Evol Ecol
DOI 10.1007/s10682-006-9142-8

ORIGINAL PAPER

Genetic variation for shell traits in a direct-developing marine snail involved in a putative sympatric ecological speciation process

Paula Conde-Padín · Antonio Carvajal-Rodríguez
Mónica Carballo · Armando Caballero · Emilio Rolán-Alvarez

Received: 7 April 2006 / Accepted: 1 November 2006

Abstract Populations of the marine gastropod *Littorina saxatilis* from exposed rocky shores of NW Spain provide one of the few putative cases of sympatric ecological speciation. Two ecotypes with large differences in shell morphology and strong assortative mating are living at different vertical levels of the shore separated by a few meters. It has been hypothesized that shell size is the main determinant for the reproductive isolation observed between the ecotypes, and that several shell shape traits are subject to divergent natural selection and are responsible for the adaptation of each ecotype to its respective habitat. Using embryos extracted from wild females we obtain estimates of genetic variation for shell size and shape and compare them with those from neutral molecular markers. Estimates of heritability are significantly larger for the ecotype found in the upper shore than for that in the lower shore, in concordance with a similar result observed for heterozygosity of neutral markers. The large genetic differentiation between ecotypes for the shell traits, contrasting the smaller close to neutral differentiation between populations of the same ecotype, supports the implication of the traits in adaptation.

P. Conde-Padín · A. Carvajal-Rodríguez · M. Carballo · A. Caballero · E. Rolán-Alvarez (✉)
Departamento de Bioquímica, Genética e Inmunología, Facultad de Biología, Universidad de Vigo, 36310 Vigo, Spain
e-mail: rolan@uvigo.es

P. Conde-Padín
e-mail: paulavig@uvigo.es

M. Carballo
e-mail: mcarballo@sescam.jccm.es

A. Caballero
e-mail: armando@uvigo.es

A. Carvajal-Rodríguez
Department of Microbiology and Molecular Biology, Brigham Young University, Provo, Utah 84602, USA
e-mail: acraaj@uvigo.es

Journal : 10682 | Dispatch : 13-11-2006 | Pages : 16
Article No. : 9142 | ☐ LE | ☐ TYPESET
MS Code : EVEC180 | ☐ CP | ☐ DISK

Keywords Genetic differentiation·Geometric morphometrics · Hybrid zone · Heritability · Phenotypic plasticity

Introduction

Ecological speciation occurs when divergent selection in contrasting environments leads to the evolution of reproductive isolation (Schluter 2001). Several studies have provided evidence of the contribution to partial or complete reproductive isolation of traits that are being affected by divergent selection (Macnair and Christie 1983; Filchak et al. 2000; Via et al. 2000; Rundle et al. 2000; Jiggins et al. 2001; Nosil et al. 2002; McKinnon et al. 2004; Rolán-Alvarez et al. 2004). However, the genetic structure of traits involved in ecological speciation, especially those affecting the premating reproductive isolation, is poorly known (reviewed in Rundle and Nosil 2005). Here we study genetic variation and differentiation of traits assumed to be involved in reproductive isolation and adaptation for one of the few model cases of putative sympatric speciation (Johannesson et al. 1993; Rolán-Alvarez et al. 1997 2004; Cruz et al. 2004a; Pérez-Figueroa et al. 2005).

Littorina saxatilis (Olivi), a dioecious gastropod from intertidal North Atlantic rocky shores, is one of the most polymorphic species in shell size and morphology within the genus *Littorina* (Reid 1996; Johannesson 2003). This species has low dispersal ability because of its internal fertilization and direct, non-pelagic, development (females carry a brood pouch with shelled embryos). These life-history characteristics promote local adaptation to habitat heterogeneity, allowing the snails to live in a wide range of different ecological niches (Raffaelli and Hawkins 1996; Reid 1996; Johannesson 2003; Rolán-Alvarez et al. 2004; Rolán-Alvarez 2006). One of the most extreme polymorphisms of this species can be found in the exposed Galician rocky shores (NW Spain; reviewed in Rolán-Alvarez 2006). Two ecotypes of *L. saxatilis* are living at different vertical levels of the shore separated by a few meters and they differ in a number of morphological characteristics associated with two different habitats. The ridged, banded and bigger form (RB) is usually found on the upper shore dominated by barnacles, while the smooth, unbanded and smaller one (SU) is found on the lower shore dominated by blue mussels (Johannesson et al. 1993; Rolán-Alvarez et al. 1997; Cruz et al. 2004a b). The SU snails, living in a high wave-action environment during the whole range of tides, tend to have more flattened thinner shells and have wider apertures to accommodate a larger foot, which have been hypothesized to be an adaptation to avoid dislodgment from wave action by providing a better grip on the substrate and for hiding in cracks and crevices. The relatively more globular, thicker shells, and smaller apertures of the RB snails from upper shores, are assumed to be an adaptation to intense predation by crabs, heat desiccation, and a lack of wave action at low tide and very slight at high tide (Johannesson and Johannesson 1996; Rolán-Alvarez et al. 1997; Guralnick and Kurpius 2001; Johannesson 2003; Rolán-Alvarez 2006). Thus, the two ecotypes of *L. saxatilis* living at different shore levels are assumed to be exposed to intense divergent selection favoring morphological differences in shell size and shape to cope with different environmental factors.

In the mid shore, upper and lower shore environments overlap (see Carballo et al. 2005), and both ecotypes meet in this area in true sympatry producing hybrids with phenotypically intermediate characteristics (Rolán-Alvarez et al. 1999). The two

Journal : 10682 | Dispatch : 13-11-2006 | Pages : 16
Article No. : 9142 | LE | TYPESET
MS Code : EVEC180 | CP | DISK

pure ecotypes maintain a substantial (though incomplete) sexual isolation (Johannesson et al. 1995; Rolán-Alvarez et al. 1999), and a link between this reproductive barrier and shell size has been demonstrated experimentally (Cruz et al. 2004a; Rolán-Alvarez et al. 2004). The two ecotypes differ substantially in shell size (mean RB is 5.15 ± 0.048 mm; mean SU is 3.20 ± 0.020 mm; Johannesson et al. 1995), and because there is a tendency for mating to occur between individuals with similar body sizes (probably because males follow preferentially female mucus-trails of similar size; Erlandsson et al. 1999), these could account for the evolution of the partial reproductive isolation in this system.

