

Genetic Differentiation and Estimation of Effective Population Size and Migration Rates in Two Sympatric Ecotypes of the Marine Snail *Littorina saxatilis*

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On exposed rocky shores in Galicia (northwest Spain), a striking polymorphism exists between two ecotypes (RB and SU) of *Littorina saxatilis* that occupy different levels of the intertidal zone and exhibit an incomplete reproductive isolation. The setting has been suggested to represent ongoing sympatric speciation by ecological adaptation of the two ecotypes to their respective habitats. In this article we address whether or not the ecotypes have developed their own population structures in response to the rigors of their corresponding environments and life histories. We analyzed four to five allozymic *loci* from three surveys of the same sites, spanning a 14-year period. An experimental design including three localities with two transects per locality and three shore levels allowed studying temporal and spatial population structure and estimation of effective population sizes (N_e), neighborhood sizes (N_n), and migration rates (m). Genetic differentiation was significantly lower in RB populations ($\theta_{ST} = 0.067$) than in SU ones ($\theta_{ST} = 0.124$). Mean estimates of N_e , N_n , and m did not differ significantly between ecotypes, but local ecotype differences in migration between the two closest localities (larger migration rates in RB than in SU populations) could explain the pattern in population differentiation.

Two ecotypes of *Littorina saxatilis* appear on the same rocky shores of Galician coasts (northwest Spain), but occupy different shore-level microhabitats separated by a few (10–25) meters. The larger ecotype is ridged and banded (RB), typically occurring among barnacles on the upper shore, whereas the smaller smooth and unbanded ecotype

(SU) lives on the lower shore among mussels. These ecotypic differences are considered adaptive (Rolán-Alvarez et al. 1997): the lower shore habitat is wet and heavily wave exposed, favoring smaller shells with larger apertures, whereas the upper shore is more air exposed and inhabited by crabs, favoring larger, more sculptured shells. The two ecotypes are found in their respective habitats at variable, but typically high densities, separated by some areas without or with low snail densities. Adult migration has been estimated to be on the order of 1 m/month (Erlandsson et al. 1998; Janson 1983), allowing the opportunity for snails to move along the physical distance separating both habitats. The ecotypes meet and produce intermediate forms or hybrids at the midshore, where both habitats (barnacles and mussels) overlap, forming a patchy distribution (Johannesson et al. 1993). RB and SU ecotypes differ in many morphological, anatomical, and behavioral traits, and exhibit strong assortative mating in the midshore (Rolán-Alvarez et al. 1999). This system has been suggested to be a case of ongoing sympatric speciation driven indirectly by natural selection adapting each ecotype to its respective ecological niche (Rolán-Alvarez et al. 2004).

Populations of the same species living in the same area are expected to show similar degrees of differentiation and gene diversity, assuming they share similar life-history and demographic characteristics. However, because the two ecotypes are adapted to different microhabitats and show a strong, but partial reproductive isolation in the hybrid zone, a question arises as to whether or not they have already developed their own population structures. In this article we

address this question by analyzing allozymic data from three localities spanning a 14-year period and estimating population genetic differentiation, effective population sizes, neighborhood sizes, and migration rates for each of the ecotypes.

Materials and Methods

Samples and Loci Studied

The three datasets for *L. saxatilis* were obtained for the same sites using the same sampling design, but in different years. The sampling design included three different localities, with two parallel transects in each locality and three different shore levels per transect. The two closest localities (Silleiro and La Cetarea, at a distance of about 25 km) belong to a nearly continuous area in which both upper and lower shore populations are common and dense (see Figures 1 and 2 from Rolán-Alvarez et al. 2004), whereas Corrubedo (at 52 km from Silleiro) is separated from the others by two estuaries in which the RB and SU ecotypes are replaced by other ecotypes. The distance between the two transects in each locality is 24 m in Corrubedo and La Cetarea, and 45 m in Silleiro. The vertical distance between the upper shore and midshore ranges between 6 m and 21 m, and that between the midshore and lower shore between 2 m and 7 m.

