

PHYLOGENETIC EVIDENCE FOR MULTIPLE SYMPATRIC ECOLOGICAL DIVERSIFICATION IN A MARINE SNAIL

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Parallel speciation can occur when traits determining reproductive isolation evolve independently in different populations that experience a similar range of environments. However, a common problem in studies of parallel evolution is to distinguish this hypothesis from an alternative one in which different ecotypes arose only once in allopatry and now share a sympatric scenario with substantial gene flow between them. Here we show that the combination of a phylogenetic approach with life-history data is able to disentangle both hypotheses in the case of the intertidal marine snail *Littorina saxatilis* on the rocky shores of Galicia in northwestern Spain. In this system, numerous phenotypic and genetic differences have evolved between two sympatric ecotypes spanning a sharp ecological gradient, and as a side effect of the former have produced partial reproductive isolation. A mitochondrial phylogeny of these populations strongly suggests that the two sympatric ecotypes have originated independently several times. Building upon earlier work demonstrating size-based assortative mating as the main contributor to reproductive isolation among ecotypes, our analysis provides strong evidence that divergent selection across a sharp ecological gradient promoted the parallel divergence of body size and shape between two sympatric ecotypes. Thus, divergent selection occurring independently in different populations has produced the marine equivalent of host races, which may represent the first step in speciation.

KEY WORDS: Allopatric, ecological speciation, *Littorina*, parallel evolution, sympatric.

Ecological speciation occurs when disruptive selection in contrasting environments leads directly or indirectly to the evolution of reproductive isolation (Schluter 2001). Several studies have shown that traits affected by divergent selection can contribute to partial or complete reproductive isolation (Johannesson 2001; Schluter 2001; McKinnon et al. 2004; Nylin et al. 2005; Sandoval and Nosil 2005; Barluenga et al. 2006; Savolainen et al. 2006). In two recent studies (Barluenga et al. 2006; Savolainen et al. 2006; but see Schlieuwen et al. 2006), a sympatric origin of two ecotypes

was deduced because the newly formed sister taxa were restricted to a small isolated area. However, a key issue in other studies involving more broadly distributed ecotypes is to distinguish the sympatric origin from an alternative scenario in which the ecotypes arose in allopatry but there is substantial gene flow between them after secondary contact (Coyne and Orr 2004). In such cases, ongoing gene flow between ecotypes can obscure their history, making allopatrically formed populations appear to have originated in sympatry. This, for example, is the case in the stickleback

Gasterosteus aculeatus (McKinnon et al. 2004; Rundle and Schluter 2004) and the Hawaiian crickets (Shaw 2002). Most possible examples of ecological speciation also suffer from insufficient knowledge about the actual mechanism of divergence (Coyne and Orr 2004). Thus, although recent theoretical studies suggest that natural selection across habitat gradients may be an important speciation mechanism (Doebeli and Dieckmann 2003), few well-supported examples exist (Coyne and Orr 2004; Rundle and Nosil 2005), and more empirical studies are needed to ascertain its generality.

A candidate example of incipient ecological speciation is the intertidal marine snail *Littorina saxatilis*, an ovoviviparous marine gastropod with low dispersal capabilities, that has received much attention as a model system for ecomorphological diversification (Johannesson 2001; Rolán-Alvarez 2007). This snail displays an extreme microhabitat-associated intraspecific dimorphism along the wave-exposed rocky shores of Galicia (northwestern Spain). In these areas, the intertidal rocky shore displays, on a scale of a few meters, sharp microhabitat differences in irradiation, salinity, and desiccation. A small and fragile ecotype with a smooth and unbanded shell (SU) lives in the lower shore on mussels (*Mytilus galloprovincialis*; Johannesson et al. 1993), whereas a large and robust ecotype with a ridged and banded shell (RB) lives in the upper shore associated to barnacles (*Chthamalus stellatus* and *C. montagui*) (see Fig. 1). In the lower shore, the main physical and ecological factor affecting survival is the strength of the waves, whereas in the upper shore the snails need to endure daily changes in salinity, heat stress and higher predation rates by crabs (*Pachygrapsus marmoratus*; Johannesson et al. 1993; Rolán-Alvarez et al. 1997). In the midshore, both habitats overlap

across a 1–5 m wide zone forming a patched mixture of barnacles and mussels (Rolán-Alvarez 2007). In some areas of the midshore, both ecotypes meet and occasionally mate producing apparently fertile intermediate morphological forms (designated hybrids) at variable frequencies (range 1–40%; Rolán-Alvarez et al. 1997), which leads to gene flow between the two ecotypes (Johannesson et al. 1993; Fernández et al., 2005). The mean migration distance for adults in Galicia is approximately 1–2 m per month (Erlandsson et al. 1998), which generates, on a geographical scale, a scarce rate of gene flow. Furthermore, the exposed rocky shores where these two ecotypes live are not continuous, because they are typically interrupted by bays, beaches, or even the inner and sheltered parts of the Galician estuaries in which they are not present. Accordingly, previous studies using different molecular markers have shown that populations from different localities are genetically distinct because of isolation by distance (Johannesson et al. 1993, 1995; Rolán-Alvarez et al. 2004).

Several sources of evidence provide a link between ecological adaptation, vertical distribution, and phenotypic differentiation between ecotypes. First, the two ecotypes display large differences for many morphological, anatomical, and behavioral traits (reviewed in Pérez-Figueroa et al. 2005; Rolán-Alvarez 2007), and there is clear evidence indicating that most of these differences (like those due to shell form and size) are genetically based (Johannesson et al. 1993, 1997; Erlandsson et al. 1998; Carballo et al. 2001; Conde-Padín et al. 2007). Second, each ecotype shows a significantly higher fitness in its own habitat. This has been demonstrated by mark-recapture experiments (Rolán-Alvarez et al. 1997; Cruz et al. 2004a, 2004b), or inferred by comparing quantitative morphological and molecular differentiation

