.05$) but not with shell shape on Ramsö. Thus, both hybrid morphology and neutral multilocus genotypes show unimodal distributions of introgressed variants and no clear signs of an admixture of two parental forms.

Discussion

Although the islands of this study were only 2 km apart, island populations were substantially differentiated, indicating independent evolution of island populations (supporting earlier results from allozyme and RAPD (random amplified polymorphic DNA) loci, Johannesson & Tatarenkov 1997; Johannesson *et al*. 2004). Furthermore, gene flow between subpopulations of contrasting ecotypes within islands was impeded, supporting the existence of local reproductive barriers, and different neutral alleles contributed to these differences on the two islands. Clustering of individuals by island rather than ecotype is consistent with a scenario of *in situ* nonallopatric evolution of ecotypes on each island (Johannesson 2001; Via 2001), although it could alternatively be a result of secondary overlap and intense introgression between ecotypes that evolved during an earlier phase of allopatric separation. However, the fact that genetic differences between ecotypes on each island is generated by completely different microsatellite alleles is a strong indication of parallel processes and is difficult to explain assuming allopatric origin of ecotypes. Indeed, if introgression has proceeded so far as to eliminate *all* earlier ('allopatric') differences, the separation that followed was a nonallopatric separation anyway. Additionally, the hypothetical secondary contact between the ecotypes on each island is recent (age of islands is less than 5000 years), so introgression of two distinct gene pools would likely have left traces of a common origin.

The low F_{ST} differences between islands (0.02–0.07) might appear to suggest substantial gene flow between them, thus indicating dependent instead of parallel evolution of the two island populations. However, the theoretical maximum F_{ST} is set by homozygosity within subpopulations ($1 - H_S$) (Hedrick 2005), that is, 0.1–0.3 for the microsatellite loci of this study. Furthermore, values of F_{ST} between island populations 125 km apart are in the range 0.03–0.09 (Mäkinen, Panova, Appelqvist, Johannesson & André, unpublished data), probably due to homoplasy and the effect of variability of the marker discussed above. Consequently, F_{ST} values cannot be used to back-calculate Nm in this case. On the other hand, a direct estimate of migration of individuals between islands can be made from an earlier observation that 12% of small island populations in the same area, wiped out by a toxic algal bloom in 1988, were re-established 4 years (eight generations) later (Johannesson & Johannesson 1995). Assuming that each island population was established by one female founder mated with, seven to eight males (Janson 1987; Mäkinen *et al*. submitted) then Nm between islands is $<< 1$. Lacking a planktonic larval stage, it is still a mystery how snails are transported between islands, but the most probable suggestion is through occasional rafting on driftwood or algae, etc. Thus our conclusion is that gene flow between the two populations of this study is low enough to allow them to evolve independently of each other.

Site-specific differentiation in genetic markers (both at allozyme and microsatellite loci) in independently evolving hybrid zones was also observed in a study of Spanish populations of *Littorina saxatilis* (Rolán-Alvarez *et al*. 2004). In that study genetic differentiation within sites between an upper and a lower shore ecotype was compared with the overall differentiation between ecotypes (subpopulations from different sites pooled). No overall differentiation between ecotypes was found, while differentiation at each site was statistically significant, a consequence of site-specific rather than ecotype-specific differences (Rolán-Alvarez *et al*. 2004). The Spanish result is similar to the result of the present study, although the Spanish data were analysed using F_{ST} equivalents rather than principal component analysis.

In situ evolution of ecotypes is an important evolutionary factor structuring the ecology and the genetics of this species. Experimental work has shown that assortative mating is substantial and strongly related to size differences between *L. saxatilis* ecotypes, the average E ecotype snail being half the size of the average S ecotype snail (Hollander *et al*. 2005). Size, in turn, is one of several traits regulated by strong divergent selection promoting large and robust shells in boulder (crab-rich) areas, and small and fragile shells on exposed cliffs (Johannesson 1986). Survival rates of the robust S ecotype in the boulder habitats are about 10 times as high as that of the fragile E ecotype, and vice versa for the exposed cliffs (Janson 1983), indicating extremely strong differential natural selection. Consequently, the impeded gene flow is a secondary effect of ecological divergence of subpopulations over habitats.

Primary hybrid zones produced by nonallopatric reproductive separation are not usually expected to show evidences of intrinsic postzygotic isolation (Coyne & Orr 2004), and thus the relative fitness of hybrids in the

intermediate habitat is expected to be at least the average of the parental types (Endler 1977; Barton & Hewitt 1985; Harrison 1993). Earlier studies in both Sweden and Spain have shown that hybrid individuals have similar fitness to individuals of parental ecotypes in the hybrid zone (Janson 1983; Rolán-Alvarez *et al*. 1997); this observation lends further support to *in situ* (primary) zones rather than secondary overlap of separate lineages. Moreover, the morphological separation of the ecotypes of the present study was of comparable size on both islands, while the genetic differentiation was of different magnitudes. A similar degree of divergence in selected characters and weak variable differentiation in neutral markers are typical of primary hybrid zones formed by ecological divergence (Barton & Hewitt 1985; Barton & Gale 1993; Harrison & Bogdanowicz 1997). No association between individual morphology and microsatellite genotypes, and absence of linkage disequilibria between neutral markers in hybrids, further support primary hybrid zones.

Extensive studies of parallel evolution of reproductive barriers have been undertaken in the three-spine stickleback species complex (*Gasterosteus aculeatus*), and this case is often mentioned as the best example of parallel speciation (Coyne & Orr 2004). However, limnetic (freshwater) sticklebacks diverged repeatedly from the same ancestral anadromous (marine) lineage, and indeed allopatric divergence and repeated colonization of a lake seems a plausible alternative to sympatric divergence within a lake (Schluter *et al*. 2001). Using the same rationale as discussed above, gene flow between sympatric ecotypes might have changed the phylogeny and masked earlier periods of allopatry, in particular in nonrecombining markers such as mitochondrial DNA (Coyne & Orr 2004). So even if the role of natural selection remains unquestioned, parallel speciation in sticklebacks is inconclusive when it comes to the origin of the reproductive barrier. In *L. saxatilis*, on the other hand, locally adapted ecotypes appear in this as in other species of the genus (Reid 1996; Johannesson 2003), showing a general capacity of littorinids, in particular those with poor dispersal, to produce ecotypes suitable for specific environments.

The Swedish populations of *L. saxatilis* are similar to Spanish and UK populations in how and when ecotypes and reproductive barriers are formed (Johannesson *et al*. 1993, 1995; Rolán-Alvarez *et al*. 1996; and see Introduction), although some major ecological differences are apparent. Spanish and UK ecotypes are, for example, distributed over vertical instead of horizontal shore gradients. Moreover, both the UK and the Spanish hybrid zones have phenotype distributions that are bimodal, with hybrids often being in a minority to parental ecotypes in the intermediate habitat (Hull *et al*. 1996; Rolán-Alvarez *et al*. 1997). Nevertheless, the magnitudes of assortative mating (Johannesson *et al*. 1995; Pickles & Grahame 1999; Hollander *et al*. 2005) and locally impeded gene flow between ecotypes (Wilding *et al*. 2001; Rolán-Alvarez *et al*. 2004; Grahame *et al*. 2006; present study) are both very similar among these geographical areas. Moreover, reproductive isolation is established through the same kind of processes in both Spain and Sweden (UK remains to be tested). Thus *Littorina saxatilis* emerges as a very promising example of parallel and nonallopatric evolution of *in situ* reproductive isolation.

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