Although the general morphological differences in shell size and shape between the ecotypes are rather distinctive for adults (Johannesson et al. 1993; Rolán-Alvarez et al. 1997, 1999), the different traits involved are confounded, and a precise disentangling with the appropriate tool has not been made until recently. Carvajal-Rodríguez et al. (2005), using geometric morphometrics techniques, were able to pinpoint the specific components responsible for the morphological differences between the adults of each ecotype. They found that RB and SU adults not only differ significantly in size, but also in one shape variable denoting uniform elongations of the horizontal axis (perpendicular to the axis of the shell) (SU are more flattened than RB), and another shape variable of local deformations involving the relative size of the shell aperture (SU have larger apertures than RB). The availability of variables accounting for specific size and shape components of morphological variation with a clear biological interpretation allows the investigation of their presumable role in adaptation.

Because shell size is assumed to be involved in the pre-mating reproductive isolation between the ecotypes (Rolán-Alvarez et al. 2004; Cruz et al. 2004a), it is important to assess the amount of genetic variation available in the populations. In addition, the amount of genetic differentiation for the traits assumed to be involved in the adaptation of the ecotypes to their corresponding habitats has not been quantified relative to neutral variation. The ovoviviparous nature of *L. saxatilis* provides the unusual advantage of allowing the estimation of genetic components for shell characters directly from wild individuals, by measuring shelled embryos taken from pregnant females. It also allows a comparison between the morphology of adults and embryos, the latter not yet being directly affected by external environmental conditions.

The objective of this work is twofold. First, to quantify the amount of genetic variation for the traits assumed to be under selection, particularly shell size, the trait presumably responsible for the pre-mating reproductive isolation between the ecotypes. Second, to assess the amount of genetic differentiation between and within ecotypes in relation to neutral differentiation, in order to quantify the implication of the traits in adaptation.

Material and methods

Sampling

Specimens were sampled from February to May 2003 in three localities, Corrubedo, Silleiro and La Cetarea, separated by 52 and 25 km, respectively (see Fig. 1 in Rolán-Alvarez et al. 2004). These three areas represent replicates of the same

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

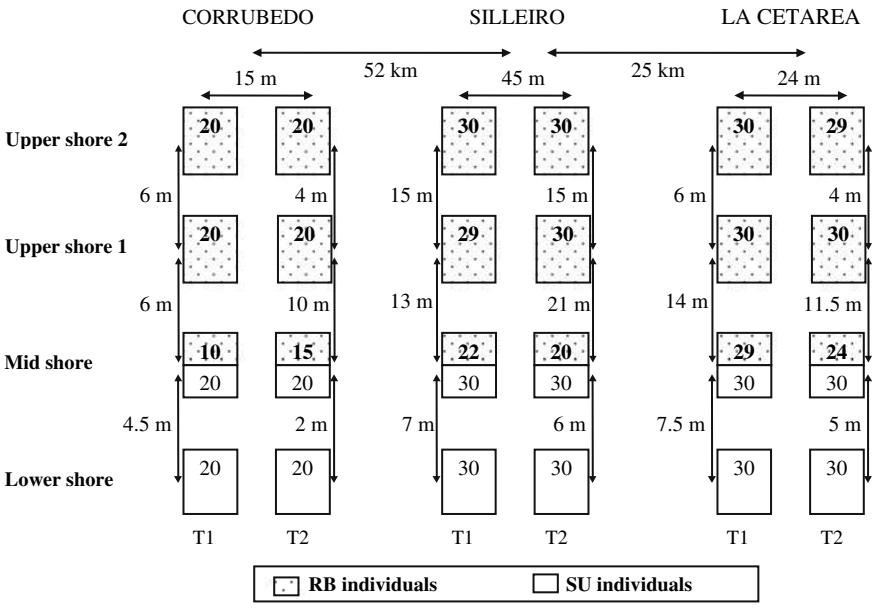

Fig. 1 Sampling design. The number of females analyzed is presented for each unit of sampling (rectangles). The samples of the upper shore 1 and 2 were collected in the barnacle belt, while the samples of the lower shore were collected in the mussel belt. The samples of the mid shore were collected in the patchy barnacle/mussels zone. T1, T2: sampling transects per locality

vertical shore gradient, therefore allowing an empirical replication of the different genetic estimates. Individuals were picked during the low tide, along two vertical transects of the intertidal rocky shore of each locality, and four different zones (quadrats) within each transect (Fig. 1). The upper (1 and 2) and lower shore samples were representative of RB and SU populations, respectively, whereas individuals of both ecotypes were also captured in the mid-shore hybridization area. In all samples the individuals were obtained within a few squared meters. Female hybrids were discarded as they did not provide enough developed embryos for analysis.

Size and shape variables

The dissection of individuals was carried out in the laboratory, and only fertilized females were used. On the whole, 438 RB and 320 SU females (families) were obtained, of which the brood pouch with their shelled embryos was extracted and kept in alcohol at 4°C. Shelled embryos in the later stage of development (with a complete shell; see Fig. 2) from each female were placed on a surface divided in numbered rows and columns, employing a paintbrush to avoid any possible fracture of the light shells. This typically represented more than 50% of the embryos within the brood pouch of the female. Three embryos were randomly chosen from each family using a pseudo-random number generator (GWBASIC), obtaining a total of 2,274 embryos. The embryo shells were examined using a Leica MZ12 stereoscopic