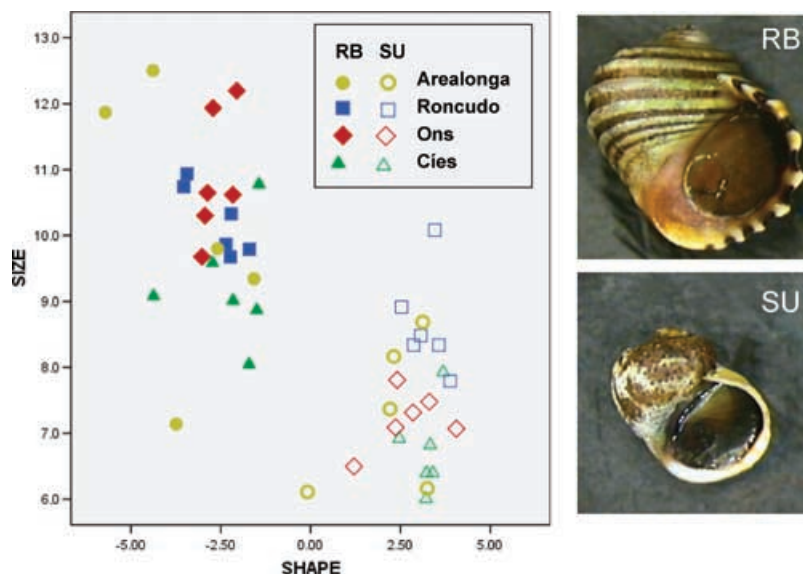


Figure 1. Individuals from different ecotypes and localities plotted for size and the first nonuniform estimates (relative warps) of shell shape. Individuals are color and symbol coded according to their geographical origin (see Fig. 3).

between ecotypes (Conde-Padín et al. 2007). Third, transplant and laboratory experiments indicate that natural selection seems to be responsible for the adaptation of each ecotype to its own habitat and shore level (Rolán-Alvarez et al. 1997; Cruz and García 2001; Cruz et al. 2001). For example, a larger shell aperture and a bigger foot in SU than in RB individuals determine higher attachment strength to the substrate in SU individuals (Rolán-Alvarez et al. 1997; Carvajal-Rodríguez et al. 2005). Thus, ecotypes of *L. saxatilis* living at different shore levels are exposed to intense divergent selection favoring morphological differences in shell size, ornamentation, color, behavior and capability to resist different environmental factors (Johannesson et al. 1993; Cruz et al. 2004c; Rolán-Alvarez et al. 1997, 2004; Conde-Padín et al. 2007).

Interestingly, an association between ecological adaptation and reproductive isolation has been also observed in the wild. This reproductive isolation is the result of two different factors. First, habitat isolation resulting from different habitat and shore level preferences contributes importantly to maintain the genetic and phenotypic cohesion of each ecotype (Johannesson et al. 1993; Erlandsson et al. 1998; Pérez-Figueroa et al. 2005), and is obviously a side effect of adaptation. Second, sexual isolation between ecotypes when they live in sympatry in the midshore results from the contribution of ecotype microdistribution, which explains up to 50% of the observed sexual isolation, and true mate choice due to shell size-based assortative mating (Johannesson et al. 1995; Erlandsson et al. 1999; Rolán-Alvarez et al. 1999, 2004; Cruz et al., 2004a; Carballo et al. 2005). Thus, in the Galician hybrid zone, divergent selection has promoted striking differences in mean size between the two ecotypes, which indirectly has produced the observed size-based assortative mating (Rolán-Alvarez et al. 1999, 2004; Cruz et al. 2004b; Conde-Padín et al. 2007).

Recent studies have shown that individuals of the same ecotype and shore level are nearly identical for several molecular markers across a geographical scale of tens of meters, but that individuals of different ecotype and shore level are slightly different at a microgeographical level (10–27 m) (Rolán-Alvarez et al. 2004). This confirms the existence of an incomplete genetic barrier between the ecotypes, obviously caused by their habitat isolation and assortative mating in the hybrid zone. However, a key question remains unanswered: did premating reproductive isolation and/or morphological differences among ecotypes evolve in sympatry, or are these differences among ecotypes the consequence of allopatric divergence followed by secondary contact between divergent populations? This question is important because it sheds light on the controversial possibility that reproductive isolation has evolved in presence of gene flow (see Coyne and Orr 2004). However, earlier attempts to assess these alternative hypotheses—although in agreement with a sympatric origin—were not fully

conclusive because of shared polymorphism in nuclear markers, lack of phylogenetic signal, or incomplete sampling (see Rolán-Alvarez et al. 2004).

The life cycle of *L. saxatilis*, characterized by low dispersal capabilities and partially isolated rocky shore populations that show a typical pattern of isolation by distance (Rolán-Alvarez 2007), provide an opportunity to test alternative phylogenetic hypotheses for the origin of the two ecotypes. Let us assume that the two ecotypes coexist in a number of localities sampled at a geographical scale large enough to make some localities independent. Under this scenario, we may examine phylogenies of alleles looking at their geographical distribution rather than analyzing the clustering pattern of populations from each ecotype in a tree. Allele trees rather than population trees should be used because the latter are inferred using the allele frequencies at each sampled location, and therefore gene flow between two populations can give the false impression that they share a most recent common ancestor when this is not true. In contrast, relationships between alleles sampled at different locations can be inferred using their nucleotide sequences, and because of this, such inference is not affected by gene flow. Thus, supposing that no ancestral alleles are still segregating at each locality, it is possible to predict three distinct phylogeographic patterns for mitochondrial alleles corresponding to three different, exclusive hypotheses about the origin of these two ecotypes (Table 1 and Fig. 2):

HYPOTHESIS A: SINGLE ORIGIN IN SYMPATRY OR ALLOPATRY

This implies that the two ecotypes became reproductively isolated either at one site (sympatry) or different sites (allopatry) from which one or both ecotypes spread out. Under this scenario, alleles should form two major phylogenetic groups (clades) owing to a common origin of alleles of the same ecotype. Within each clade, alleles can be shared between ecotypes, depending on whether gene flow between ecotypes has already occurred. In the absence of gene flow between ecotypes, phylogenetic and geographical distance between alleles should be correlated. However, this correlation is not expected after gene flow between ecotypes, because spreading patterns of shared alleles are unlikely to be coincident in time and space for each ecotype.

HYPOTHESIS B: MULTIPLE ORIGIN IN SYMPATRY

Under this scenario, alleles of the two ecotypes evolved from a common ancestor independently at each geographical locality. Thus, alleles originated at the same locality should form monophyletic groups in a phylogeny, regardless to whether they are shared between ecotypes. Moreover, we expect a correlation of phylogenetic and geographical distances both with and without gene flow between ecotypes, so that clades that are more related in the tree contain alleles that occur at localities that are geographically closer.

