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DETECTING SHAPE DIFFERENCES IN SPECIES OF THE *LITTORINA SAXATILIS* COMPLEX BY MORPHOMETRIC ANALYSIS

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ABSTRACT

We investigated variation in shell size and shape of marine snail species of the *Littorina saxatilis* complex (*L. saxatilis*, *L. compressa* and *L. arcana*) using geometric morphometric methods. These morphologically similar periwinkle species that are common in the European intertidal have presented many problems for diagnosis based on morphology alone. A discriminant analysis demonstrated that geometric morphometrics is very efficient for diagnosing individuals to species among sympatric populations. We successfully diagnosed an average of 96% of the specimens (with 85.7–100% correct diagnosis for specific comparisons). The diagnosis capability of this method is absolute at the population level. This makes the technique potentially useful for the design of manipulative field or laboratory experiments. Moreover, a geometric-morphometric analysis was also accomplished in two snail ecotypes (H and M) of *L. saxatilis* from rocky shores of NE England which are apparently adapted to different degrees of wave exposure. We found that the H (exposed) ecotype has a relatively rounded shell shape with a bigger aperture, whereas the M (protected) ecotype has a smaller aperture.

INTRODUCTION

The study of shape is an indispensable technique in the identification of species and in quantifying the nature of morphological variation within a species (e.g. Caley, Grahame & Mill, 1995). In *Littorina*, studies of shell shape variation within a species indicate a correlation with environmental factors such as wave exposure, predation or isolation (Newkirk & Doyle, 1975; Janson, 1982; Johannesson, Grahame, Mill & Brown, 1986; Carvajal-Rodríguez, 1990; Conde-Padín & Rolán-Alvarez, 2005), which may be the result of ecophenotypic variation, natural selection or genetic drift.

Snails belonging to the 'rough periwinkle' complex form a group of three closely related species (Small & Gosling, 2000), and are important members of rocky-shore intertidal communities in the North Atlantic. There has been a history of taxonomic confusion (Reid, 1996). The shells of *Littorina saxatilis* (Olivi, 1792) and *L. arcana* Hannaford Ellis, 1978 are relatively similar in shape and cannot be reliably discriminated in the field (see Fig. 1). For these species, the most reliable diagnosis rests on female reproductive tract characters. In the ovoviviparous *L. saxatilis*, females possess a brood pouch in which embryos develop as far as the shelled stage, while *L. arcana* lays eggs on the shore. *Littorina compressa* Jeffreys, 1865 is usually the easiest of the three species to recognize in the field; its shell has flattened spiral ridges, wider than the intervening grooves (Fig. 1). Like *L. arcana*, it lays eggs on the shore, and in males the penis has a characteristic form (Reid, 1996).

In Britain, *L. saxatilis* and *L. arcana* occupy a broad range of the upper littoral zone (Smith, Mill & Grahame, 1995). *Littorina saxatilis* is widespread, located on bedrock, boulders, small stones and gravel (on both open shores and in estuaries). *Littorina arcana* has a more limited distribution (rock and large stable boulders on exposed shores), while *L. compressa* occurs rather lower on the shore and rarely also in estuarine locations (Warmoes, Dumoulin & Reid, 1992; Mill & Grahame, 1995), and its geographic distribution is even more restricted.

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A major influence on shell shape in *L. saxatilis* appears to be the degree of wave exposure of the shore (Janson & Sundberg, 1983; Grahame & Mill, 1989). On British rocky shores, the shell polymorphism of *L. saxatilis* may be relatively great, no doubt in part because the tidal range is greater than is the case for the well-studied Swedish populations. There are some examples of extreme intraspecific polymorphism in *L. saxatilis*. Thus, Hull, Grahame & Mill (1996) reported two morphological forms, H (on the upper shore, thin shelled and with a wide aperture for adhesion in strong waves) and M (on the mid shore, thicker shelled, with a smaller aperture to reduce the risk of crab predation) (Johannesson, 1986; Wilding, Butlin & Grahame, 2001). In these cases, relative physical isolation due to microgeographical separation associated with habitat choice may be involved in the maintenance of the polymorphism (Johannesson, Johannesson & Rolán-Alvarez, 1993; Grahame, Wilding & Butlin, 2006; Rolán-Alvarez, 2007). This variation is at least partly paralleled in the Tjärnö region of Sweden, where similar morphs are referred to as E (resembling H) and S (resembling M) forms (Hollander, Lindegarth & Johannesson, 2005).

