

Revealing the mechanisms of sexual isolation in a case of sympatric and parallel ecological divergence

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Two ecotypes of a marine intertidal snail (*Littorina saxatilis*), living at different microhabitats and shore levels, have evolved in sympatry and in parallel across the Galician rocky shore. These ecotypes differ in many traits (including size) due to differential adaptation. They meet, mate assortatively, and partially hybridize at the mid shore where the two microhabitats overlap. The partial sexual isolation observed is claimed to be a side-effect of the size differences between ecotypes combined with a size assortative mating found in most populations of this species. We investigated this hypothesis using three complementary experimental approaches. First, we investigated which of the different shell variables contributed most to the variation in individual sexual isolation in the field by using two new statistics developed for that purpose: (1) pair sexual isolation and (2) r_i , which is based on the Pearson correlation coefficient. We found that size is the most important trait explaining the sexual isolation and, in particular, the males appear to be the key sex contributing to sexual isolation. Second, we compared the size assortative mating between regions: exposed rocky shore populations from north-western Spain (showing incomplete reproductive isolation due to size assortative mating) and protected Spanish and Swedish populations (showing size assortative mating but not reproductive isolation between ecomorphs). Most of the variation in size assortative mating between localities was significantly explained by the within-population level of variation on size. Third, we performed a laboratory male choice experiment, which further suggested that the choice is made predominantly on the basis of size. These results confirm the mechanism proposed to explain the sexual isolation in the Galician hybrid zone and thus support this case as a putative example of parallel incipient speciation. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **94**, 513–526.

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INTRODUCTION

There is little doubt that natural selection, directly or indirectly, has been a main force responsible for the origin of species (Turelli, Barton, & Coyne, 2001; Coyne & Orr, 2004; Hoskin *et al.*, 2005). Some controversy remains about the importance of reinforcement (direct selection against hybridization; but see also Coyne & Orr, 2004), but it is widely accepted that traits involved in speciation can be indirectly driven

by natural selection during the course of adaptation to new habitats (Schluter, 2001; Coyne & Orr, 2004; Rundle & Nosil, 2005; Funk, Nosil, & Etges, 2006). Indeed, there are a number of convincing experimental cases in support of this hypothesis (Schluter & Nagel, 1995; Via, Bouk, & Skillman, 2000; Nosil, Crespi & Sandoval, 2002, 2003; McKinnon *et al.*, 2004; Hoskin *et al.*, 2005; Joyce *et al.*, 2005; Rolán-Alvarez, 2007). Moreover, this mechanism may function irrespective of the gene flow existing between incipient species (i.e. in sympatry as well as in allopatry; Turelli *et al.*, 2001), although the empirical

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demonstration of ecologically driven sympatric speciation has proven more elusive (Coyne & Orr, 2004; but see also Savolainen *et al.*, 2006; Schlieven *et al.*, 2006). For example, what kinds of traits cause reproductive isolation and how do they evolve as an indirect result of natural selection?

Different alternative strategies have been used to distinguish between adaptative and non-adaptative speciation (Rundle & Nosil, 2005). One of the most successful was the finding of parallel patterns of differentiation in those traits responsible for the sexual isolation (i.e. if the sexual isolation is found irrespective of geographical origin) because parallel patterns support the hypothesis of adaptation (Schluter & Nagel, 1995; Nagel & Schluter, 1998; Schluter, 2001; Nosil *et al.*, 2002). However, such an approach is limited to species in which mating pairs can be obtained in laboratory conditions, although little is known about how reproductive isolation is achieved in the wild. Fortunately, in some organisms, it is possible to estimate sexual isolation directly in the wild, as well as inferring the mechanisms responsible for it, as we will show below for a marine snail. Understanding the biological mechanisms causing sexual isolation is essential for explaining how reproductive isolation evolves between incipient species and thus how speciation can be achieved (Coyne & Orr, 2004).

Rolán-Alvarez & Caballero (2000) developed sexual isolation coefficients for each mate-pair combination separately: the pair sexual isolation (PSI) coefficient estimates mating preferences (deviations from random mating) for every mate combination, exclusively using data taken from mating pairs. Rolán-Alvarez & Caballero (2000) suggest the use of PSI coefficients under a multiple regression approach to predict which variables (environmental, morphological, etc.) can significantly account for individual contributions to the sexual isolation (PSI coefficients). This approach is similar to the use of multiple regression for identifying components of natural selection (Arnold & Wade, 1984a, b). Recently, Pérez-Figueroa, Conde-Padín P, Uña-Alvarez J, & Rolán-Alvarez E (unpublished) presented a new statistic (r_i) based on the Pearson correlation coefficient, which also allows investigation of the causes of assortative mating or sexual isolation directly in the wild in the same manner as the PSI coefficient. In the present study, we use, for the first time, both r_i and PSI coefficients in a multiple regression framework to test a potential mechanism of sexual isolation, as suggested by Cruz *et al.* (2004a) and Rolán-Alvarez *et al.* (2004), in a model system of parallel ecological differentiation.

On exposed rocky shores in Galicia (north-west Spain), a remarkable polymorphism exists, where two ecotypes of *Littorina saxatilis* adapted to different

shore levels and habitats are found (Rolán-Alvarez, 2007). The ridged and banded (RB) ecotype lives preferentially on the upper part of the shore associated with a barnacle belt (*Chthamalus* spp.), whereas the smooth and unbanded (SU) form is found associated with a mussel belt (*Mytilus galloprovincialis*) on the lower shore (Johannesson, Johannesson & Rolán-Alvarez, 1993). Mussels and barnacles overlap on the mid shore, forming a patchy microhabitat, where both 'pure' forms and a variable percentage of intermediates (putative hybrids) can be found living in true sympatry (Rolán-Alvarez *et al.*, 1999, 2004). These pure forms mate partially assortatively at the mid shore (Rolán-Alvarez *et al.*, 1999) but, because some gene flow exists between them, they are considered as conspecifics (Johannesson *et al.*, 1993; Rolán-Alvarez, Rolán & Johannesson, 1996; Rolán-Alvarez *et al.*, 2004). However, these two ecotypes differ in many morphological, behavioural, and even life-history characteristics, mostly due to the existence of disruptive selection acting across the vertical environmental gradient (Johannesson *et al.*, 1993; Rolán-Alvarez *et al.*, 1996; Rolán-Alvarez, Erlandsson & Johannesson, 1997; Cruz, Rolán-Alvarez & García, 2001; Cruz *et al.*, 2004b). Most of these traits are genetically determined (Rolán-Alvarez, 2007), and the estimated heritability for shell size and shape traits is approximately 0.6 (Carballo, García & Rolán-Alvarez, 2001; Conde-Padín *et al.*, 2007). The pattern of quantitative genetic differentiation of shell traits compared with molecular differentiation supports the hypothesis that natural selection plays a fundamental role in maintaining shell differences between ecotypes. In addition, the main differences in shape between these ecotypes can be interpreted in adaptive terms. The shell aperture of RB is smaller to better resist crab attacks and environmental stress, whereas the larger shell aperture in the SU morph has evolved to accommodate a larger muscular foot to better attach to the substratum in the most extremely wave-exposed habitat (Rolán-Alvarez *et al.*, 1997; Carvajal-Rodríguez, Conde-Padín & Rolán-Alvarez, 2005; Conde-Padín *et al.*, 2007).