Table 1. Predictions of alternative hypotheses for the origin of RB and SU *Littorina saxatilis* ecotypes.

Hypothesis	No gene flow between ecotypes		Gene flow between ecotypes	
	Phylogenetic clustering	Isolation by distance	Phylogenetic clustering	Isolation by distance
(A) Single origin in sympatry or allopatry	Two major clades, ecotype especific	YES	Two major clades, neither ecotype nor locality specific	NO
(B) Multiple origin in sympatry	Multiple clades, locality specific	YES	Multiple clades, locality specific	YES
(C) Multiple origin in allopatry	Multiple clades, neither locality nor ecotype specific	YES ¹	Multiple clades, neither locality nor ecotype specific	YES ¹

¹Weaker than in hypothesis B.

HYPOTHESIS C: MULTIPLE ORIGIN IN ALLOPATRY

In this case, each ecotype has arisen several times as a result of multiple and independent divergence events in allopa-

try. Under this scenario, alleles from each ecotype are located essentially randomly within the phylogeny. Assuming the less favorable situation to distinguish between the three alternative

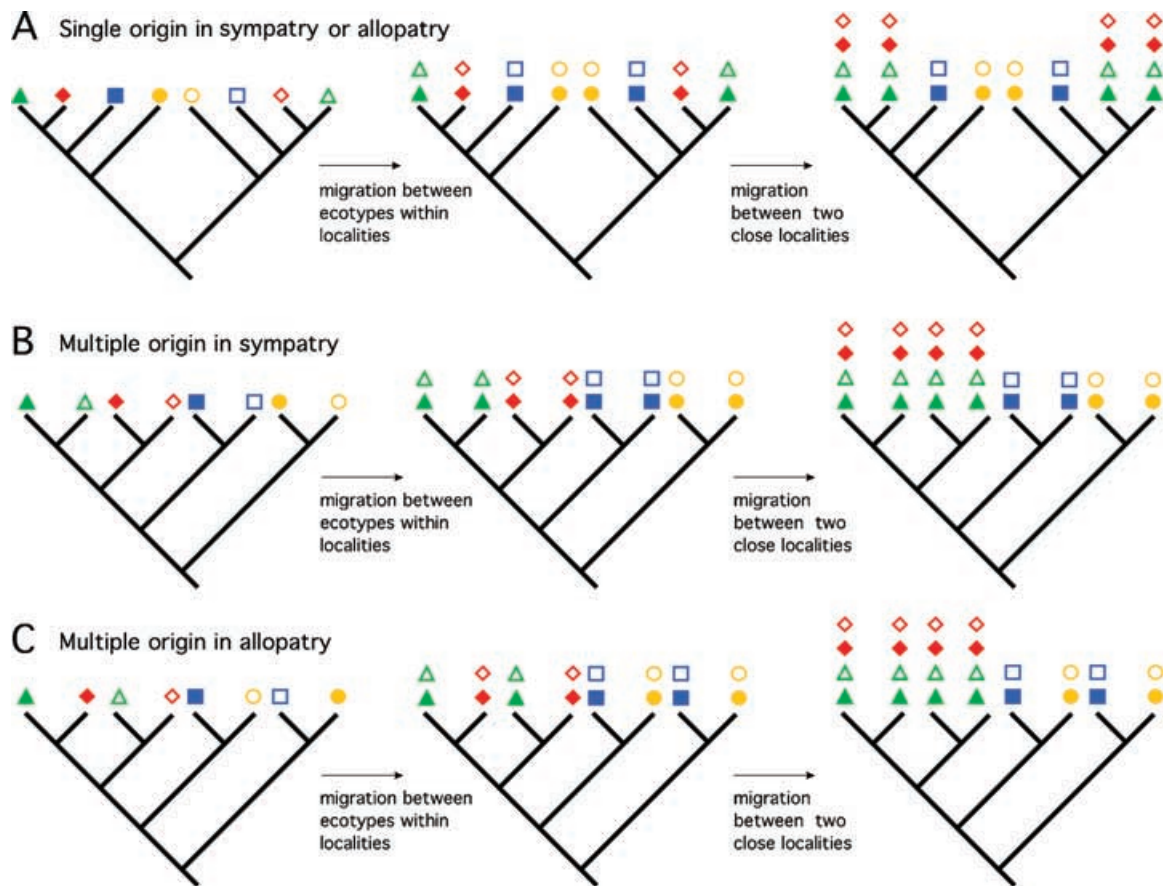


Figure 2. Alternative phylogenetic hypotheses for the origin of two ecotypes (open and closed circles). Colors and symbols represent geographical localities. (A) Single origin in sympatry or allopatry. Alleles are clustered in two distinct major phylogenetic lineages. (B) Multiple origin in sympatry. Alleles group by locality. (C) Multiple origin in allopatry. Alleles do not cluster by locality nor by ecotype. In the three scenarios three situations are considered: (1) No introgression between ecotypes; (2) Introgression between ecotypes within each locality; (3) Gene flow between two localities. Note that alleles do not cluster by ecotype under any of the hypothesis when there is introgression between ecotypes.

hypotheses (Fig. 2C), phylogenetic and geographical distances would be correlated regardless of whether or not there is gene flow between ecotypes.

To evaluate the above hypotheses in the *L. saxatilis* model, we obtained sequences of a 1.83 Kb mitochondrial DNA (mtDNA) region in samples of RB and SU ecotypes from northwestern Spain. Our rationale for using mtDNA was that low levels of recombination in this molecule would let us resolve intact haplotypes, which would then permit historical inferences to be made for hypothesis testing. We also studied morphological variation between the two ecotypes to quantify this type of differentiation between ecotypes and localities.

Methods

SAMPLING

Forty-eight adult snails were obtained between July and September 2004 from four different wave-exposed localities encompassing the entire distribution of the two *L. saxatilis* ecotypes. Individuals of the two ecotypes were collected from geographically distant regions (Arealonga and Roncudo) and from islands separated 15–20 km from each other and from the continent (Ons and Cies) (see Fig. 3). Such a sampling design was chosen to maximize the probability that the ecotypes living at a particular locality had evolved independently and that *L. saxatilis* populations were physically isolated, minimizing gene flow between them. At each

locality, 6 RB and 6 SU individuals were collected from 1 m² plots of the upper and lower shore levels, respectively, along a vertical transect 10–20 m long.