This variable shell morphology has been extensively studied in rough periwinkles by using distance variables in classical multivariate analyses (Janson & Sundberg, 1983; Johannesson, 1986; Grahame *et al*., 1990; Caley *et al*., 1995; Mill & Grahame, 1995; Johannesson & Johannesson, 1996; Cruz, Rolán-Alvarez & García, 2001). Using both bivariate and multivariate analyses with classical morphometric techniques (Grahame & Mill, 1986, 1989) or Fourier analysis (Dytham *et al*., 1992), it has been shown that there are significant shell differences between some populations of *L. saxatilis* and *L. arcana* (Grahame & Mill, 1986) and size differences between populations of *L. saxatilis* (Sundberg, 1988). Both local and regional scale variation in the shapes of shells of the two species in southern Britain have been shown (Grahame & Mill, 1989, 1992; Caley *et al*., 1995). In fact, Caley *et al*. (1995) made the first cross-validation study on the diagnosis of *L. compressa*, *L. saxatilis* and *L. arcana*, using discriminant functions calculated from distance measurements. This approach, however, gave only a moderate accuracy in the

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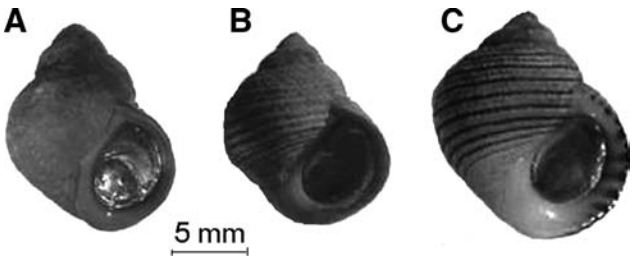

Figure 1. Female specimens representative of the three species of the *Littorina saxatilis* complex at the locality Watwick. **A**, *L. saxatilis*.; **B**, *L. arcana*. **C.**, *L. compressa*.

diagnosis of these species. For example, *L. arcana* and *L. saxatilis* were overall correctly classified for only 49% and 54% of the specimens, respectively. Morphometric studies across a range of sizes can confound size and shape unless specific analytical attempts are made to separate these essential variables of form, and it has been argued that the results may be very sensitive to the particular distances chosen in each study (Bookstein, 1991; Johnston, Tobachnick & Bookstein, 1991; Stone, 1998; Zelditch *et al*., 2004; Carvajal-Rodríguez *et al*., 2005). The new landmark-based technique of geometric morphometrics is argued to be a highly effective way of capturing information about the shape of an organism, especially when combined with multivariate statistical procedures (Rohlf & Marcus, 1993; Rohlf, Loy & Corti, 1996; Zelditch *et al*., 2004). It also very effectively separates size and shape variation as a standard part of the analyses.

Here we report a study of shell size and shape in the rough periwinkles at different localities in Britain, using landmark variables and geometric morphometric analyses to investigate the potential of this technique in discriminating between and within species. As an example of this application, we compared discrimination of female shells (which can be diagnosed *a priori* by anatomical traits) with that for *L. saxatilis* and *L. arcana* males (which cannot be reliably diagnosed *a priori*). Additionally, we investigated the main differences in shape between H and M ecotypes of *L. saxatilis* in order to explore the biological meaning of such differences.

MATERIAL AND METHODS

During September 2004, 526 snails belonging to the three species of the *Littorina saxatilis* complex (*L. arcana*, *L. saxatilis* and *L. compressa*) were collected from four localities around Britain: Watwick (British National Grid Reference SM 817039), Westdale (SM 797056) and The Gann (SM 813062) (all on the St Ann's Head Peninsula, Pembrokeshire, Wales) and Pettico Wick (NT 907692) (southeastern Scotland), (Fig. 2, Table 1).