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

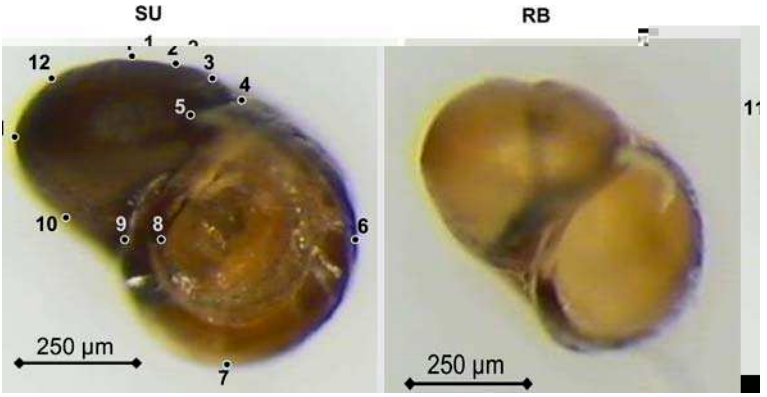

Fig. 2 Representative embryos of SU and RB individuals positioned with the axis of the shell on the *y*-axis and the aperture in the same plane as the objective, showing the location of the 12 landmarks used in the study

microscope, and color images were captured and digitized using a Leica digital ICA video camera and QWin Lite version 2.2 software, with the specimens always placed in the same position, with the axis of the shell on the *y*-axis and the aperture in the same plane as the objective (Fig. 2).

The geometric morphometrics approach studies the changes in size and shape from the displacement in the plane or in the space of a set of morphometric points or landmarks (LM), to be combined with statistical multivariate procedures (Cavalcanti et al. 1999; Adams et al. 2004; Zelditch et al. 2004). Twelve representative LM of the shell were used in this study (Fig. 2), and distances among them were measured with the Image Tool 3.0 (available at http://ddsdx.uthscsa.edu/dig/itdesc.html). We used a grill to locate the LM following Carvajal-Rodríguez et al. (2005), except for LM12, which marks the intermediate position between LM1 and LM11 along the curvature of the whorl (see Fig. 2).

The estimate of shell size was the centroid size (CS), which is the square root of the sum of squared distances of a set of LM from their centroid, this being the center of gravity of a configuration of points (Bookstein 1991). The shape (geometric information that remains after eliminating the effects of translation, rotation and scale), can be decomposed into uniform and non-uniform components (Bookstein 1991; Rohlf and Bookstein 2003; Zelditch et al. 2004). The uniform component describes the global variation of the shell (affecting all LM simultaneously) and, in turn, is decomposed into the first uniform component (U1), that shows changes or deviations in the horizontal axis of the shell (*x*-axis in Fig. 2), and the second uniform component (U2), that represents changes in the vertical axis (*y*-axis in Fig. 2). The non-uniform components (relative warps, RW) describe local shape deformations of a reference configuration at different spatial scales (representing local changes in the LM). These were computed with the scale options of `a` = 0, 1 and –1, but the results were basically the same in all cases. Thus, results are only shown for `a` = 1, that emphasizes those LM separated by longer distances (Rohlf 1993). There were 18 RWs arising from the analysis, but we focused mainly on the first two, RW1 and RW2, that explained 53 and 17%, respectively, of the overall variation for non-uniform components. All calculations

Journal : 10682 | Dispatch : 13-11-2006 | Pages : 16
Article No. : 9142 | LE | TYPESET
MS Code : EVEC180 | CP | DISK

were performed with the program MODICOS, developed by Carvajal-Rodríguez and Rodríguez (2005), and available at http://www.uvigo.es/webs/c03/webc03/XENETICA/XB2/antonio/modicos/Modicos.zip and the program TPSrelw, developed by Rohlf (1998) and available at http://life.bio.sunysb.edu/morph/morphmet/tpsrelww32.exe.

We estimated the mean error attached to variation in the establishment of LM, by repeating five times the orientation and location of the 12 LM on the shell of 10 different embryos. The errors of the variables were obtained analyzing these 50 repeated measurements along with the whole experimental data (2,274 embryos). The mean errors were 0.0016 mm for CS, 0.0019 mm for the uniform components, and 0.0011 mm for the first six RW, representing a 2.0% of the overall variation on average (range from 0.43% to 4.53%).

In order to compare the variation in shape between embryos and adults, some analyses were also carried out on data from 60 adults of Silleiro and La Cetarea populations (data from Carvajal-Rodríguez et al. 2005 reanalyzed with the option a = 1).

Data from laboratory breeding

An estimate of heritability for the same morphometric traits was carried out using RB individuals, bred and mated in the laboratory. Sixty virgin females were obtained from a sample of hundreds of juveniles collected from the upper shore 1 zone of Silleiro in September 1999. Juveniles were sexed every 2 weeks during 8 months, to exclude incipient developed males, and females were kept isolated during that period. Then, crossings were carried out with RB males collected from the same sampling point, a single male being mated to each female. Sixty breeding pairs separated in independent flasks were established. After a year, the appearance of embryos was observed in 14 flasks and three embryos from each of the fertilized females were analyzed.

Estimation of genetic components and statistical analysis

Estimates of genetic variation were obtained for shell size and for each of the geometric morphometrics shell size components. The analysis is focused on specific shape features rather than using a multivariate global approach. As suggested by Klingenberg and Leamy (2001), the former is an appropriate procedure when the main interest is on the comparison of specific shape components between two species (e.g. Zeng et al. 2000) or, in the present case, ecotypes. The heritability was estimated for all variables using a full-sib (correlation) design analysis (Falconer and Mackay 1996, p. 163) for each combination of ecotype, locality and shore level (for a total of 30 estimates), as $h^2 = 2V_{bf} / (V_{bf} + V_{wf})$, where V_{bf} and V_{wf} are the between and within-family components of variance, respectively. This design provides estimates of an upper limit of the narrow sense heritability, $h^2 + d^2/2 + 2c^2$, where h^2 is the narrow sense heritability, and d^2 and c^2 are the ratios of the dominance and common environmental variances, respectively, to the phenotypic variance (Falconer and Mackay 1996, p. 158). The use of three individuals per family is optimum from the standpoint of the precision of estimation of heritability with values around 0.6 (Falconer and Mackay 1996, p. 180).