MORPHOMETRIC ANALYSIS

Sampled specimens were examined using a Leica MZ12 stereoscopic microscope before molecular analysis. Color images were captured by a Leica digital ICA video camera. Adult shell images ($n = 46$) were analyzed using 12 landmarks (coordinate points) positioned on the digitalized shell image (online Appendix A1 and Carvajal-Rodríguez et al. 2005). Size was measured by the centroid size, the square root of the sum of squared distances of landmarks to their centroid (mean X and Y coordinate points), whereas shape was measured by both the uniform component and the relative warps (Zelditch et al. 2004). The uniform component was computed using the space complement to the relative warps, and the relative warp was obtained excluding the uniform component with a scaling option of $\alpha = 0$. Landmarks were recorded for each specimen with the program TPSDIG, whereas size and shape components were computed with TPSRELW (available from <http://life.bio.sunysb.edu/morph/soft-tps.html>). Mean differences between groups in shell size and shape were assessed with a two-way ANOVA using the factors ecotype (fixed; RB vs. SU) and locality (random; the four localities). The analysis was performed using SPSS/PC version 12.01.

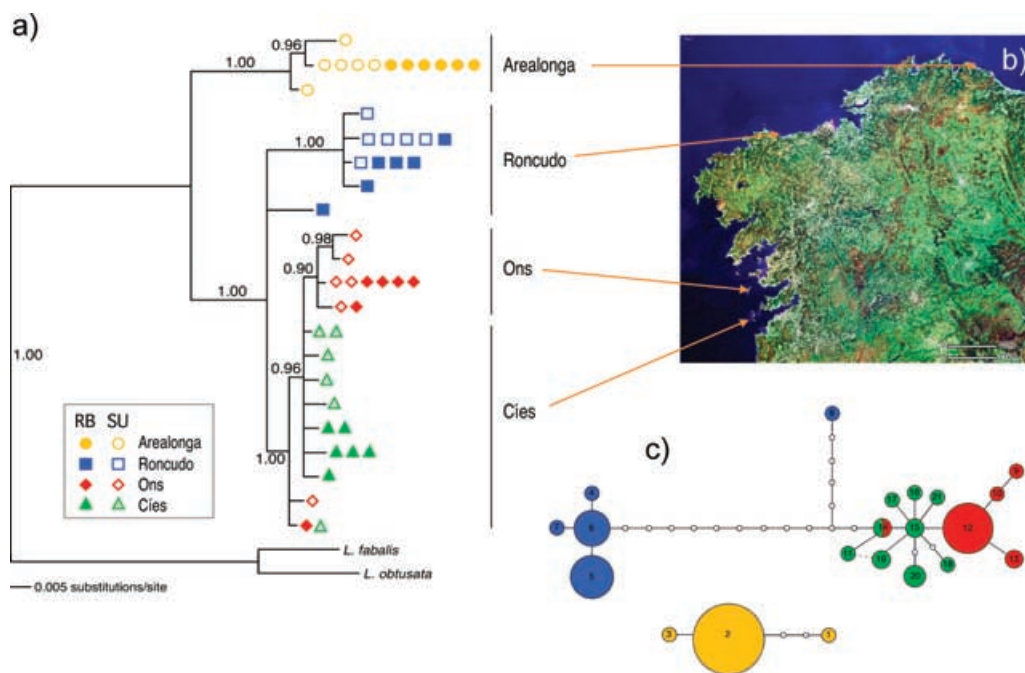


Figure 3. (a) Bayesian allele tree. Numbers above branches indicate posterior probabilities. RB and SU ecotypes are depicted as close and open circles, respectively. (b) Location of sampling sites. (c) Statistical parsimony network. Each line represents one mutational step connection between two haplotypes. Small empty circles represent missing intermediate haplotype states. Haplotypes numbered as in online appendix A2. Pie charts represent the frequencies at each locality. In all panels, color and symbol designation indicates geographic origin.

DNA EXTRACTION AND SEQUENCING

The shells of the 48 specimens of *L. saxatilis* and two outgroup taxa (*Littorina fabalis* and *L. obtusata*) were broken and the head-foot tissue was used for extracting total genomic DNA, according to a standard small-scale procedure (Wilding et al. 1999). Primers designed from the annotated *L. saxatilis* partial mtDNA sequence (AJ132137 from Wilding et al. 1999) were used to amplify a 1.83 Kb fragment encompassing the *ND6* and *tRNA_{Pro}* mitochondrial genes, as well as the 3' end of the *ND1* gene and the 5' end of the *Cyt-b* gene. After PCR purification using GFX columns (Amersham Biosciences, Piscataway, NJ), both strands were sequenced directly with internal primers using an ABI 310 automated sequencer. Newly reported DNA sequences are deposited in the EMBL nucleotide sequence database under accession numbers AM500945–AM500967.

SEQUENCE ANALYSIS

Contigs were assembled using Seqman (DNASTAR; Madison, WI) and aligned with ClustalX (Thompson et al. 1997). The resulting alignment (1832 bp) was inspected by eye and needed no further refinements. Data was searched for evidence of recombination using the programs MaxChiGlobal (Posada and Crandall 2001), Geneconv (Sawyer 1989), and Reticulate (Jacobsen et al. 1997). Several tests were used to detect a possible departure from a neutral equilibrium (Tajima 1989; Fu and Li 1993; Fu 1996, 1997). Estimates of polymorphism, neutrality tests, and other population genetic analyses were carried out in DnaSP 4.0 (Rozas et al. 2003). The effective number of migrants (N_m) was estimated using the N_{ST} statistic (Lynch and Crease 1990). We performed a molecular variance analysis considering a two-level hierarchical partition (localities and morphs within localities) using Arlequin 2.0 (Schneider et al. 2000). This program was also used to compute Mantel tests between genetic and geographical distance matrices. Genetic differentiation between populations was also assessed using the S_{nn} statistic, which is a measure of how often the nearest neighbors of sequences are from the same locality in geographical space (Hudson 2000). Divergence times between ecotypes were obtained for each population using the program IM (Hey and Nielsen 2004), assuming a $1.83 \pm 0.21\%$ population divergence per Mya (Wilke and Pfenninger 2002). In each case, a minimum of five Markov chains were run independently with different conditions and checked for convergence using the autocorrelation and effective sample size values, and comparing the resulting posterior distributions for the different parameters. The length of each chain was always above 250 million generations, without heating. Burn-in was 10,000 generations. We tried different upper bound thetas, different maximum migration rates and several upper bounds on the prior distribution for divergence time, always resulting in very similar posterior distributions for the divergence time estimates.