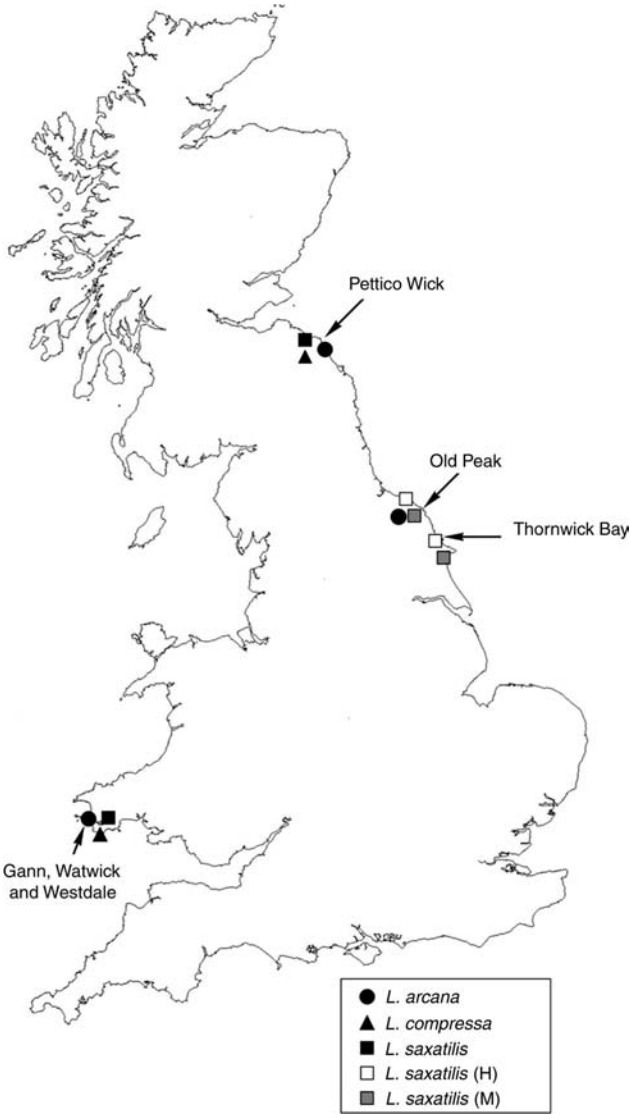

Figure 2. Map of localities sampled on the shores of Britain. The different symbols show the species present at each locality.

The H and M ecotypes of *L. saxatilis* were sampled on upper and mid rocky shores, respectively, in two localities typical of wave-exposed or moderately wave-exposed conditions, Old Peak (NZ 982024) and Thornwick Bay (TA 233724), but not

Table 1. Number of adult females and males (in brackets) of the three species and the ecotypes H and M of *Littorina saxatilis* from four localities in Britain.

	Watwick			Gann	Westdale	Pettico Wick	Old Peak	Thornwick Bay
	Upper	Mid	Lower					
L. arcana	35	11			10	67	9	
L. saxatilis	11	5		12	13	24	15 (Morph H)	16 (Morph H)
	(34)	(20)		(6)	(12)	(71)	15 (Morph M)	17 (Morph M)
L. compressa	1	9	5	9	9	22		
		(13)	(13)	(22)	(4)	(16)		
Total	47	25	5	21	32	113	39	33

Littorina saxatilis and *L. arcana* cannot be diagnosed in males based on shell or anatomical differences, and so they are pooled within *L. saxatilis*. In Old Peak and Thornwick Bay no males were recorded.

at the sites for interspecific comparison. Only adult snails were used in this study, and were recognized by dissection in the laboratory, so that only fertile females or males with a well-developed penis were analysed.