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

Accordingly, genetic differentiation for the different quantitative traits was obtained as $Q_{ST} = V_{bp} / (V_{bp} + 2V_{wp})$ (Wright 1951; Spitze 1993), where V_{bp} and $V_{wp} = 2V_{bf}$ archical26351d791]lev-0

Classical parametric tests were computed by the SPSS/PC package version 12.0.1. A one-way randomization ANOVA following the protocol of Peres-Neto and Olden (2001) was used (available as free software at http://webs.uvigo.es/c03/webc03/XENETICA/XB2/ anova.zip) to test for differences in heritability between ecotypes and to compare molecular and quantitative genetic differentiation estimates.

Results

Embryos of the two ecotypes did not present systematic differences for CS (SU embryos were significantly larger than RB ones in two localities, but the opposite was observed in the third). However, they showed significant differences for the first uniform (U1) and non-uniform (RW1) components of shape variation. The means of these variables for each of the ecotypes and localities are plotted in Fig. 3. A clear differentiation between the two ecotypes is observed for the uniform U1 component, showing that SU embryos present larger values (greater elongation for the *x*-axis) than RB ones. A minor differentiation between localities is also present, particularly among SU populations. This variable basically describes the more flattened form of SU shells than that of RB ones, a difference also evident in adults (Carvajal-Rodríguez et al. 2005).

Differences between ecotypes were also observed for the first non-uniform component RW1 (Fig. 3). The meaning of this trait is shown in Fig. 4 by means of a thin-plate spline, an interpolating function to describe shape changes with respect to the reference configuration. The figure shows the deformations implied by displacements for this shape component, where SU embryos presented the most negative deformations, whereas the RB showed the most positive ones. These shape

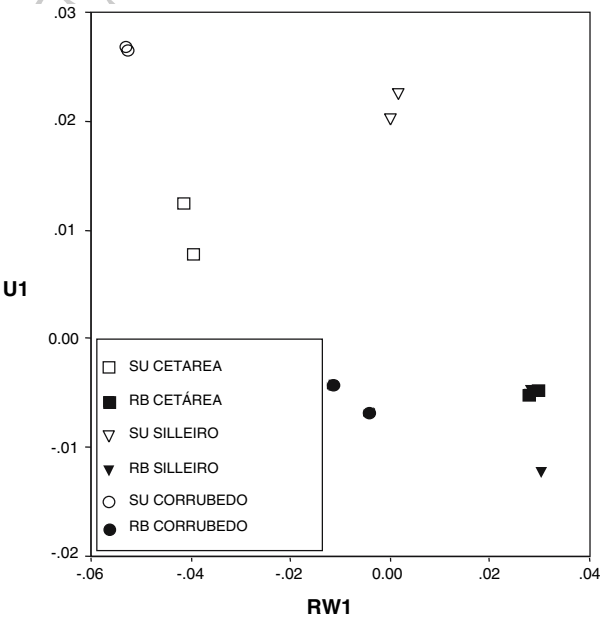

Fig. 3 Average values for the first uniform component (U1) and the first non-uniform component (RW1) of shell shape, for each ecotype and locality obtained with a = 1. The two replicates correspond to the two transects per sample

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

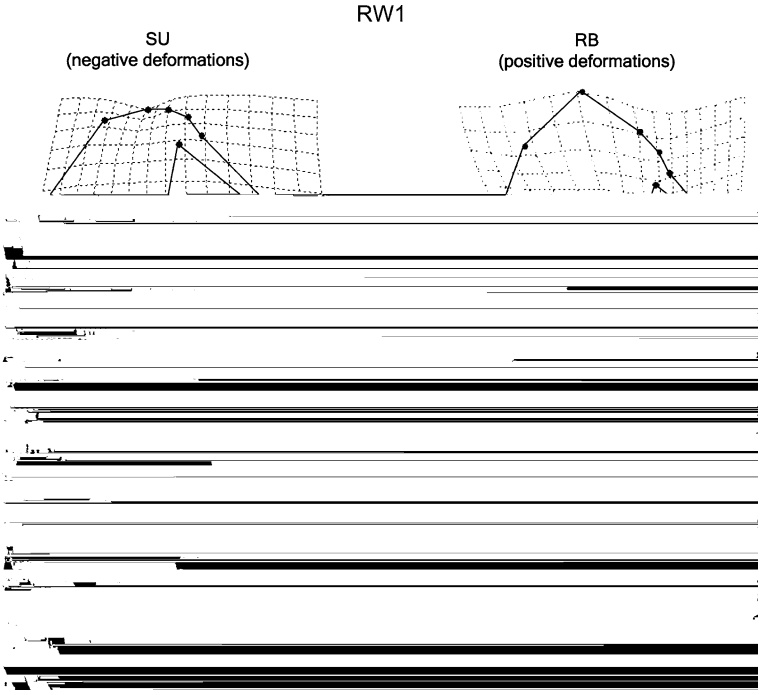

Fig. 4 Thin-plate spline representation, from the TPSrelw software (Rohlf 1998), showing the most extreme positive (RB) and negative (SU) deformation of the landmarks (within the 95% confidence interval within each group) with respect to the reference configuration (using $a = 1$). Some landmarks are connected by lines to facilitate the meaning of the differences between ecotypes. Note the larger relative aperture area (percentage of the total shell area) of the SU ecotype both for adults and embryos

differences between ecotypes are rather similar both for shelled embryos and adults, and denote a larger aperture area for the SU than the RB ecotype (see Fig. 4).

Average estimates of heritability and their standard errors are shown in Table 1 for the components explaining most of the variation. Typically, the heritability was intermediate to high (0.533 ± 0.036 on average) and significant (108 significant estimates out of 150 samples studied). A consistently larger heritability for all traits was shown for the RB ecotype (average 0.609 ± 0.036) relative to the SU ecotype (average 0.458 ± 0.039). For the RB ecotype, laboratory estimates of heritability were generally somewhat larger (average 0.742 ± 0.080) than field estimates, all being significantly different from zero. Similar results and trends were observed for estimates from other RW with smaller but substantial contribution to shape variation (RW3–RW6; not shown). Estimates for simple shell measures were also obtained for comparison, showing similar results. Heritabilities for maximum height of the shell (distance between LM 1 and 7; see Fig. 2) were 0.574 ± 0.068 (SU), 0.720 ± 0.052 (wild RB), and 0.699 (lab RB), and for width of the aperture (distance between LM 6 and 8) were 0.581 ± 0.045 (SU), 0.804 ± 0.100 (wild RB), and 1.162 (lab RB).