In spite of strong differentiation in morphology (genetically based), behaviour, and other traits, these ecotypes maintain a strong genetic homogeneity for molecular (putatively neutral) variation at a geographical scale of dozens of kilometres (Johannesson *et al.*, 1993; Rolán-Alvarez *et al.*, 2004; Fernández *et al.*, 2005). This has been interpreted as a consequence of an intraspecific polymorphism that has arisen *in situ* at each locality (Rolán-Alvarez, 2007). In addition, the phylogenetic pattern of mitochondrial genes in both ecotypes from geographically distinct localities can only be explained assuming parallel and sympatric origin of the ecotypes in each locality (Quesada *et al.*, 2007). At a vertical microgeographical

scale of tens of metres, however, these ecotypes show some genetic (neutral) differentiation, which supports the hypothesis that some partial isolation barrier is contributing to their differentiation (Rolán-Alvarez *et al.*, 2004; Fernández *et al.*, 2005). Such an isolation barrier is produced by two mechanisms: first the two ecotypes preferentially live at different shore levels and habitats (upper versus lower shore) and, to a lesser extent at mid shore (ecological isolation *sensu* Coyne & Orr, 2004) and, second, even when they meet in sympatry at the mid shore, they show partial (70% of the maximum possible on average) sexual isolation (*sensu* Coyne & Orr, 2004; see also Johannesson, Rolán-Alvarez & Ekendahl, 1995; Rolán-Alvarez *et al.*, 1999; Cruz *et al.*, 2004a; Rolán-Alvarez *et al.*, 2004). We have previously suggested that sexual isolation was caused by a shift in average size between ecotypes (the RB ecotype is twice as large as the SU morph) because of disruptive natural selection with respect to size at mid shore (Cruz *et al.*, 2001, a; Rolán-Alvarez *et al.*, 2004; Conde-Padín *et al.*, 2007), and by size assortative mating, which exists in many populations of this species (Saur, 1990; Reid, 1996; Erlandsson, Rolán-Alvarez & Johannesson, 1998; Hull, 1998; Pickles & Grahame, 1999). If true, such a hypothesis suggests two additional predictions. First, most of the sexual isolation will be caused exclusively by size rather than by shell shape differences between ecotypes (RB versus SU). This hypothesis can be tested using new morphometric techniques that are able to disentangle size and shape variables from shell morphology (Carvajal-Rodríguez *et al.*, 2005; Conde-Padín *et al.*, 2007) and by using new statistical tools that have been developed to infer the individual contribution to the population assortative mating (and sexual isolation) (Rolán-Alvarez & Caballero, 2000). Second, the hypothesis predicts that the behaviour of mate choice based on size is similar in both the exposed Galician shores and other populations where differentiation of ecotypes has not occurred. As a result, we expect the degree of size assortative mating in each population to correlate with the intrapopulation variation in size. Thus, following the former sexual isolation mechanism in this species, it would be possible to predict the degree of size assortative mating in every population.

In the present study, we investigated the mechanism contributing to sexual isolation in the Galician *L. saxatilis* model system by means of three different experimental approaches. First, we investigated which of the different shell (size and shape) variables contributed most to the variation in individual sexual isolation in the field (r_1 and PSI). We expect a priori that size would be the variable contributing most to the sexual isolation, although we do not know which sex will be the choosy sex. Second, we extended the

study of size assortative mating outside the hybrid zone of Galician to Swedish shores to determine whether size variation within population correlates with size assortative mating among localities. Under this approach, our prediction is that a large proportion of the variation in size assortative mating among populations will be based on the level of size variation within each population. Third, we also conducted laboratory behavioural experiments to assess whether the proposed mechanism would explain the mating behaviour in controlled conditions.

MATERIAL AND METHODS

SAMPLING OF MATING PAIRS

The mating pairs of *L. saxatilis* were obtained in three localities (Baiona, Barra, and Cabo Estai) outside of the Galician hybrid zone (here we meant by hybrid zone those localities where RB and SU ecotypes are present in sympatry; Rolán-Alvarez, 2007) during November 2001 to February 2002, and two extra localities of the protected (S) Swedish ecotype (Vinnarstrand and Ängklåvebukten) during July/August 2002 (Fig. 1). In these samples, approximately 100 mating pairs were captured and brought to the laboratory for morphological analyses. In addition, mating pairs from two localities (Silleiro and Centinela) from the *L. saxatilis* hybrid zone at Galicia (north-west Spain) were also obtained during May/June 2006 (Fig. 1). In this later sampling, a plastic circle, 20 cm in diameter, centred on the captured mating pair was used to obtain a sample of noncopulating snails as well as some environmental/demographic variables (see below) associated with the mid shore area (for further details, see Johannesson *et al.*, 1995). The mating pairs (i.e. a male with the penis introduced inside the mantle cavity of the partner; *sensu* Saur, 1990; Johannesson *et al.*, 1995) were obtained during low tide directly on the rocky shore. Some of the pairs (6.5–38.9%) were male–male or male–immature pairs and were discarded from analyses. The final sample size used in each locality is shown in Table 1.