In the 1989 dataset, a total of 24 samples (RB individuals from the upper shore and midshore and SU individuals from the lower shore and midshore, in two transects per locality and three localities), with 48 individuals on average (range 4–100 individuals) per sample, were analyzed for four polymorphic loci (Johannesson et al. 1993): phosphoglucose isomerase (*Pgi*, 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 methods used to run and score these allozymes are detailed in Janson and Ward (1984). A second similar sampling scheme was carried out during June–July 1999 (Rolán-Alvarez et al. 2004). Again 24 samples were taken, with 25.6 individuals per sample on average (range 15–67 individuals), analyzing five polymorphic loci: *Pgi*, *Aat-1*, phosphoglucose mutase (*Pgm-2*, EC 5.3.1.9), leucine aminopeptidase (*Lap*, EC 3.4.1.1), and arginine kinase (*Ark*, EC 2.7.3.3). We followed Janson and Ward (1984) in the running and scoring of *Pgi*, *Pgm-2*, and *Aat-1*, whereas *Lap* and *Ark* were based on Rolán-Alvarez et al. (1995) protocols. Finally, a third identical sampling scheme was carried out during January 2003. The 24 samples consisted of 26 individuals per sample on average (range 22–30 individuals), and four polymorphic loci were analyzed—*Pgi*, *Mpi*, *Aat-1*, and *Pnp*—following the methods of the previous study.

Analyses of Geographical Variability

We estimated the heterozygosities of RB individuals in the upper shore and their differentiation among localities, and analogously for the SU individuals in the lower shore. A similar analysis was done for the RB and SU individuals living in the midshore. Thus we used six different

pseudoreplicates (upper/lower and midshore data in three sampling periods: 1989, 1999, and 2003) for comparing population genetic differentiation and expected heterozygosity between ecotypes. We also obtained some estimates of differentiation at a microgeographical scale: between transects (within ecotype) in each locality and between shore levels (i.e., between RB and SU individuals) in each locality.

We estimated the amount of population genetic differentiation using the theta estimator (θ_{ST} ; Weir and Cockerham 1984), which shows some statistical advantages over Wright's (1951) fixation index, F_{ST} . Analysis of expected levels of unbiased heterozygosity (H_c ; Nei 1987) and estimates of population genetic differentiation (θ_{ST}) were performed using GENEPOP 3.3 (Raymond and Rousset 1995). The randomization test to check the significance of different statistics between groups of samples (RB versus SU) was calculated using FSTAT 2.9.3.2 (Goudet 2000).

We also estimated the neighborhood size (Wright 1969, p. 303), a parameter expected to be directly related to effective population size. For this we analyzed unpublished data from Carballo (2002), which show snail density in the midshore for 110 squares (32 cm \times 32 cm) pseudoreplicated across a 3-year study, as well as data from Erlandsson et al. (1998), to obtain the average and variance of dispersal rates of the two ecotypes at the midshore. In this latter study, about 8000 snails representing different ecotypes, shore levels, and localities were marked and released; 1625 (the survivors) were recaptured 1 month later. Under a geographical bell-shaped curve of migration in two dimensions, the neighborhood size is $N_n = 4\pi\delta\sigma^2$ (Wright 1969), where δ is the number of breeding individuals per unit area and σ^2 is the variance of distance between birth and breeding sites.

Analyses of Temporal Variability

To estimate effective population sizes at different localities and shore levels, we used allelic frequencies from four allozymic loci (*Pgi*, *Aat-1*, *Mpi*, and *Pnp*) common to the two datasets more separated in time (samples from 1989 and 2003, about 28 generations apart). We could also obtain effective population size estimates from two allozymic loci (*Pgi* and *Aat-1*) common to the three datasets. Because we could not guarantee that we sampled exactly the same sites (at a microgeographical scale) in the three datasets, we pooled samples of different transects within localities and shore levels. This pooling is supported by the almost complete absence of significant differences in allelic frequencies between transects within different shore levels and localities in the three datasets (not shown).

We estimated the effective population size (N_e) from the standardized variance of allelic frequencies across generations (Krimbas and Tsakas 1971). The variance of allelic frequencies, and the corresponding N_e , was estimated as suggested by Nei and Tajima (1981; F_c) and by Pollak (1983; F_k). In addition, a maximum-likelihood framework (Wang 2001; Williamson and Slatkin 1999) was also used. Briefly, the idea is to calculate, through transition matrices, the probabilities of the different alleles' configurations and

Table 1. Expected heterozygosities (H_e) within ecotype (RB or SU) and population genetic differentiation between samples of the same ecotype over different localities (θ_{ST}) for the six datasets