The larger heritabilities observed for the RB ecotype relative to the SU one are consistent with the corresponding higher heterozygosity of neutral molecular markers (significant only for allozymes; Table 1). However, the estimated

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

Table 1 Average estimates of heritability and their empirical standard errors indicating variation among samples, for the two ecotypes analyzed

	Trait	Ecotype			Randomization ANOVA[d]
		SU[a]	RB[a]	RB laboratory[b]	
Heritability ± SE	CS	0.601 ± 0.055	0.700 ± 0.051	0.879	$F = 7.0$ $P = 0.0203$
	U1 (59%)	0.418 ± 0.066	0.612 ± 0.078	0.486	
	U2 (29%)	0.363 ± 0.059	0.479 ± 0.105	0.713	
	RW1 (53%)	0.449 ± 0.088	0.614 ± 0.092	0.941	
	RW2 (17%)	0.460 ± 0.067	0.640 ± 0.094	0.692	
Heterozygosity ± SE	Allozymes[c]	0.291 ± 0.014	0.365 ± 0.009		$F = 20.8$ $P = 0.0001$
	Microsatellites[c]	0.681 ± 0.025	0.719 ± 0.016		$F = 1.6$ $P = 0.2280$
	MtDNA[c]	0.182 ± 0.067	0.198 ± 0.064		$F = 0.1$ $P = 0.8702$

CS: centroid size, measure of shell size . U1, U2: uniform components of shell shape (in parenthesis, percentage of variation explained). RW1, RW2: main non-uniform components of shell shape relative warps

[a] Average from 12 estimates for SU and 18 estimates from RB samples (see Fig. 1)

[b] Estimates obtained from 14 full-sib families bred at the laboratory

[c] Allozymic (average for 12 samples), microsatellite (average from 6 samples), and mtDNA (average from 12 samples) gene diversities reanalyzed from data of Rolán-Alvarez et al. (2004)

[d] Randomization ANOVA to test significant differences between ecotypes in the wild for different gene diversity estimates. The degrees of freedom are 1/23 for quantitative traits, 1/22 for allozymes and MtDNA, and 1/11 for microsatellites

upper-limit additive genetic variance for the main traits analyzed (CS, U1, U2, RW1 and RW2) was not significantly correlated with the gene diversity for allozymes ($r = 0.152$, $n = 24$, $P > 0.05$) and microsatellites ($r = -0.226$, $n = 12$, $P > 0.05$), and borderline significant for mtDNA ($r = 0.402$, $n = 24$, $P = 0.051$).

Table 2 shows the average estimates of population genetic differentiation at different hierarchical levels for both quantitative (Q_{ST}) and neutral molecular (F_{ST}) traits. At a macro-geographical scale, quantitative genetic differentiation (Q_{ST}) between localities was significantly larger for SU than RB populations, in an agreement with a similar significant difference for neutral F_{ST}. The magnitude of differentiation for the non-uniform component RW1 was, in all cases, considerably larger than that for the remainder traits. In fact, excluding this trait, the average differentiation within ecotypes (both within and between localities) was of the same order as that for neutral markers (Table 2), suggesting a quasi-neutral behavior of the traits within each ecotype. The average differentiation between ecotypes was, however, about three times larger than that for neutral markers, suggesting that the traits studied are subject to diversifying selection.

Discussion

There is some direct evidence in support of the adaptive explanation for the morphological differences between the ecotypes of *L. saxatilis* living in Galician shores (Johannesson et al. 1993; Rolán-Alvarez et al. 1997, 1999; Cruz et al. 2004a b) as well as in other mollusks (Pfenninger et al. 2003; Schilthuizen et al. 2005). However, only recently and with the powerful tool of geometric morphometrics it

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

Table 2 Average genetic differentiation for quantitative (Q_{ST}) and neutral molecular (F_{ST}) traits, and their standard errors

	Between localities[a]		Within localities	
	Within RB	Within SU	Within ecotypes[b]	Between ecotypes[c]
Morphological Q_{ST}				
CS	0.10 ± 0.03	0.13 ± 0.19	0.01 ± 0.01	0.19 ± 0.03
U1	0.00 ± 0.01	0.07 ± 0.01	0.05 ± 0.03	0.25 ± 0.08
U2	0.06 ± 0.04	0.11 ± 0.06	0.06 ± 0.02	0.14 ± 0.10
RW1	0.27 ± 0.02	0.61 ± 0.03	0.09 ± 0.04	0.59 ± 0.05
RW2	0.07 ± 0.07	0.19 ± 0.01	0.02 ± 0.01	0.10 ± 0.05
Average (excluding RW1)	0.06 ± 0.02	0.13 ± 0.02	0.03 ± 0.01	0.17 ± 0.03
Molecular F_{ST}	0.07 ± 0.00[d]	0.12 ± 0.01[d]	0.02 ± 0.01[e]	0.05 ± 0.01[e]

Definitions for traits as in Table 1

[a] Differentiation between localities (see Fig. 1)

[b] Differentiation between transects of the same locality and ecotype

[c] Differentiation between ecotypes of the same locality and transect

[d] Average of allozymes from Fernández et al. (2005)

[e] Average of microsatellites and allozymes from Rolán-Alvarez et al. (2004)

has been possible to disentangle size and shape components of morphological variation between the ecotypes (Carvajal-Rodríguez et al. 2005). One of our findings is that the differences between both ecotypes appear for the same shape components (in the *x*-axis elongation of the shell, U1, and the relative size of the aperture, RW1) and in the same direction for embryos and adults. The fact that the embryos have not been directly exposed to external environmental conditions suggests that shape differences between the ecotypes are not just the result of phenotypic plasticity. This is corroborated by the observation that RB and SU progeny hatched in the laboratory resembled their own ecotype when they achieved 3 mm of shell height, and also that juveniles of both ecotypes fully grown in the laboratory maintained the same phenotypic characteristics as wild individuals (Johannesson et al. 1993; this study).