MORPHOLOGICAL AND ENVIRONMENTAL MEASUREMENTS

The morphological measurements differ in the populations obtained inside (Silleiro and Centinela) and outside (Baiona, Barra and Cabo Estai, Vinnarstrand and Ängklåvebukten) of the hybrid zone. All adult shells were examined using a Leica MZ12 stereoscopic microscope, and colour images were captured and digitized using a Leica digital ICA video camera and QWin Lite, version 2.2 software, with the specimens placed in the same position, with the axis of the shell on the y-axis and the aperture in the same

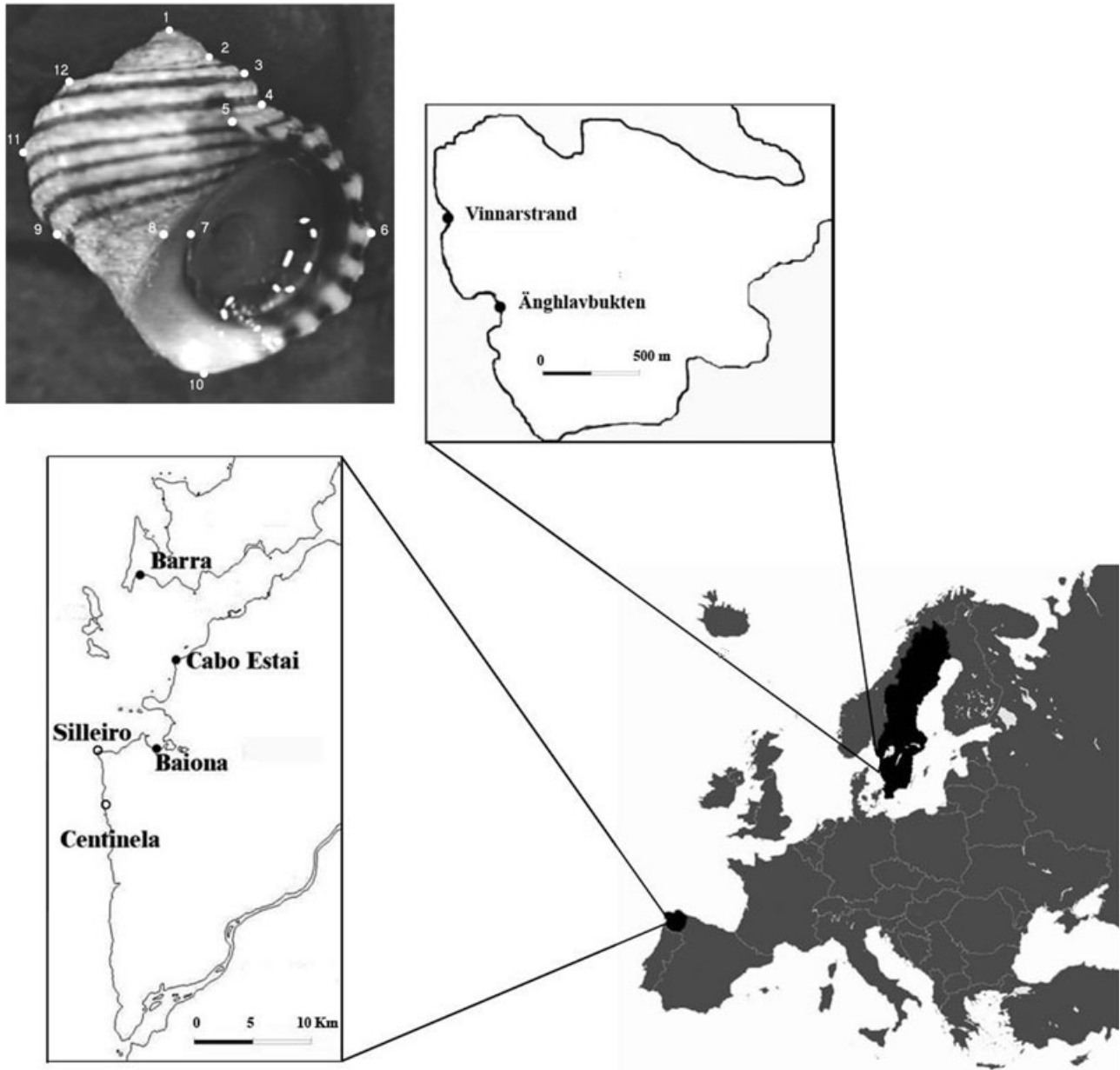


Figure 1. Sampling sites and landmarks studied on a ridged and banded individual.

plane as the objective (Fig. 1). Twelve representative landmarks (coordinates) of the shell were used (Fig. 1) for the specimens captured inside the hybrid zone following the procedure detailed in Carvajal-Rodríguez *et al.* (2005) and Conde-Padín *et al.* (2007). Because populations outside the hybrid zone experience no sexual isolation (only size assortative mating), a simpler size estimate equivalent to shell height was obtained in this case (the distance between landmark 1 and landmark 10; Fig. 1). Nevertheless, inside the hybrid zone, we could verify that shell height was highly correlated with the best esti-

mate of size (centroid size; $r = 0.99$, d.f. = 81; $P < 0.001$).

For the specimens inside the hybrid zone (from Silleiro and Centinela), we disentangled shell morphology for size and shape components using geometric-morphometric methods (Bookstein, 1991; Rohlf & Bookstein, 2003; Zelditch *et al.*, 2004). The estimate of shell size was centroid size (CS), which is the square root of the sum of squared distances of a set of landmarks from their centroid, with this being the centre of gravity of a configuration of points (Bookstein, 1991). The shape (geometric information that

Table 1. Sample size (number of male-female mating pairs), mean size (and standard deviation and error, SE and SE, respectively) of the male in mating pairs, a Pearson correlation coefficient of the shell size in pairs (r) and I_{PSI} sexual isolation statistics in the different populations studied inside and outside of the Galician Hybrid zone

	Outside the hybrid zone					
	Hybrid zone			Galicia		
	Silleiro	Centinela	Baiona	Barra	Cabo Estai	Vinnarestrand
Number of mating pairs	41	42	66	101	88	81
Male size (mean \pm SE)	4.01 \pm 0.192	3.6 \pm 0.157	8.39 \pm 0.150	4.59 \pm 0.060	4.97 \pm 0.083	12.43 \pm 0.136
Size assortative mating (r) [†]	0.632***	0.685***	0.458***	0.414***	0.441***	0.385**
Sexual isolation (I_{PSI}) \pm SD [‡]	0.804*** \pm 0.098	0.391 ^{NS} \pm 0.486	-	-	-	-
Sexual isolation (r_{10}) \pm SE [†]	0.723*** \pm 0.196	0.582* \pm 0.245	-	-	-	-

NS, nonsignificant.