PHYLOGENETIC ANALYSIS

The best-fit model of nucleotide substitution was selected using the Akaike Information Criterion in Modeltest 3.6 (Posada and Crandall 1998). Bayesian phylogenetic analyses were performed with MrBayes 3.1 (Huelsenbeck and Ronquist 2001) under the best-fit nucleotide substitution model and also under a combined model including nucleotide, codon, and doublet substitution schemes. Four chains (three heated and one cold) were run for 6×10^6 generations. Trees were sampled every 1000 generations. The initial 10^6 generations were discarded for burn-in. Every analysis was repeated at least twice, and checked for convergence. In addition, a statistical parsimony network (Templeton et al. 1992) was reconstructed using TCS 1.18 (Clement et al. 2000). The molecular clock hypothesis was tested with a likelihood ratio test (Felsenstein 1981).

Results

Despite the fact that the different geographical regions are physically isolated from each other by large expanses of land, sea, or both, morphology is consistent among regions within each ecotype but differs dramatically between ecotypes, as expected if ecotypes reflect a similar type of divergent selection among localities (Fig. 1). In fact, the two-way ANOVA shows that two traits (size and the first relative warp) display significant differences between ecotypes ($P_{\text{SIZE}} = 0.010$; $P_{\text{RW1}} = 0.042$), but not between localities ($P_{\text{SIZE}} = 0.312$; $P_{\text{RW1}} = 0.295$). Differences between ecotypes are also summarized by a canonical discriminant function using all the shape variables studied (see online Appendix A1). These results are consistent with previous morphological studies indicating that each ecotype shows a cohesive size and shape across localities sampled at a smaller geographical scale (Johannesson et al. 1993; Carvajal-Rodríguez et al. 2005; Conde-Padín et al. 2007).

DNA sequence data reveals little variation in the assayed mtDNA fragment among the 48 specimens examined. There were 21 distinct *L. saxatilis* haplotypes, with a total of 55 variable sites out of the 1832 sites surveyed (online Appendixes A2 and A3). Nucleotide changes were found mostly at synonymous sites (42 out of 55), and we observed no significant differences in overall nucleotide diversity between RB ($\Theta = 0.0067 \pm 0.0024$) and SU ($\Theta = 0.0069 \pm 0.0024$) ecotypes. Nucleotide variation of RB and SU ecotypes does not deviate from an equilibrium neutral model at any sampling site (online Appendix A4). Similarly, substitution rates do not deviate significantly from a molecular clock (LRT; $P = 0.2000$), and we found no evidence for recombination. These results suggest that *Littorina* mtDNA is a suitable marker for our purposes here.

A hierarchical analysis of molecular variance reveals that most of the variation (89%) is accounted for by location

($P = 0.0039$), whereas differences between ecotypes only represent a minor proportion of the molecular variance (0.71%; $P = 0.0469$). We also found that geographical distance is positively correlated with genetic distance between ecotypes ($r = 0.937$, $P = 0.001$, Mantel test) but not with morphological divergence ($r = -0.218$, $P = 0.095$). These findings indicate a close relationship between geographically adjacent populations rather than between ecotypes, as expected if *L. saxatilis* populations have evolved repeatedly from an ancestral population inhabiting the same region.

The phylogenetic analysis (Fig. 3) clearly indicates that alleles group by sampling location with very high posterior probabilities. Importantly, relationships among these clades correlate very well with their geographical distribution. That is, clades that are closer in the tree are also closer geographically. In addition, the tree topologies of the RB and SU alleles are almost perfectly concordant (Fig. 4; Pearson correlation coefficient among RB and SU distance matrices $r = 0.99$).

The results above allow us to reject Hypothesis A, which predicts only two major phylogenetic clades without any geographical ascription (Table 1 and Fig. 2) and Hypothesis C, which predicts multiple clades with no geographical patterning. Indeed,

our results agree very well with the phylogenetic pattern expected under the multiple sympatric scenario (Hypothesis B), because this hypothesis predicts multiple clades, locality specific. Thus, when dispersal is low (as in *L. saxatilis*), and some locations are independent (as in our samples, given their large geographical distance), only a multiple sympatric origin explains the pattern we observe. Not even a random fixation of alleles after a single origin could explain the phylogenetic pattern we detect, because in that case any correlation between phylogenetic and geographical distances among clades would disappear. Furthermore, one of the advantages of the Bayesian analysis is that allows to calculate posterior probabilities for any phylogenetic hypothesis given the data. Given our sample of trees from this analysis, the phylogenetic hypothesis in which every allele clusters by location (Hypothesis B), except for a distinct allele from Roncudo that is very likely an immigrant from a nearby location, and allowing for gene flow between Ons and Cíes, has a posterior probability of 0.9895. Therefore, any other phylogenetic hypothesis (A, C) will have a posterior probability smaller than 0.0105.

Interestingly, the geographically proximate Ons and Cíes populations share a small and divergent separate clade. We suspect that insufficient time for full lineage sorting rather than recent