Dataset (sample size)		H_e			θ_{ST}		
		RB	SU	Test ^d	RB ^c	SU ^c	Test ^d
1989 (4 loci)	Upper/lower ^a (499)	0.350	0.383	ns	0.076 ^{***}	0.102 ^{***}	ns
	Midshore ^b (381)	0.382	0.388	ns	0.067 ^{***}	0.113 ^{**}	ns
1999 (5 loci)	Upper/lower ^a (217)	0.445	0.291	**	0.069 ^{**}	0.167 ^{**}	*
	Midshore ^b (417)	0.380	0.283	**	0.056 ^{**}	0.128 ^{**}	ns
2003 (4 loci)	Upper/lower ^a (264)	0.367	0.347	ns	0.066 ^{***}	0.138 ^{***}	ns
	Midshore ^b (360)	0.353	0.363	ns	0.068 ^{***}	0.096 ^{***}	ns
Mean		0.380	0.342	ns^e	0.067	0.124	***^e

^a Upper/lower (sample size in parenthesis): average heterozygosity and differentiation between samples of the same ecotype at different localities (RB samples from the upper shore, or SU samples from the lower shore).

^b Midshore: average heterozygosity and differentiation between samples of the same ecotype (RB or SU) in different localities at the midshore.

^c Significance of the θ_{ST} within ecotypes was performed by randomizing genotypes among samples, not assuming Hardy-Weinberg, by FSTAT after 10,000 permutations.

^d The test column within each pseudoreplicate represents a randomization test performed by FSTAT after 10,000 permutations.

^e The significance of the test column for the mean differences across pseudoreplicates was done by a randomization ANOVA (program available at <http://webs.uvigo.es/genxb2>).

* $P < .05$, ** $P < .01$, *** $P < .001$; ns, not significant.

estimate N_e from the one with maximum likelihood. This approach has been claimed to be more robust against bias produced by rare alleles, and it allows for the use of samples at more than two different times (Wang 2001).

From the estimates of effective population size and genetic differentiation, we obtained approximate migration rates (m), assuming the island model of Wright (1951), $F_{ST} = 1/(4N_e m + 1)$. To calculate the migration rate between two transects (microgeographical migration rates), we used the N_e estimated by pooling data from both transects, whereas to calculate the migration rate between localities (macrogeographical migration rates), we used the harmonic mean of the respective N_e estimates. Wang and Whitlock's (2003) maximum likelihood approach to jointly estimate effective population size and migration rate was not used for the analysis, as it does not seem appropriate for our data. This method was designed for an island-continent model or a model where all populations serve as a migrant source, and the structures of the populations involved in the present study do not fit these requirements. As will be shown, migration rates differ between ecotypes and between vertically and horizontally separated populations.

Results and Discussion

Heterozygosity and Population Differentiation

There were no significant differences between the mean heterozygosities of the two ecotypes, except in the two pseudoreplicates from 1999 (Table 1). Genetic differentiation at a macrogeographical scale was significant for both ecotypes in the six pseudoreplicates. The differences in genetic differentiation between ecotypes were only statistically significant in the 1999 upper/lower shore data. However, a general trend, SU showing a larger mean

genetic differentiation than RB, could be shown across pseudoreplicates using a randomization analysis of variance (ANOVA), suggesting that the two ecotypes have distinct genetic structures over the geographical scale studied. The simplest hypothesis to explain such a difference could be that the two ecotypes have different effective population sizes, different migration rates, or both (see Wang and Caballero 1999). Thus we estimated effective population sizes from time variation in allelic frequency and neighborhood sizes in each ecotype at the same localities.

Effective Population Sizes and Neighborhood Sizes

The pseudolikelihood estimates (Wang 2001) for the different shore levels and localities ranged between 66 and infinity (this latter being, in fact, $N_e > 5 \times 10^4$) (Table 2). Similar results were obtained when the Nei and Tajima (1981) or Pollak (1983) estimators were used (not shown), although the pseudolikelihood estimator usually gave slightly larger N_e values. The likelihood confidence intervals obtained by iteration were typically too wide, but three estimates from Silleiro were significantly smaller than the others. No other trends were found, and the mean effective sizes of RB and SU ecotypes were not significantly different. Estimates of N_e obtained from the two loci common to the three sampling times were similar to those shown in Table 2, but generally with larger confidence intervals. Thus the results, though not conclusive, suggest the two ecotypes do not have radically different effective population sizes.