In the laboratory, all specimens were imaged with the specimens always placed in a consistent orientation with the axis of the shell on the *y*-axis and the apertural lateral axis in the same plane as the objective, i.e. the shell axis was placed parallel to the horizontal plane (Figs 1, 3). A grid was used to facilitate the specimen orientation and landmark location following Carvajal-Rodríguez *et al.* (2005). We then sorted individuals on the basis of shell characteristics which discriminated specimens of *L. compressa*. We discriminated females of *L. arcana* from *L. saxatilis* by dissection and noting, respectively, the absence or presence of developing embryos in the brood pouch. No morphological or anatomical diagnostic tool is available for the discrimination of male *L. saxatilis* from male *L. arcana*.

The placement of 17 landmarks (LM) followed the perimeter of the shell, to capture differences in all regions of the shell (Fig. 3). LM1 was placed at the apex of the shell. LM2 was on the right border of the profile of the shell at the end of the upper suture of the penultimate whorl. LM3 was on the right border of the profile of the shell at the end of the upper suture of the last whorl. LM4 was on the right border of the profile of the shell at the end of the lower suture of the last whorl. LM5 was at the end of the suture. LM6 was the outermost point of the external part of the outer lip. LM7 and LM8 were placed respectively on the internal and external border of the columella on a perpendicular line to the axis from LM6. LM9 was the most external point on the last whorl at the left profile of the shell on a perpendicular line to the axis from LM6. LM10 was the lowest point of the base. LM11 with a line from LM1, touching the internal columella, was the lowest point at the base. LM12 with a line from LM5, touching the internal columella, was the left profile point. LM13 was the most external point on the last whorl at the left profile of the shell. LM14 was on the left border of the profile of the shell at the end of the upper suture of the last whorl. LM15 was on the left border of the profile of the shell at the end of the upper suture of the penultimate whorl. LM16 was the most external point on the last whorl at the right profile of the shell on a perpendicular line to the axis from LM13. LM17 was the most external point on the last whorl at the right profile of the shell on a perpendicular line to the axis from LM12. The original data set used in this study can be obtained from the authors to generate a comparative basis for other studies.

For each specimen, centroid size and uniform (affine) and nonuniform (nonaffine) components of shell shape were obtained. Centroid size is the square root of the sum of squared distances of landmarks from their centroid (the average x and y coordinate points) of the landmark configuration (Bookstein, 1991). The estimation of shell shape components was accomplished by aligning the raw coordinates of the specimens using the Procrustes generalized orthogonal method (GLS; Rohlf & Slice, 1990), which determines a reference configuration by minimizing the sum of squared distances between homologous landmarks from different specimens. The uniform components account for shell variation at a global scale (producing parallel deformations affecting to all landmarks simultaneously). The first uniform component (U1) expresses changes at the horizontal scale of Figure 1 or 3, while the second component (U2) represents changes at the vertical scale. On the other hand, nonuniform (local) components describe local shape deformations of the reference configuration at different spatial scales.

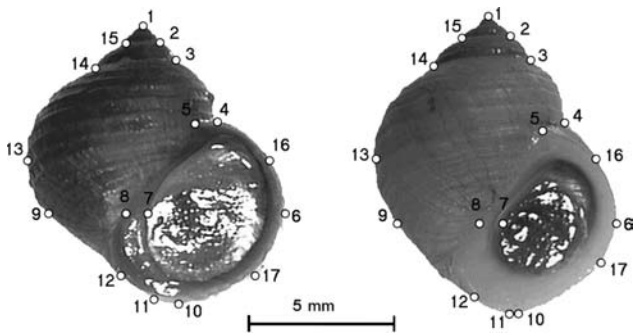

Figure 3. Female specimens representative of both ecotypes of *Littorina saxatilis* at Thornwick Bay. The H morph has a thin shell (left) and the M morph has a thicker shell (right). The landmarks (1–17) used in the morphological study to describe shell size and shape are represented by points.