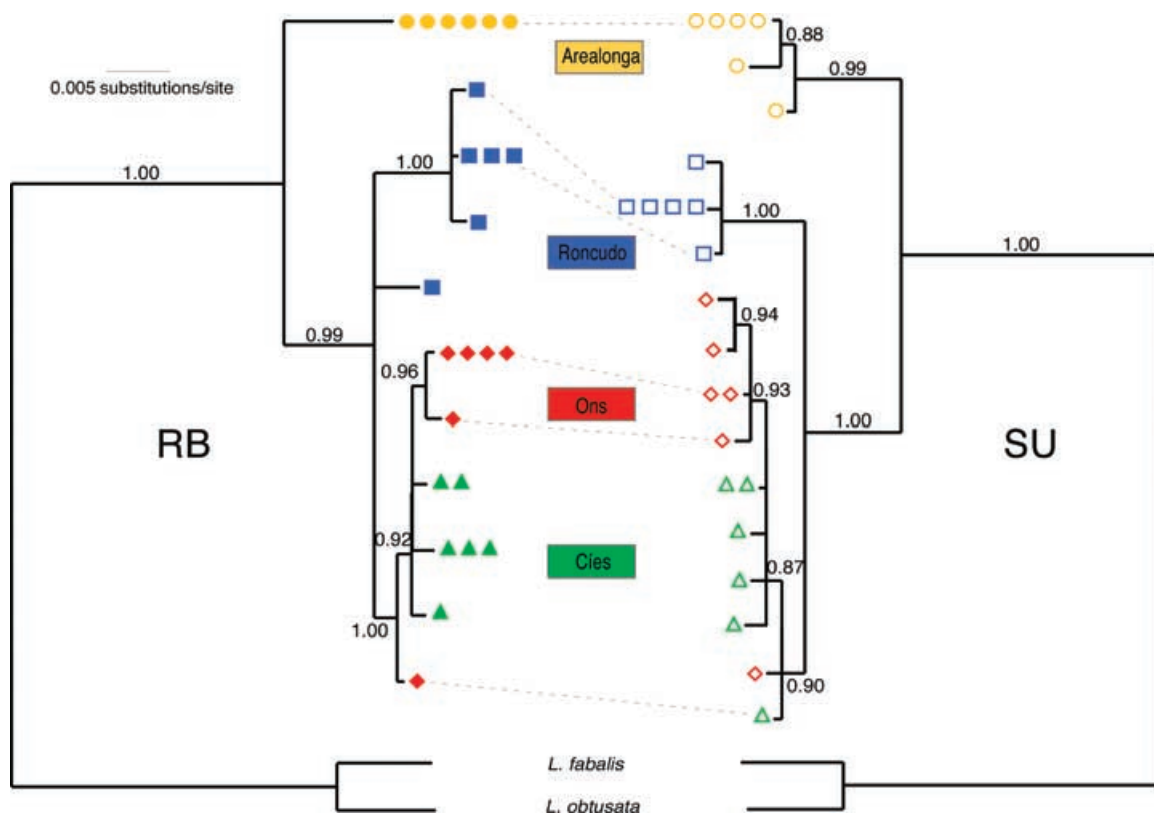


Figure 4. Bayesian tree for each ecotype. Numbers above branches indicate posterior probabilities. RB and SU ecotypes are depicted as close and open circles, respectively. Color and symbol designation indicates geographic origin. Dotted lines link shared haplotypes between RB and SU ecotypes.

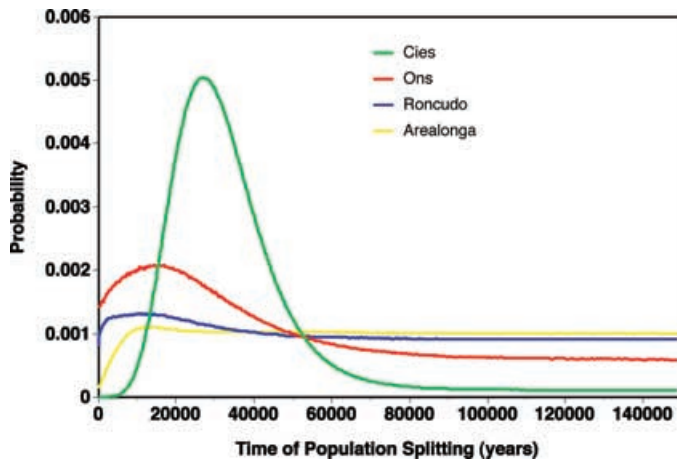


Figure 5. Probability distributions for divergence times of the ecological diversification within each of the four populations studied estimated with the program IM (Hey and Nielsen 2004). Time scale converted to years assuming a $1.83 \pm 0.21\%$ population divergence per Mya (Wilke and Pfenninger 2002).

gene flow between these two populations has contributed to this pattern. Another observation consistent with the parallel origin hypothesis is that the haplotype network contains strong signal of local radiations, with many closely related haplotypes arising from a small number of divergent haplotypes in the same population (Fig. 3). The recent origin of the RB and SU ecotypes is underscored by the finding that the average divergence per site

between ecotypes is only 0.001—as much as 17 times smaller than the divergence among localities. Approximate estimates for the origin of the ecological diversification within each of the four populations studied suggest that divergence between ecotypes, at least for some populations, occurred within the last 40,000 years (Fig. 5).

Our conclusion that different populations have independent histories is corroborated by the number of shared polymorphisms and fixed differences (Table 2). These analyses consistently reveal that distant populations have diverged substantially from each other, have a high number of fixed differences, and do not share polymorphisms. These patterns are consistent with the assumption that these populations have little or no shared ancestry and are not exchanging genes. In contrast, divergence between sympatric RB and SU ecotypes is low, they do not show fixed differences, and about 11% of all polymorphisms are shared, as expected in lineages that have recently split from a common ancestor or are exchanging genes. Despite this, RB and SU ecotypes remain distinct in sympatry: sites being polymorphic in one or the other ecotype indicate independent mtDNA histories. Estimates of gene flow strongly support that sharp gene flow restrictions occur and that they are predominantly geographically driven (Table 2). These data indicate that gene flow among geographically distant regions ceased or was drastically reduced long time ago, supporting that parallel evolution, and not shared history, is responsible for ecotype diversification.

Table 2. Distribution of variable sites in pairwise comparisons. The first column gives the items compared. The next five columns indicate the average number of differences, number of fixed nucleotide differences, sites that are polymorphic in only one of the items compared (each item appearing in the same order as referred in the first column), and sites that are polymorphic in both items.

Comparison	Avg. no. differences	Fixed differences	Exclusive polymorphism		Shared polymorphism	Nm^a	$P(Snn)^b$
			Polym.1	Polym. 2			
Between localities ^c							
Arealonga-Roncudo	31.417	21	4	17	0	0.03	0.0000
Arealonga-Ons	26.000	22	4	6	0	0.02	0.0000
Arealonga-Cíes	26.000	22	9	4	0	0.03	0.0000
Roncudo-Ons	13.083	1	17	6	0	0.11	0.0000
Roncudo-Cíes	13.250	2	17	9	0	0.13	0.0000
Cíes-Ons	2.611	0	7	4	2	1.18	0.0000
Between ecotypes							
RB-SU Arealonga	0.667	0	0	4	0	5.00	0.0000
RB-SU Roncudo	3.278	0	15	1	1	3.72	0.0420
RB-SU Ons	1.500	0	0	3	3	8.00	0.3524
RB-SU Cíes	2.333	0	4	5	0	2.00	0.0000

^aEffective number of migrants.