Estimates of heritability for shell size (centroid size; CS) and the most representative shape variables (U1, U2, RW1 and RW2) from wild females were intermediate, with values ranging between 0.4 and 0.7, a typical outcome for many quantitative traits (Riska et al. 1989; Falconer and Mackay 1996). The values were in agreement with preliminary estimates from some of the populations studied using classical morphometric distances and ratios (Carballo et al. 2001), and were remarkably consistent among traits and populations, in spite of the fact that they were obtained directly from families taken from the wild.

Several sources of bias may be affecting the estimates. The upwardly biasing factors are non-additive and common environmental components of variation. There are, however, several arguments suggesting that these sources of bias may not be substantial. First, morphological traits, in contrast to life-history traits, usually show low levels of non-additive (dominance and epistatic) variance components (Crnokrak and Roff 1995; DeRose and Roff 1999). Second, the analysis was carried out in embryos that had not yet been released to the external medium and, therefore, they are less likely to be affected by external environmental conditions. Third, maternal

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

effects may be a source of bias for shell size, but they are unlikely to be so relevant for shell shape components. Even so, a higher heritability estimate for CS relative to that of shape components was not observed (Table 1). And fourth, and more important, the lack of a main source of bias from common environmental sources specific to wild families is supported by the similar average heritabilities estimated in laboratory and wild conditions. In fact, phenotypic variances for CS were remarkably similar between wild samples (RB: 0.0034 ± 0.0006; SU: 0.0036 ± 0.0014) and the more environmentally homogeneous laboratory sample (0.0037).

A possible downwardly biasing factor for the estimates of heritability would occur if some half sibs (rather than full sibs) were in fact present in the families analyzed. Heritability values could then be underestimated by as much as one half (observed values would be really estimating $\frac{1}{2}h^2 + 2c^2$). Estimates from laboratory, though, were free from this possible source of bias, as individual matings were carried out between single pairs. The latter (average 0.742 ± 0.080) were only slightly (non-significantly) larger than the corresponding ones from the wild (average 0.609 ± 0.036; Table 1). Therefore, the results suggest that multiple paternity, if exists, only affects to a low proportion of the embryos analyzed. Note, finally, that all sources of bias are expected to be the same in RB and SU populations, so the relative comparison between them is useful even if the magnitudes are biased.

The existence of a significant genetic variation for the main traits involved in the morphological differentiation between the ecotypes is important, as the contribution of some of these traits to the variation for fitness has been well established (Rolán-Alvarez et al. 1997; Cruz et al. 2001, 2004b). The finding of a substantial amount of genetic variation for shell size is particularly relevant, as this is the trait assumed to be responsible for the pre-mating reproductive isolation between the two ecotypes (Rolán-Alvarez et al. 2004; Cruz et al. 2004a). The potentiality of the system to achieve further differences in size between the ecotypes and a correspondingly larger reproductive isolation may be a prerequisite for a putative completion of speciation.

The average estimates of heritability were significantly higher for RB than for SU populations, in agreement with the tendency observed for neutral molecular markers, which was also significant in the case of allozymes. Estimates of effective population size and neighborhood size were not significantly different between RB and SU populations, but estimated rates of migration were larger for RB populations, suggesting a larger fragmentation of SU ones (Fernández et al. 2005). This could be an explanation for the observed lower diversity of the SU populations both for quantitative and neutral molecular traits.

A comparison between the estimates of quantitative (Q_{ST}) and neutral molecular (F_{ST}) differentiation is becoming the standard procedure for inferring diversifying selection among populations (Merilä and Crnokrak 2001; McKay and Latta 2002; Le Corre and Kremer 2003; Toro and Caballero 2005). We obtained estimates of quantitative (Q_{ST}) and neutral molecular (F_{ST}) differentiation at micro-geographical (within localities) and macro-geographical (between localities) scales. Several sources of bias can also affect the estimates of quantitative genetic differentiation (Falconer and Mackay 1996; Whitlock 1999; Merilä and Crnokrak 2001; Hendry 2002; López-Fanjul et al. 2003). The possibility that half sibs are included in the analysis would produce an upward bias in Q_{ST}, as the within-population (between-family) variance component would have been underestimated. However, as noted above, the results from heritability estimates do not give much credit to this possibility. The most likely consequence of dominance and epistasis is that $Q_{ST} < F_{ST}$

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

(Whitlock 1999; López-Fanjul et al. 2003), and an analogous outcome would occur from maternal effects and common environmental effects specific to families. In contrast, environmental effects specific to populations would produce overestimations of Q_{ST}. The fact that estimates of quantitative differentiation are similar to those from neutral molecular markers within ecotypes (except for RW1) suggests that large overestimations are not taking place for populations of the same ecotype. For populations of different ecotype a larger bias may be occurring, as these live in different environments. Because RB and SU individuals living sympatrically in the mid shore are subject to similar environmental variables, a comparison between the differentiation of upper/lower shore samples versus mid-shore samples can give an idea of the magnitude of this source of bias. The estimate of Q_{ST} between ecotypes, averaged over all traits shown in Table 2, was 0.279 for upper/lower samples and 0.227 for mid-shore samples. In particular, for the main non-uniform shape variable, RW1, both estimates were very similar (0.610 and 0.578, respectively). Therefore, there is no evidence that a substantial bias from common environmental sources specific to RB and SU populations should be attached to the estimates.

Differentiation between localities was significantly larger for SU than RB populations, in agreement with the results from neutral molecular differentiation and the aforementioned hypothesis that SU populations are subjected to a larger fragmentation than RB ones (Fernández et al. 2005). Differentiation within localities was higher between ecotypes than within ecotypes, both comparisons involving populations separated by similar physical distances (see Fig. 1). For neutral molecular markers, this is likely to be the result of the reduced gene flow between the ecotypes, caused by the strong assortative mating in the zone of overlap (average isolation index 0.77; Rolán-Alvarez et al. 1999). For quantitative traits, however, the considerably larger genetic differentiation between ecotypes than within ecotypes of the same locality should be explained, not only by the barrier to gene flow but also by the adaptive nature of the traits.