From data on mean and variance of dispersal rates and snail densities we also obtained estimates of the neighborhood size, a parameter related to the effective population size, in RB and SU populations of the midshore. Snail densities (48.5 RB and 317.4 SU snails/m² on average)

Table 2. Pseudolikelihood estimates of the effective population size and their 95% confidence intervals (in parenthesis) per locality, shore level, and ecotype, and estimated migration rates between localities

Level	Ecotype	Corrubedo		Silleiro		La Cetarea
		N_e (95% CI)	m	N_e (95% CI)	m	N_e (95% CI)
Upper	RB	677 (137–∞)	0.005	305 (52–∞)	0.047	3205 (231–∞)
Midshore	RB	4872 (279–∞)	0.012	100 (39–247)	0.085	257 (75–1100)
	SU	339 (104–1568)	0.010	95 (42–205)	0.017	∞ (—)
Lower	SU	429 (125–3519)	0.012	66 (31–143)	0.017	380 (93–3705)

differed significantly between ecotypes, as shown by a paired Wilcoxon rank test across the 110 squares studied ($Z = -8.2$, $P < .001$). Mean adult dispersal rates were 2.14 and 1.04 m/month for RB and SU, respectively (significantly different using a Mann-Whitney rank test; $Z = -4.83$, $P < .001$), with variances of dispersal rates of 2.52 and 0.44 for RB and SU, respectively. However, similar neighborhood sizes were obtained for the two ecotypes (1536 for RB and 1755 for SU), supporting the above observation that the two ecotypes do not differ drastically in effective population size.

Migration Rates

The former effective population sizes and the population genetic differentiation estimates between groups of samples (averaged across datasets, not shown) allowed the estimation of migration rates (Table 2). There were smaller migration rates between Corrubedo and Silleiro (0.009 on average) than between Silleiro and La Cetarea (0.042 on average) ($F_{\text{randomization}} = 3.8$, $P = .013$), in accordance with the larger geographical distance and the presence of two estuaries between the former pair of locations. Migration estimates did not differ significantly between ecotypes ($F_{\text{randomization}} = 1.6$, $P = .435$), but the average SU migration rate (0.014) was half that estimated between RB populations (0.037), a trend similar to that observed for adult dispersal rates. This also agrees with the fact that lower shore (SU) populations are apparently a little more fragmented than upper shore (RB) ones (personal observation). Whereas migration rates between Silleiro and Corrubedo were about the same for RB and SU populations, the migration rate between the closest Silleiro and La Cetarea was 0.066 for RB and 0.017 for SU. This explains the larger mean population differentiation between Silleiro and La Cetarea in the SU ($\theta_{\text{ST}} = 0.058$) than in the RB ecotype ($\theta_{\text{ST}} = 0.008$). Migration ability seems to differ between ecotypes at a microgeographical scale as well as to an intermediate geographical scale (within an area with no or low habitat fragmentation), but it does not clearly differ between ecotypes when the localities are separated by different estuaries. Thus rare, long-distance dispersal events would perhaps be more similar between these ecotypes, but not dispersion at a more local scale.

We could also obtain microgeographical estimates of migration rates (from samples tens of meters apart, between transects in each locality, and between shore levels in each transect). The mean migration estimate between transects (within ecotypes) of the same locality ($m = 0.087$, $n = 9$) and

between upper/lower shore levels (between ecotypes) of the same locality ($m = 0.016$, $n = 11$) were significantly different ($F_{\text{randomization}} = 4.0$, $P = .006$), in spite of the fact that both kinds of samples were separated by similar physical distances. This is in agreement with the partial assortative mating existing between these ecotypes at the midshore (Johannesson et al. 1995; Rolán-Alvarez et al. 1999). In addition, the mean migration rate between ecotypes of upper/lower shore levels ($m = 0.016$) was significantly smaller than the mean migration rate between ecotypes in sympatry at the midshore level ($m = 0.065$, $n = 12$; $F_{\text{randomization}} = 4.3$, $P = .044$). This supports the view that the two ecotypes are conspecific and still maintain some gene flow. It has been hypothesized that the two ecotypes of this species are undergoing sympatric speciation driven by ecological factors (Rolán-Alvarez et al. 2004). The possibility of a real difference in the genetic structure of the ecotypes seems to support this view.

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