^bProbability of the Snn test statistic of genetic differentiation (Hudson 2000).

^cRB and SU individuals were pooled within localities.

Discussion

Parallel evolution of similar traits in populations that inhabit similar environments strongly implicates natural selection, as genetic drift is unlikely to produce concerted change, correlated with the environmental differences, in multiple independent lineages. An important distinction between parallel speciation and the more general concept of parallel evolution is that parallel speciation requires independent and repeated evolution of the same isolation barriers (Schluter and Nagel 1995; Schluter 1998; Nosil et al. 2002). This implies that natural selection has caused the divergence. In some model systems this has been deduced by showing that reproductive isolation between ecotypes is observed independently of the geographical origin of the ecotypes (Schluter and Nagel 1995; Nosil et al. 2002). In our model system this has been deduced by studying the biological mechanism responsible for the reproductive isolation in the species. This reproductive isolation is the result of two different factors. First, ecological isolation resulting from different habitat and shore level preferences (Erlandsson et al. 1998). Second, sexual isolation between ecotypes in the midshore, which is a side effect of ecotype microdistribution and size-assortative mating (Cruz et al. 2004a; Rolán-Alvarez et al. 2004).

Size-based assortative mating is a common phenomenon in many populations of *L. saxatilis* within and outside the hybrid zone studied here (Saur 1990; Reid 1996; Erlandsson and Rolán-Alvarez 1998). In the present case, divergent natural selection has produced striking differences in mean size and shape between the two ecotypes which indirectly has produced the partial sexual isolation observed (Rolán-Alvarez et al. 1999, 2004; Cruz et al. 2004b; Conde-Padín et al. 2007). If the shifts in morphology are a consequence of divergent selection on traits that are relevant to mate choice through size-based assortative mating, the dynamics of the *L. saxatilis* system might eventually lead to parallel speciation. However, we do not know yet whether the incomplete reproductive isolation between these two ecotypes will ever become complete. Indeed, computer simulations suggest that the observed pattern of variation could be maintained as an intraspecific polymorphism given the estimated levels of migration, assortative mating and divergent selection (Pérez-Figueroa et al. 2005). If the two ecotypes finally speciate or not is of secondary relevance, as the most interesting aspect is that we are observing a mechanism able to cause speciation (we only need to imagine a further amplification of the environmental differences affecting to these two ecotypes to complete the speciation process). The sympatric distribution of RB and SU ecotypes, the high gene flow estimated between ecotypes ($Nm > 4$), the evidence of reproductive isolation, the monophyly within regions, and the rejection of a putative allopatric scenario, satisfy all the criteria required to conclude a multiple sympatric origin of both ecotypes (Coyne and Orr 2004).

A hypothetical scenario for the origin of RB and SU ecotypes considers that an ancestral morph arrived to Galician coasts from the North, because this species shows a preferentially northern distribution in Europe (Reid 1996). The ancestral morph then spread to the South, splitting into the two ecotypes every time that a new exposed site was reached. Indeed, the allele tree shows an ordered or directional trend (Fig. 3): alleles from the most northern population (Arealonga) form the most basal clade, and then clades containing alleles from more southern populations gradually follow as subsequent nested branches, supporting that Galician territories were colonized in a north-to-south fashion. Because natural selection is extremely strong and local (the environmental gradients are not the same in every shore), the fixation of alleles in geographically distant and partially isolated localities was in practice an independent process.

An alternative hypothesis of a multiple origin of RB and SU ecotypes in microallopatry (Rolán-Alvarez et al. 2004) seems very unlikely. This hypothesis posits that alleles from each ecotype evolved repeatedly and independently in allopatry in each locality. Thus, alleles originated in each locality should form monophyletic groups (regardless to whether they belong to one or both ecotypes), and phylogenetic proximity of clades should be correlated with their geographical distance. The phylogenetic predictions of this scenario are thus essentially the same as those of Hypothesis B (Fig. 2). Two observations, however, make the microallopatric hypothesis seem unrealistic for these rocky shore populations. First, we can think of no geological or oceanographic mechanism that could have kept the two ecotypes physically separated *within each locality* over long enough periods to permit the observed genetic differentiation between ecotypes. Indeed, in the midshore, the habitats that are characteristic of each ecotype overlap across a 1–5 m wide zone forming a mosaic distribution of mussel and barnacle patches in which RB and SU ecotypes meet and occasionally mate. Second, the mean migration distance for adults is approximately 1–2 m per month (Janson 1983; Erlandsson et al. 1998), and previous studies have shown a relatively large gene flow at a local scale, particularly in the midshore (Johannesson et al. 1993; Rolán-Alvarez et al. 1996; Fernández et al. 2005). These considerations indicate that a microallopatric scenario is unlikely, and that our results are more parsimoniously explained under a multiple sympatric origin of ecotypes with some introgression between them within each locality (intermediate scenario in Fig. 2).

Other potential alternative explanations for the observed mtDNA tree under a single origin of ecotypes scenario are very unlikely for several reasons. We can rule out weak phylogenetic signal generating misleading phylogenetic relationships because the Bayesian tree is very well supported, with high posterior probabilities (Fig. 3). This implies that alternative phylogenetic hypotheses are not supported by the data. In addition, the collected

snails are likely to adequately represent the genetic variation of each exposed site as a whole, because previous studies indicate that individuals of the same ecotype and shore level are nearly identical for several molecular markers across a geographical scale of tens of meters (Rolán-Alvarez et al. 2004). Fortuitous misclassification of individuals within localities can be excluded given the sharp morphological differences among ecotypes (Fig. 1), and because RB and SU individuals were collected from the upper and lower shore levels, respectively, where intermediate forms are unlikely to occur (Johannesson et al. 1993). Amplification of pseudogenes resulting from nuclear integrations of mtDNA fragments (Bensasson et al. 2001) also seems a remote possibility, as the unusual patterns of molecular evolution that are consistent with the reduced functional constraint (e.g., elevated frequencies of nonsynonymous substitutions, frameshifts, and stop codons), or nuclear location (slowed rates of substitution) were not detected. Lineage sorting (ancestral polymorphism; Pamilo and Nei 1988) among haplotypes in the ancestral population is not consistent with the data, because the geographical distribution of mtDNA haplotypes in the tree is not random. Indeed, we repeatedly observe locality-specific clades including individuals from the two ecotypes. Moreover, geographically proximate clades are closer in the tree than are geographically distant clades. Such observations are better explained assuming that nucleotide substitutions have accumulated independently in each clade after its separation from the ancestor, as predicted under the hypothesis of parallel evolution (see Table 1 and Fig. 2).