** $P < 0.01$; *** $P < 0.001$.

[†]Standard errors (SE) and significance were obtained by classical parametric tests (Pearson correlation coefficient).

[‡]Standard deviation (SD) and significance were obtained by bootstrapping the mating pairs.

remains after eliminating the effects of translation, rotation, and scale), can be decomposed into uniform and non-uniform components (Bookstein, 1991; Rohlf & Bookstein, 2003; Zelditch *et al.*, 2004). They were computed with the Procrustes generalized orthogonal method (Rohlf & Slice, 1990), which determines a reference configuration minimizing the sum of squared distances between homologous landmarks from all specimens. The uniform component describes the global variation of the shell (affecting all landmarks simultaneously) and, in turn, is decomposed into the first uniform component (U1), and the second uniform component (U2), which could represent compression/dilation or shear deformations (Zelditch *et al.*, 2004). The non-uniform components (relative warps, RW) describe local shape deformations of a reference configuration at different spatial scales (representing local changes in the landmarks). These were computed excluding the uniform components and with the scale option of $\alpha = 0$. There were 18 noncorrelated RW, each successively explaining a decreasing percentage of the overall local variation, arising from the analysis of 12 landmarks (Zelditch *et al.*, 2004). The digitalized images were prepared with the software TPSutil, and the landmarks located with the TPSdig, both developed by Rohlf (1998, 2004). All calculations were performed with MODICOS, developed by Carvajal-Rodríguez & Rodríguez (2005) and TPSrelw, developed by Rohlf (1998).

The plastic circle defining the microareas surrounding mating pairs was used to obtain some environmental/demographic variables inside those microareas. One was the frequency of RB and the frequency of SU ecotypes. We captured all snails within the microarea and classified them according to the presence/absence of ridges and bands (Johannesson *et al.*, 1993). We also took a digital photograph of the microarea and, in the laboratory, we divided the photograph into 16 large quadrates, each one again divided into 16 smaller quadrates (256 small squares in total). Thus, we obtained from such images the relative abundance of mussels (i.e. the number of small quadrates fully covered by mussels), and accordingly the relative abundance of barnacles within the microarea. The aggregation of mussels and the aggregation of barnacles were obtained by dividing the mean number of small quadrates by their variance across large quadrates (*sensu* Taylor, 1984; Margalef, 1991). We also obtained two linear profiles (horizontal and vertical profiles in relation to the shore line) of the surface of the microarea *sensu* Kostylev, Erlandsson & Johannesson (1997). We used the length of these profiles as an estimate of the surface complexity. Surface complexity has been estimated by means of the fractal dimension of the surface and the rugosity (Kostylev *et al.*, 1997).

However, in a pilot study on our shores, we previously observed that such a profile length was highly correlated with the fractal dimension of the surface ($r = 0.64$, $N = 20$, $P < 0.01$) and the rugosity ($r = 0.90$, $N = 20$, $P < 0.001$), and thus we used the horizontal and vertical length profiles as a simple estimate of those other measures. Some of those environmental variables could partially contribute to the existing sexual isolation between RB and SU ecotypes on the hybrid zone.

ESTIMATING INDIVIDUAL CONTRIBUTIONS TO SEXUAL ISOLATION

We used the I_{PSI} sexual isolation estimator as a global estimate of sexual isolation between RB and SU ecotypes exclusively in those populations inside the hybrid zone (Silleiro and Centinela) (Rolán-Alvarez & Caballero, 2000; Coyne, Elwyn & Rolán-Alvarez, 2005), which is considered as one of the most valuable statistics for estimating sexual isolation (Pérez-Figueroa, Caballero & Rolán-Alvarez, 2005; Carvajal-Rodríguez & Rolán-Alvarez, 2006). This index ranges from -1 to 1 , with zero equivalent to random mating and 1 equivalent to complete sexual isolation. However, this index is not valid for estimating the individual contribution to the sexual isolation. Instead, we employ the PSI coefficients with such a purpose (i.e. defined for every pair combination as the number of observed pair types divided by the expected pair types from mates), which estimates mating preferences for every mate combination (Rolán-Alvarez & Caballero, 2000; Cruz *et al.*, 2004a; Coyne *et al.*, 2005). Such a strategy, however, would give exactly the same contribution to sexual isolation in all individuals belonging to a particular mating pair combination (e.g. male RB and female SU).

We also used a complementary strategy that should be preferentially applied in hybridizing species or ecotypes, such as in our RB and SU ecotypes. We attempted to obtain a continuous variable describing each specimen from a phenotypic point of view in relation to the RB and SU differentiation (including both size and shape information). A discriminant analysis employing all the shell size and shape variables was then used, and the discriminant scorings (individual predicted values obtained from the discriminant function) of each individual represented a single continuous phenotypic variable separating the two ecotypes. Such discriminant scoring allows us to significantly differentiate RB and SU subpopulations in both Silleiro (mean RB = 1.88, mean SU = -4.75 ; Wilks' $\lambda = 0.098$; $P < 0.001$) and Centinela (mean RB = 0.59, mean SU = -3.78 ; Wilks' $\lambda = 0.304$; $P < 0.001$) with a 100% and 92% individual cross-validation per locality, respectively. The Pearson cor-

relation coefficient of this variable, among male and female in mating pairs, would represent an estimate of the level of assortative mating for both size and shape and, because these ecotypes differ in average in both size and shape (Carvajal-Rodríguez *et al.*, 2005), it also represents an estimate of sexual isolation in our populations (notice that, although this correlation is another estimate of sexual isolation, the type of information that we used was completely different from the I_{PSI} mentioned above).

Instead of the Pearson correlation coefficient, we used the r_i index, which is an additive decomposition of the former that estimates for a particular trait the individual contribution of each pair to the population assortative mating. This statistic is obtained as the product of the standardized values (for each sex separately) of a mating pair, $r_i = Z_m \times Z_f$, where Z_m and Z_f are the standardized values of the variable within each sex and sample (each z -value from an x -value requires knowing the mean \pm SD for males and females independently from each sample (locality) obtained; so, for males: $Z_m = \frac{x_m - \mu_m}{\sigma_m}$, and similar for

females). The mean r_i in a population is algebraically equal to the parametric Pearson correlation coefficient (r) in the population. The use of standardized variables has another advantage because it allows the same variable to be compared in populations differing in their mean and variances (Sokal & Rohlf, 1995). The product of the standardized discriminant scorings (per sex and population) in each mating pair gives the r_{id} coefficient, which represents the contribution of each mating pair to the overall sexual isolation in this species to be used under multiple regression following the method described by Pérez-Figueroa *et al.* (unpublished).