Differentiation within ecotypes, both between and within localities, was of the same order as that of neutral molecular differentiation for all quantitative traits, except RW1 (see Table 2), suggesting that the traits show quasi-neutral behavior within ecotypes, but are subject to strong diversifying selection between ecotypes. RW1, accounting for the relative aperture of the shell (Fig. 4), presented the highest differentiation between ecotypes, but also a substantial amount of differentiation between populations of the same ecotype (particularly for the SU) at different localities. Because the ability of the snails to remain attached to the substrate must be proportional to the shell aperture, it is possible that differences in the shore between localities (inclination level, amount of cracks and crevices, density of barnacles and mussels, etc.) are responsible for the observed differentiation between localities.

The *L. saxatilis* system, showing partial assortative mating for size, with two ecotypes differing in size and morphology because of differential adaptation to distinct habitats, strongly resembles the polymorphism existing between anadromous and stream-resident threespine stickleback populations (Nagel and Schluter 1998; McKinnon et al. 2004). In both cases it has been suggested that the reproductive isolation has evolved as an indirect consequence of the divergent selection on size in addition to the size-assortative mating occurring in the species (McKinnon et al. 2004; Rolán-Alvarez et al. 2004; Cruz et al. 2004a). Here we provide evidence that the size difference has a strong genetic basis. The high Q_{ST} values observed for CS

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 LE TYPESET
MS Code : EVEC180 CP DISK

between ecotypes within locality (compared to the equivalent molecular F_{ST}) further supports the adaptive differences in size between them (Table 2). The pattern of genetic variation for other shape traits is also compatible with their presumable role in the adaptation of the ecotypes to their respective habitats at different shore levels.

Acknowledgements We thank Pilar Alvariño and Nieves Santamaría for technical help, and Carlos López-Fanjul, Dr. Jormalainen, and two anonymous referees for useful comments on the manuscript. This works has been partially funded by Ministerio de Educación y Ciencia (CGL2004-03920 BOS; VEM2003-20047) and Fondos Feder, Xunta de Galicia (PGIDT05PXIC31002PN), and Universidade de Vigo. P.C-P. was supported by a research fellowship from Ministerio de Educación y Ciencia.

References

Adams DC, Rohlf FJ, Slice DE (2004) Geometric morphometrics: ten years of progress following the "revolution". Italian J Zool 71:5–16

Bookstein FL (1991) Morphometric tools for landmark data. Cambridge University Press, New York

Carballo M, Caballero A, Rolán-Alvarez E (2005) Habitat-dependent ecotype micro-distribution at the mid-shore in natural populations of *Littorina saxatilis*. Hydrobiologia 548:307–311

Carballo M, García C, Rolán-Alvarez E (2001) Heritability of shell traits in wild *Littorina saxatilis* populations: results across a hybrid zone. J Shellfish Res 20:415–422

Carvajal-Rodríguez A, Conde-Padín P, Rolán-Alvarez E (2005) Decomposing shell form into size and shape by geometric morphometric methods in two sympatric ecotypes of *Littorina saxatilis*. J Molluscan Studies 71:313–318

Carvajal-Rodríguez A, Rodríguez MG (2005) MODICOS: morphometric and distance computational software oriented for evolutionary studies. Online J Bioinform 6:34–41

Cavalcanti MJ, Monteiro LR, Duarte Lopes PR (1999) Landmark-based morphometric analysis in selected species of serranid fishes (Perciformes: Teleostei). Zoo Studies 38:287–294

Crnokrak P, Roff DA (1995) Dominance variance: associations with selection and fitness. Heredity 75:530–540

Cruz R, Carballo M, Conde-Padín P, Rolán-Alvarez E (2004a) Testing alternative models for sexual isolation in natural populations of *Littorina saxatilis*: indirect support for by-product ecological speciation? J Evol Biol 17:288–293

Cruz R, Rolán-Alvarez E, García A (2001) Sexual selection on phenotypic traits in a hybrid zone of *Littorina saxatilis* (Olivi). J Evol Biol 14:773–785

Cruz R, Vilas C, Mosquera J, García C (2004b) Relative contribution and dispersal and natural selection to the maintenance of a hybrid zone in *Littorina*. Evolution 58:2734–2746

DeRose MA, Roff DA (1999) A comparison of inbreeding depression in life-history and morphological traits in animals. Evolution 53:1288–1292

Erlandsson J, Kostylev V, Rolán-Alvarez E (1999) Mate search and aggregation behaviour in the Galician hybrid zone of *Littorina saxatilis*. J Evol Biol 12:891–896

Falconer DS, Mackay TFC (1996) Introduction to Quantitative Genetics, 4th edn. Longman, Harlow

Fernández J, Galindo J, Fernández B, Pérez-Figueroa A, Caballero A, Rolán-Alvarez E (2005) Genetic differentiation and estimation of effective population size and migration rates in two sympatric ecotypes of the marine snail *Littorina saxatilis*. J Heredity 96:1–5

Filchak KE, Roethele JB, Feder JL (2000) Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. Nature 407:739–742

Guralnick RP, Kurpius J (2001) Spatial and temporal growth patterns in the phenotypically variable *Littorina saxatilis*: surprising patterns emerge from chaos. In: Zeldich M (ed) Beyond heterochrony. John Wiley and Sons, New York, pp 195–228

Hendry AP (2002) $Q_{ST} > = \neq < F_{ST}$? Tren Ecol Evol 17:502

Jiggins CD, Naisbit RE, Coe RL, Mallet J (2001) Reproductive isolation caused by colour pattern mimicry. Nature 411:302–305

Johannesson K (2003) Evolution in *Littorina*: ecology matters. J Sea Res 49:107–117

Johannesson B, Johannesson K (1996) Population differences in behaviour and morphology in the snail *Littorina saxatilis*: phenotypic plasticity or genetic differentiation? J Zool 240:475–493

Journal : 10682 | Dispatch : 13-11-2006 | Pages : 16
Article No. : 9142 | ☐ LE | ☐ TYPESET
MS Code : EVEC180 | ☐ CP | ☐ DISK