We recognize that mtDNA represents a single line of phylogenetic evidence. Nonetheless, as shown here, mtDNA may be an important component in the identification of evolutionary lineages when the history of alleles rather than the history of populations is considered, allowing one to discriminate between common ancestry and gene flow. This is particularly true given the low levels of recombination in the mitochondria (but see Tsaousi et al. 2005). We note that the asymmetric mtDNA introgression from one ecotype to another could confound the allele phylogeny by removing the phylogenetic information of one ecotype. Thus, alleles from each ecotype could cluster by sampling location after the origin of the two ecotypes in allopatry in one location followed by asymmetric hybridization and the subsequent spread of the two morphs. However, under this scenario, we do not expect concordant tree topologies between RB and SU ecotypes, because their colonization patterns are unlikely to be concordant in time and space. In clear contrast, we did observe completely concordant tree topologies between the two ecotypes (Fig. 4; Pearson correlation coefficient among RB and SU distance matrices $r = 0.99$), as predicted under a multiple and sympatric origin of RB and SU ecotypes. The alternative hypothesis of a single allopatric origin in one location followed by secondary contact and asymmetric hybridization at each locality after the spread of both

ecotypes also appears unlikely. This is because a specific mechanism has to be invoked to set apart each ecotype until secondary contact in each locality, and to ensure the asymmetric introgression from one ecotype to another in each independent locality. Indeed, the possibility of asymmetric mtDNA introgression contrasts with the fact that RB and SU ecotypes remain distinct in sympatry due to the occurrence of sites being polymorphic in one or other ecotype but not both (Table 2), thus indicating separate mtDNA histories.

In recent years, a few investigators have used methods that combine distributional data with explicit hypotheses of species relationships to infer the prevalence of allopatric versus sympatric speciation (Lynch 1989; Frey 1993; Chesser and Zink 1994; Berlocher 1998; Chan and Moore 1999; Barraclough and Vogler 2000; Johnson and Cicero 2002; Fitzpatrick and Turelli 2006). These studies show the pervasiveness of allopatric speciation (Barraclough and Vogler 2000; but see Lossos and Glor 2003). The accuracy of these methods depends critically on the initial assumptions, and debate exists on how often these assumptions are met (Avice 2000; Lossos and Glor 2003; Fitzpatrick and Turelli 2006). However, research integrating phylogenetic hypotheses with additional evidence that argues against a scenario of nonsympatric speciation can provide compelling evidence for sympatric divergence, particularly when referring recent divergence events (Schlieven et al. 1994; Berlocher 1998; Avice 2000). In our study, we do not aim to introduce a general method for disentangling sympatric versus allopatric divergence for any species and situation, but a phylogeographic approach tailored for a particular species with low dispersal capabilities, and sampled at a geographical scale large enough to make some localities independent. Both assumptions are fully satisfied in our study. Although we have assumed isolation by distance, the specific predictions for the tree topologies are independent of this assumption provided that at least some localities are independent. Thus, the observation that in the phylogenetic tree haplotypes group by location, when dispersal is low and some locations are independent, is most parsimoniously explained by a multiple sympatric origin, particularly when coupled with ecological and field experimental data.

In North Atlantic shores, *L. saxatilis* populations also display extreme intraspecific polymorphisms across sharp ecological gradients. Distinct pairs of morphs are found on shores in England (Hull et al. 1996) and Sweden (Janson 1983). In England, the two morphs live separately in the upper- and mid-intertidal shore levels, respectively, and show partial reproductive isolation, although intermediates between the two morphs are very rare (Hull et al. 1996). The lack of intermediates and the discontinuous distribution suggests that the observed differentiation could be attributed to secondary contact between populations after allopatric divergence (Hull et al. 1996; Wilding et al. 2001; Grahame et al. 2006). In Sweden there is evidence for habitat related variation in

morphology, survivorship and in enzyme polymorphism, although the environmental gradient is horizontal (exposed vs. sheltered) rather than vertical due to the short tide ranges (Janson 1983; Johannesson and Tatarenkov 1997). Nevertheless, the two morphs rarely overlap in the field, and there is not clear evidence of a partial reproductive barrier between them (Janson 1983; Johannesson 2003; but see Hollander et al. 2005 and Panova et al. 2006). Thus, in clear contrast to the situation in northwestern Spain, in both Sweden and England sexual isolation is not able to contribute to the maintenance of the polymorphism, because there are not stable hybrid zones in which different morphs can meet and mate (Hull 1998; Johannesson 2003; Rolán-Alvarez 2007).

Several similar studies to ours suggest that reproductive isolation also may have evolved in parallel (reviewed in Coyne and Orr 2004; Rundle and Nosil 2005). Of the accepted demonstrations of parallel evolution, at least three of the four cases discussed by Rundle and Nosil (2005) represent early stages of speciation in which hybrids can still occur, genetic incompatibilities are weak or absent, and restricted gene flow exists between ecotypes (e.g., Funk 1998; Rundle et al. 2000; Nosil et al. 2002; see also Coyne and Orr 2004). However, in the majority of cases reporting parallel evolution, the reproductive isolation has been studied in the laboratory, and so it is difficult to know the real contribution of the ecological trait to reproductive isolation in the wild (Coyne and Orr 2004). A few exceptions exist, such as the size-based reproductive isolation described in natural populations of the threespine stickleback (*Gasterosteus aculeatus*), where it is known that size differences between ecotypes are caused by differential adaptation and also yield partial reproductive isolation (Nagel and Schluter 1998; McKinnon et al. 2004; Boughman et al. 2005). However, *L. saxatilis* is unusual in that most other putative cases of parallel evolution do not involve a sympatric origin of independently evolved ecotypes (e.g., Funk 1998; Nosil et al. 2002; McPeck and Wellborn 1998; Schluter et al. 2001; Rundle and Schluter 2004; see also Coyne and Orr 2004). Thus, *L. saxatilis* might represent a singular model system to assess the link between reproductive isolation and adaptation.

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Supplementary Material

The following supplementary material is available for this article:

Appendices S1–S6

Data matrix (news format)

This material is available as part of the online article from:

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1558-5646.2007.00135.x>

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