Sexual isolation statistics (I_{PSI} and PSI coefficients) and their bootstrap significances were obtained using the software JMATING (Carvajal-Rodríguez & Rolán-Alvarez, 2006). Discriminant analyses, individual scoring values and their corresponding r_{id} coefficients were calculated with the SPSS/PC, version 14.0.

UNDERSTANDING THE CAUSES OF SEXUAL ISOLATION IN THE HYBRID ZONE

As mentioned above, in the littorinid hybrid zone, the size assortative mating has been proposed as the mechanism for sexual isolation (Cruz *et al.*, 2004a; Rolán-Alvarez *et al.*, 2004) and this hypothesis predicts that, among all shell variables, shell size will contribute most to the individual variation in sexual isolation. Alternatively, if sexual isolation is not the result of size assortative mating, we would expect that other variables (such as shape variables) would contribute more to sexual isolation. The individual

variation in sexual isolation was estimated by two different approaches. First, as the PSI mating preferences, obtained for each mating pair combination (Cruz *et al.*, 2004a). Second, as the r_i estimate of the discriminant scores described above (r_{id}). Both estimates were used as dependent variables in two multiple linear regression analyses. As independent variables, we used all environmental variables obtained from microareas as well as morphological ones (shell size and shape variables) obtained from each particular specimen. When the multiple regressions on environmental variables were significant, we also used the residuals of the regression as dependent variables to investigate the contribution of the morphological ones (independent of the environmental variation) to the sexual isolation (the portion of sexual isolation not explained by the environmental ones) following Rausher (1992). However, any study on linear multiple regression need to be cautious with the number of independent variables used, especially at small sample sizes, because too many independent variables may produce spurious relationships (Endler, 1986; Mitchell-Olds & Shaw, 1987). Because, in our case, the number of variables is doubled (we use both male and females shell variables), we reduced the shell variables to the most important ones: the size (CS), the two uniform (U1 and U2) and the two main non-uniform (RW1 and RW2) shape components (ten shell variables in total). Moreover, we also used the squares of the independent variables in order to find quadratic relationships between the variables and the dependent variable (Lande & Arnold, 1983; Brodie, Moore & Janzen, 1995). Thus, in total, we could use up to 20 independent variables in the regression analysis. These calculations were computed using SPSS/PC, version 14.0.

UNDERSTANDING THE CAUSES OF THE DIFFERENTIAL SIZE ASSORTATIVE MATING AMONG POPULATIONS

Size assortative mating is a widespread phenomenon in wild populations of *L. saxatilis*, although different populations do not show the same level of size assortative mating (Saur, 1990; Johannesson *et al.*, 1995; Reid, 1996; Erlandsson & Rolán-Alvarez, 1998). If mate choice is exclusively (or mostly) based on size and following a similar and consistent mate preference function among populations (Cruz *et al.*, 2004a; Rolán-Alvarez *et al.*, 2004), the degree of assortative mating observed in most populations of *L. saxatilis* could be predicted based exclusively on the size variability within each population studied. This is the case because a higher size assortative mating would be predicted in those populations with a larger variance in shell size, as the larger the difference between individuals becomes within a particular population

the larger the chance to cause size assortative mating. The alternative hypothesis would be that ecotype assortative mating is caused by a different mechanism than size assortative mating or that the mechanism differs across populations. This second alternative would not predict a significant relationship between within-population size variation and size assortative mating when populations inside and outside the hybrid zone are included. The variance of a particular variable in a population, however, is influenced by the mean of the value in such a population, and thus was used instead the coefficient of variation for shell size ($SD/mean \times 100$; Sokal & Rohlf, 1995). We used the population size assortative mating (Pearson correlation coefficient) as the dependent variable and the coefficient of variation of males in the populations as the independent one to investigate, by linear regression, the amount of variability in the dependent variable explained by the independent one across all localities studied (two inside and five outside of the hybrid zone). The calculations were performed with SPSS/PC, version 14.0.

UNDERSTANDING THE CAUSES OF MATE CHOICE IN THE LABORATORY

In other geographical areas, such as in the Swedish populations, it is considerably easier to perform behavioural experiments because the populations show a certain tendency to mate in early spring (Saur, 1990; Hollander, Lindegarth & Johannesson, 2005). However, to use RB and SU specimens from Galicia in the laboratory when conducting behavioural experiments is a formidable task because, in this case, the specimens do not show seasonality in mating behaviour. In addition, RB specimens are very sensitive to manipulation because they live in habitats with abundant predators. Initially we carried out preliminary experiments to maximize the number of mating pairs obtained in controlled conditions aiming to investigate mating preferences. We used the methods described by Conde-Padín (2006) for maximizing the number of mating pairs obtained in the laboratory. Accordingly, we used five SU males (with an average shell size of 3.42 ± 0.57 mm) surrounded by four groups of five females each, all placed in a round plastic container (diameter 36 cm) with seawater to allow free movement of snails. The four groups of females were small SU (4.61 ± 0.54), large SU (5.75 ± 0.63), small RB (6.63 ± 0.62), and large RB (8.71 ± 0.89). It should be noted that this experimental design is also very convenient if the male is the main determinant of the mate choice. Each trial was observed for 80 min and any copulations recorded. All 20 trials (replicates) were accomplished within 48 h, and thus we could observe a maximum of 100 mating