Johannesson K, Johannesson B, Rolán-Alvarez E (1993) Morphological differentiation and genetic cohesiveness over a microenvironmental gradient in the marine snail *Littorina saxatilis*. Evolution 47:1770–1787

Johannesson K, Rolán-Alvarez E, Ekendahl A (1995) Incipient reproductive isolation between two sympatric morphs of the intertidal snail *Littorina saxatilis*. Evolution 49:1180–1190

Klingenberg CP, Leamy LJ (2001) Quantitative genetics of geometric shape in the mouse mandible. Evolution: 55:2342–2352

Le Corre V, Kremer A (2003) Genetic variability at neutral markers, quantitative trait loci and trait in a subdivided population under selection. Genetics 164:1205–1219

López-Fanjul C, Fernández A, Toro MA (2003) The effect of non-additive gene action on the neutral quantitative index of population divergence. Genetics 164:1627–1633

Macnair MR, Christie P (1983) Reproductive isolation as a pleiotropic effect of copper tolerance in Mimulus guttatus? Heredity 50:295–302

McKay J K, Latta RG (2002) Adaptive population divergence: markers, QTL and traits. Tren Ecol Evol 17:285–291

McKinnon JS, Mori S, Blackman BK, David L, Kingsley DM, Jamieson L, Chou J, Schluter D (2004) Evidence for ecology's role in speciation. Nature 429:294–298

Merilä J, Crnokrak P (2001) Comparison of genetic differentiation at marker loci and quantitative traits. J Evol Biol 14:892–903

Nagel L, Schluter D (1998) Body size, natural selection, and speciation in sticklebacks. Evolution 52:209–218

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Newkirk GF, Doyle RW (1975) Genetic analysis of shell-shape variation in *Littorina saxatilis* on the environmental cline. Marine Biol 30:227–237

Nosil P, Crespi BJ, Sandoval C (2002) Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature 417:441–443

Peres-Neto PR, Olden JD (2001) Assessing the robustness of randomization tests: examples from behavioural studies. Animal Behav 61:79–86

Pérez-Figueroa A, Cruz F, Carvajal-Rodríguez A, Rolán-Alvarez E, Caballero A (2005) The evolutionary forces maintaining a wild polymorphism of *Littorina saxatilis*: model selection by computer simulations. J Evol Biol 18:191–202

Pfenninger M, Eppenstein A, Magnin F (2003) Evidence for ecological speciation in the sister species *Candidula unifasciata* (Poiret, 1801) and *C. rugosiuscula* (Michaud, 1831) (Helicellinae, Gastropoda). Biol J Linnean Soc 79:611–628

Raffaelli D, Hawkins S (1996) Intertidal ecology. Chapman and Hall, London

Raymond M, Rousset F (1995) GENEPOP (version 1.2): a population genetics software for exact tests and ecumenicism. J Heredity 86:248–249

Reid DG (1996) Systematics and evolution in Littorina. The Ray Society, Dorchester

Riska B, Prout T, Turelli M (1989) Laboratory estimates of heritabilities and genetic correlations in nature. Genetics 123:865–871

Rohlf FJ (1993) Relative warp analysis and an example of its application to mosquito wings. In: Marcus LF, Bello E, García Valdecasas A (eds) Contributions to morphmetrics, Vol. 8. Museo Nacional de Ciencias Naturales, Madrid, pp 131–159

Rohlf FJ (1998) TPSrelw: RW, version 1.20. New York State University, Stony Brook

Rohlf FJ, Bookstein FL (2003) Computing the uniform component of shape variation. Syst Biol 52:66–69

Rolán-Alvarez E (2006) Sympatric speciation as a by-product of ecological adaptation in the Galician *Littorina saxatilis* hybrid zone. J Molluscan Studies (in press)

Rolán-Alvarez E, Carballo M, Galindo J, Morán P, Fernández B, Caballero A, Cruz R, Boulding EG, Johannesson K (2004) Nonallopatric and parallel origin of local reproductive barriers between two snail ecotypes. Mol Ecol 13:3415–3424

Rolán-Alvarez E, Erlandsson J, Johannesson K, Cruz R (1999) Mechanisms of incomplete prezygotic reproductive isolation in an intertidal snail: testing behavioural models in wild populations. J Evol Biol 12:879–890

Rolán-Alvarez E, Johannesson K, Erlandsson J (1997) The maintenance of a cline in the marine snail *Littorina saxatilis*— the role of home site advantage and hybrid fitness. Evolution 51:1838–1847

Rundle HD, Nagel L, Boughman JW, Schluter D (2000) Natural selection and parallel speciation in sympatric sticklebacks. Science 287:306–308

Rundle HD, Nosil P (2005) Ecological speciation. Ecol Lett 8:336–352

Journal : 10682 | Dispatch : 13-11-2006 | Pages : 16
Article No. : 9142 | LE | TYPESET
MS Code : EVEC180 | CP | DISK

Schluter D (2001) Ecology and the origin of species. Tren Ecol Evol 16:372–380
Spitze K (1993) Population structure in Daphnia obtuse: quantitative genetic and allozymic variation. Genetics 135:367–374
Schilthuizen M, Cabanban AS, Haase M (2005) Possible speciation with gene flow in tropical cave snails. J Zoolog Syst Evol Res43:133–138
Toro MA, Caballero A (2005) Characterization and conservation of genetic diversity in subdivided populations. Phil Trans Roy Soc, Series B 360:1367–1378
Via S, Bouk AC, Skillman S (2000) Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. Evolution 54:1626–1637
Wright S (1951) The genetic structure of populations. Ann Eug 15:323–354
Whitlock MC (1999) Neutral additive genetic variance in a metapopulation. Genet Res 74:215–221
Zelditch ML, Swiderski DL, Sheets HD, Fink WL (2004) Geometric morphometrics for Biologists. A primer. Elsevier Academic Press, London
Zeng Z, Liu J, Stam LF , Kao C, Mercer JM, Laurie CC (2000) Genetic architecture of a morphological shape difference between two Drosophila species. Genetics 154:299–310

Journal : 10682 Dispatch : 13-11-2006 Pages : 16
Article No. : 9142 □ LE □ TYPESET
MS Code : EVEC180 □ CP □ DISK