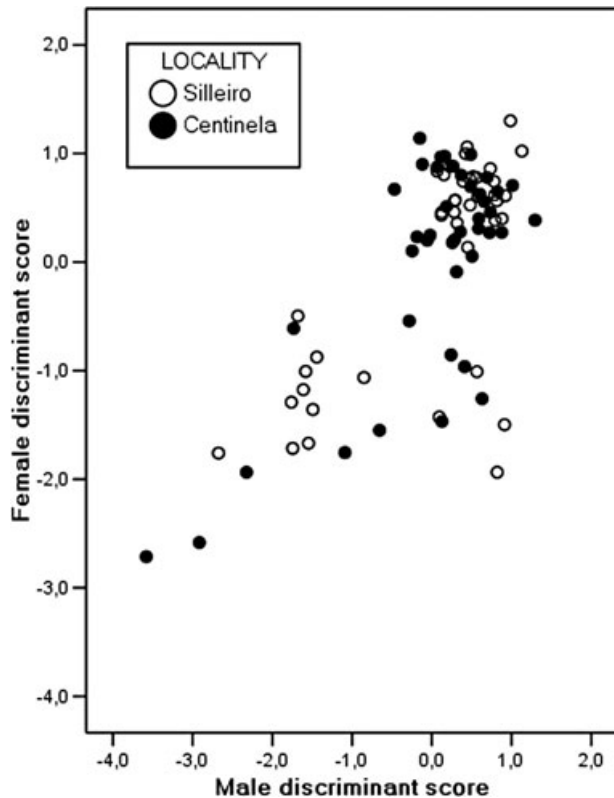


Figure 2. Distribution of the standardized discriminant scorings of males and females in mating pairs in the two populations studied. The product of the male and female values is the r_{id} , an estimate of the individual contribution to sexual isolation.

pairs. All statistical analyses were performed with SPSS/PC, version 14.0.

RESULTS

THE CAUSES OF SEXUAL ISOLATION

The level of sexual isolation was high and significant in Silleiro for the two sexual isolation estimators (mean r_{id} and I_{PSI}), but only significant in Centinela for the mean r_{id} estimator (Table 1). This suggests that, in this particular case, r_{id} may be a more powerful estimator of sexual isolation effects because it uses more detailed information (individual size and shape information) than its alternative I_{PSI} . Notice that, in this case, mean r_{id} is almost an average between the I_{PSI} (using exclusively ecotype information) and the Pearson correlation coefficient for size in pairs (using exclusively size information) because r_{id} uses both size and ecotype information. The relationship between male and female standardized discriminant scoring in pairs is shown in Figure 2.

Thus, we could study the causes of sexual isolation (using the r_{id} coefficient) by multiple linear regres-

sions. We examined whether the environmental variables could explain any significant part of the individual variation in the estimate of sexual isolation (r_{id}) or in PSI . Only one variable (horizontal profile; horizontal surface complexity in relation to the shore line; see Material and methods) significantly explained 11% of the variation in r_{id} coefficients in Centinela ($P < 0.05$) but, in Silleiro, environmental variables did not explain a significant portion of the sexual isolation. Thus, the studied environmental variables only described a minor part of the sexual isolation (5.5% in average). Moreover, environmental variables did not explain any portion of the PSI .

Furthermore, we investigated whether shell morphology could explain sexual isolation (r_{id} or PSI) and, more importantly, which trait (shell size or shape) is the most accurate predictor. Shell morphology explained a significant part (76–92%) of the variability in sexual isolation (r_{id}) in both localities (Table 2). Interestingly, the most important traits contributing to sexual isolation were quadratic shell size (CS2) and shell size (CS) (representing 72–85% of the above regression model), as predicted a priori by our hypothesis. In Centinela, when the dependent variable is corrected for the environmental variables, the result is very similar: the regression explains the 77% of the variability and the variables that entered in the model were CS2 (50%) and CS (19%) from males and U2 (8%) from females. More important, male size is the relevant trait for predicting the level of sexual isolation in our populations. These trends are maintained when all samples are pooled (Table 2), or when more independent shell shape variables (RW3 to RW18) are included (not shown). Minor contribution of female choice cannot be definitively excluded because a few female shell traits (e.g. CS and U2) appear to contribute significantly to a minor proportion of the variation in sexual isolation. It could be argued that, because male and female size is correlated in pairs, the regression model is preferentially choosing those male traits that are only due to chance. However, when the regression model is accomplished for males and females separately, the same trends were observed: the percentage of r_{id} explained by CS2 in both samples pooled was two-fold greater in males (52%) than in females (26%).

The graphical representation of the individual contribution to sexual isolation (r_{id}) and male size is shown in Figure 3. Male RB appears to be more involved in explaining the linear relationship with sexual isolation, which is expected due to size variability being larger in the RB than in any other category of males. The other estimate of sexual isolation gave somewhat contradictory results. A larger percentage of variation in PSI coefficients was explained in Silleiro than in Centinela. In Silleiro, the

Table 2. The stepwise linear regression of the morphological variables explaining the individual sexual isolation (r_{id} and PSI coefficients). The percentage of variance explained by the whole regression model (r^2) or each variable introduced (%) and the coefficient of partial regression are shown for each analysis

Coefficients	Locality	Regression analysis			
		r^2	Morphological variables	(%)	Coefficient of partial regression
r_{id}	Silleiro	0.763***	(CS male) ²	65.3	2.721***
			CS male	7.0	-1.777**
			CS female	4.0	-0.253*
	Centinela	0.925***	(CS male) ²	75.7	2.934***
			CS male	9.9	-2.149***
			(U2 male) ²	5.5	0.172**
			(RW1 female) ²	1.5	0.142*
	Pooled	0.684***	(CS male) ²	52.2	1.696***
			RW2 male	4.9	0.365***
			(RW2 male) ²	6.8	0.306***
CS male			2.7	-1.083*	
U2 female			1.8	-0.137*	
PSI	Silleiro	0.574***	(CS male) ²	57.4	0.758***
	Centinela	0.11*	RW2 female	11.0	-0.332*
	Pooled	0.459***	(CS male) ²	38.9	0.742***
			(CS female) ²	4.0	-0.315**
			(RW1 female) ²	3.1	0.200*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

results were similar to those of the r_{id} coefficient (the quadratic male CS was the most important trait), although only one female trait contributed to variation in PSI coefficients in Centinela. The rare pattern observed in Centinela for PSI values could be due to the I_{PSI} estimator not being significant in Centinela (Table 1).

THE REASONS FOR SIZE ASSORTATIVE MATING DIFFERENCES AMONG POPULATIONS

We predicted that shell size in males would account for both size assortative mating outside the hybrid zone and sexual isolation in the hybrid zone (basically size assortative mating involving two ecotypes differing in size). To test this prediction, we estimated the strength of the relationship between the coefficient of variance for male shell size (estimated as the mean shell height within population) and the degree of size assortative mating (as the Pearson correlation for size among male and female partners in pairs) across samples (Table 1). Note, however, that, in this analysis, we cannot investigate whether the male or the female contributed most to the size assortative mating because variability in size for males and females are absolutely correlated within populations. The male size variability was significantly correlated

with the level of size assortative mating using parametric ($r = 0.94$; $P < 0.01$) and nonparametric (Kendall's tau-b, $\tau_b = 0.79$; $P < 0.01$) tests. The relationship between both variables and the significant regression line is shown in Figure 4. Variability within populations for male (or female) size alone could explain up to 88% of variability in size assortative mating among populations. This result strongly suggests that, in this species, it is the degree of size variation within a population that determines the level of size assortative mating everywhere (inside and outside of the hybrid zone), and thus ecotype differences do not contribute to the level of size assortative mating in the hybrid zone, as expected under the alternative hypothesis.

CAUSES OF MATE CHOICE IN LABORATORY CONDITIONS

Typical SU males were placed in the laboratory with different groups of females (SU and RB, small and large) surrounding them. The results of this mate choice experiment are shown in Table 3. The number of mating pairs observed was very low in any of the different female groups. There were no significant differences between female groups in mating frequency or mating duration by analysis of variance.

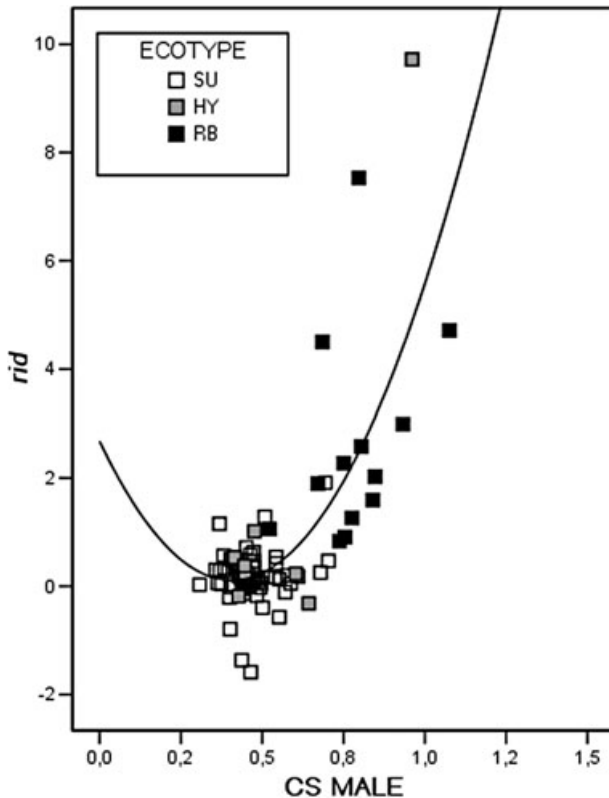


Figure 3. Relationship between r_{id} , an estimate of the individual contribution to sexual isolation, and male size. The curve represents a quadratic regression. CS, centroid size; SU, smooth and unbanded; HY, hybrid; RB, ridged and banded.

Interestingly, however, mating frequency was significantly and highly correlated with the mean size of each female class ($r = -0.96$; $P = 0.044$), but it was not significantly correlated with ecotype ($r = 0.83$; $P = 0.171$). This suggests that the size of the female, rather than the female's ecotype, is the focal trait being used by the male during mate choice.

DISCUSSION

The Galician RB and SU ecotypes have evolved in sympatry and in parallel (Rolán-Alvarez *et al.*, 2004; Quesada *et al.*, 2007; Rolán-Alvarez, 2007). Although these two ecotypes cannot be considered as true species yet, they are different evolutionarily stable entities that have evolved an almost complete isolation barrier. Consequently, understanding the biological mechanism that has produced the sexual isolation in this case will surely help to explain how speciation can occur in *L. saxatilis* and other species in allopatry, parapatry, or sympatry.

We have already described the two main processes contributing to the incomplete reproductive isolation

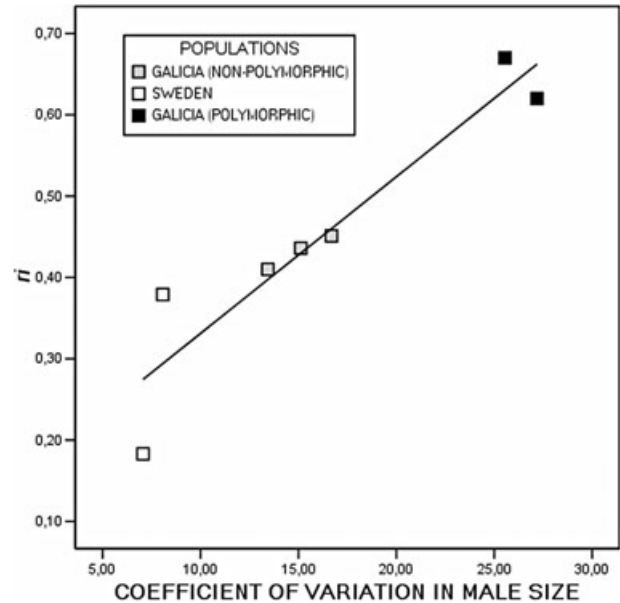


Figure 4. Relationship between male size variability (coefficient of variation) and the level of size assortative mating (the significant regression line is included).

between RB and SU ecotypes: habitat isolation and sexual isolation. The first is obviously a side-effect of adaptation because each ecotype adequately chooses its own habitat (Erlandsson *et al.*, 1998; Cruz *et al.*, 2004b) to avoid an unfit genotype being in the wrong habitat (Rolán-Alvarez *et al.*, 1997; Cruz *et al.*, 2004b). There is little doubt that habitat isolation is typically originated by natural selection (Coyne & Orr, 2004: 188). A few well known examples of habitat isolation mechanisms contributing to the reproductive isolation have been described in plants, such as in sagebrush (Wang *et al.*, 1997) or buckwheat (Coyne & Orr, 2004), and animals, such as the host-specific parasite *Rhagoletis pomonella* inhabiting apples and hawthorn and its close relative *Rhagoletis mendax* living on blueberries (Feder & Bush, 1989). More recently, two ecotypes of the stick insect *Timena cristinae* were described living and typically mating in alternative plant species (Nosil *et al.*, 2002). In all these cases, natural selection has been the principal force during the evolution of the habitat isolation (Feder & Bush, 1989; Wang *et al.*, 1997; Nosil *et al.*, 2002; Coyne & Orr, 2004).

Furthermore, the observed sexual isolation between *L. saxatilis* ecotypes from Galicia at the mid shore has also been claimed as an indirect consequence of the adaptive responses of each ecotype to its own habitat (Rolán-Alvarez *et al.*, 2004; Cruz *et al.*, 2004a). In other words, sexual isolation is caused by a previous size assortative mating linked to an important shift in mean size between ecotypes, due to

Table 3. Description of sex, ecotypes and mean shell sizes (and standard errors) of the specimens used (100 of each ecotype, sex and size class) in the laboratory behavioural experiment (see text)

Sex	Ecotype	Size (class) and shell height (mm)		Number of observed mating pairs	Duration of mating (min) Mean \pm SE
		Class	Mean \pm SE		
Males	SU	Small	3.42 \pm 0.057	12	23.7 \pm 3.375
	SU	Small	4.61 \pm 0.054	5	19.2 \pm 5.358
Females	SU	Large	5.75 \pm 0.063	4	35.9 \pm 9.425
	RB	Small	6.63 \pm 0.062	2	9.7 \pm 2.107
	RB	Large	8.71 \pm 0.089	1	25.6

The number of observed mating pairs and its duration is also given. SE, standard error; SU, smooth and unbanded; RB, ridged and banded.

divergent natural selection on size in these populations (Johannesson *et al.*, 1993, 1995; Cruz *et al.*, 2001; Conde-Padín *et al.*, 2007). In the present study, we show that sexual isolation is a side-effect of size assortative mating in these populations by means of three complementary tests. First, male (not female) size was the most important trait for explaining up to 70% of the overall individual variation in sexual isolation. Second, size variation within population explains almost 90% of the overall variation in size assortative mating between populations both inside and outside the hybrid zone. Third, the laboratory experiment suggests that males prefer to choose by female size rather than female ecotype.

Our explanation, however, needs to account for all existing observations on the mating behaviour in this species, and a particular problem still remains: how can it be possible that sexual isolation originates with males if a large proportion of maladaptative pairs are typically found? (male–male and male–juvenile pairs are in the range 4–58%; Johannesson *et al.*, 1995; Rolán-Alvarez *et al.*, 1999). *Littorina saxatilis* males have been occasionally observed copulating with different species (Raffaelli, 1977; Saur, 1990). It appears that the mate recognition system in *L. saxatilis* does not allow a precise choice of the sex, or even between species. In other littorinid species, such as *Littorina littorea*, individuals have a capability of distinguishing the sex of the snails responsible for the mucus trail that remains after a conspecific specimen has passed on any surface, which explains the low rate of male–male (or interspecific) mating pairs observed in the wild (Erlandsson & Kostylev, 1995). However, *L. saxatilis* has no such ability, although males follow the mucus trail left by specimens of a similar size significantly more frequently (Erlandsson *et al.*, 1998; Hollander, 2005). In summary, *L. saxatilis* has the capability of following mucus trails presumably based on the physical size of the trail itself, but it does not

have the ability to detect the sex (or ecotype) of the snail being followed (Erlandsson *et al.*, 1998). Such an ability appears to be sufficient for mating in typically high density populations, although, from time to time, they may wrongly follow a male or a juvenile, or find a different specimen than the one they intend to follow. The high frequency of maladaptative pairs does not suggest that they are evolutionarily costly, and so these errors only produce some minor loss of time or energy. This would explain why maladaptative pairs also show significant and high levels of size assortative mating and sexual isolation (Johannesson *et al.*, 1995). Such a hypothesis would explain both the existing size assortative mating outside the hybrid zone, the sexual isolation inside the hybrid zone, and, at the same time, the high frequency of maladaptative pairs.

Size-based male behaviour, during trail following and mating, is the key for understanding sexual isolation in the wild, but it may not be the unique mechanism. We also observed some contribution from females when more independent variables are used (which could explain 4–5% of the variability in sexual isolation; data not shown). Moreover in the case of PSI coefficients in Centinela, only female traits contributed to sexual isolation. The mating duration was apparently dependent on the relative size of the female-within ecotype (Table 3). Some behavioural experiments performed in the laboratory using Swedish populations also suggest a role for both shape (affecting to the copulation duration) and size (contributing to the frequency of mates) in determining the pattern of assortative mating (Hollander *et al.*, 2005). It was concluded that the mate choice is based in two processes: one causing the size assortative mating (probably produced by the male) and, second, a choice based on ecotype characteristics, such as shell shape (Hollander *et al.*, 2005). The first of these processes is the most relevant in determining the observed patterns of size and ecotype assortative

mating in the wild (as we have shown above), although some caution is needed in that we have not studied the Swedish hybrid zone using our methods. Another well studied example of the male–female interactions during mating is available. Both males and females make some contribution to the sexual isolation detected between *Drosophila yakuba* and *Drosophila santomea* under laboratory conditions (Coyne *et al.*, 2005). However, even in this case, what happens in the wild in the only known hybrid zone affecting *Drosophila* species is not known (Coyne *et al.*, 2005; Llopart, Lachaise & Coyne, 2005). However, with the methodology proposed in the present study, it will be possible to study and infer the causes of mate choice directly in the wild.

In *L. saxatilis*, sexual isolation is a side-effect of the ecotype (size) adaptation to its respective habitat (Cruz *et al.*, 2001, 2004a; Rolán-Alvarez *et al.*, 2004; Conde-Padín *et al.*, 2007). Additionally, these ecotypes have originated in sympatry and in parallel at localities geographically separated (Quesada *et al.*, 2007). Thus, this comprises a good example of the ecological evolution of partial reproductive isolation in sympatry. Other examples are known in which sexual isolation has evolved as a side-effect of adaptation (Nagel & Schluter, 1998; Nosil *et al.*, 2002; Coyne & Orr, 2004). The case of the stickleback fish found in many rivers and fresh water lagoons from North America and other regions is particularly clear (Schluter & Nagel, 1995; Nagel & Schluter, 1998). In this case, limnetic and benthic ecomorphs show different characteristics (size among them) as a consequence of adaptation to their different habitats (Nagel & Schluter, 1998). These cases are not, however, identical. The stickleback has probably evolved in allopatry (Coyne & Orr, 2004), whereas reinforcement is contributing to the evolution of sexual isolation in the stick insect (Nosil *et al.*, 2003). We believe that the PSI and r_i coefficient and multiple regression analysis, as used in the present study, could help to reveal the mechanisms of sexual isolation in several other evolutionary examples, which would further increase our understanding of how this process evolves in nature.

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