Local shape measurements were computed by relative warp analysis (RWA) (Bookstein, 1991; Rohlf, 1993) and the uniform part of shape variation was computed using the space complement of the nonuniform component (Rohlf & Bookstein, 2003). The relative warp analysis consists of fitting an interpolating function (thin-plate spline) to all homologous landmarks for each specimen in a sample (Rohlf, 1993). The local shape is decomposed in partial warps, each being a component of change in shape. The contribution of each individual partial warp to the total shape change (from the reference configuration) for one specimen is given by its partial warp scores. The relative warps (RW) are the principal components of the variation among specimens in the space of the principal warps (see Bookstein, 1991; Rohlf, 1993). The relative warps were computed with the scaling option $\alpha = 0$ which weights all landmarks equally, for studying differences in shell shape among samples following Rohlf *et al.* (1996). We obtained landmark data by digitizing images of the shells as in Figure 3, and using the software TPSDIG (http://life.bio.sunysb.edu/morph/morphmet/tpsdig2w32.exe) to generate coordinates. Geometric-morphometric analysis was performed by the programs TPSRELW developed by Rohlf (1998; http://life.bio.sunysb.edu/morph/soft-tps.html) to obtain the thin-plate spline representation, and MODICOS (Carvajal-Rodríguez and Rodríguez, 2005; http://life.bio.sunysb.edu/morph/soft-comprehensive.html) to get the size and shape variables.

Firstly, we restricted our analysis to female shells (315 adult specimens studied), but after this, we used the morphometric information obtained from females to analyse secondarily the male shells (211 adult specimens studied; see Table 1 and below). Moreover, specimens of both ecotypes (H and M) of *L. saxatilis* (15–17 per locality; Table 1) were analysed separately in a second study to investigate differences within species in two localities (Old Peak and Thornwick Bay).

In the interspecific study in females, the first eight relative warps (RW1–8, explaining more than 90% of the local variation) in addition to centroid size (CS) and the two uniform components (U1 and U2) of shell shape were used to detect differences between species (fixed factor) and localities (random factor) by two-way ANOVA and by multivariate ANOVA (MANOVA; Sokal & Rohlf, 1995; Zelditch *et al.*, 2004). Partial eta-squared (η^2) coefficients were used to approximate the relative importance of the independent factors in MANOVA (Pierce, Block & Aguinis, 2004).

We also used a canonical discriminant analysis with the 31 derived variables (CS, U1 and U2, RW1–28) to compare the shell shape and size for females of the three species (diagnosed *a priori* based on anatomical differences; not available for males) as well as for any pair of species living in sympatry (see Zelditch *et al.*, 2004). The possibility of discriminating these species when they live in sympatry is the most interesting

application of the method, as it could address a limitation of studies in those areas arising from the difficulty of diagnosing living males and females of *L. saxatilis* and *L. arcana*. Starting with all the variables, we used the stepwise method in order to find those which significantly contributed to the discrimination. The discriminant analysis allows diagnosis of the species *a posteriori* (using shell size and shape information), using the discriminant scores, and reports the reliability of such diagnosis (Manly, 1986). We used the predictive equation for females on the male data set to distinguish between *L. arcana* and *L. saxatilis*. Thus, we assumed that this diagnosis would be equivalent to *a priori* diagnosis in the male shells. All morphometric univariate and multivariate analyses were computed with the SPSS/PC statistical package version 12.0.1.

RESULTS

A MANOVA revealed significant differences between species (Wilks' $\lambda = 0.381$; $F_{(22,\ 418)} = 11.776$, $P < 0.001$) for the first eight relative warps, the uniform and the centroid size variables in the female data set. Thus, it was possible to distinguish some of the groups using the derived morphometric variables studied. However, the MANOVA could not distinguish the three species at the same time by a SNK post hoc test. Therefore, we investigated the role of each variable separately by a two-way univariate ANOVA which showed which of the variables has the largest effect distinguishing between the three species, independent of the variation between localities. Only RW2 and RW5 showed significant differences between species, while they did not show significant effects for the factor locality or the interaction (see Table 2). This suggested that RW2 and RW5 are the best shape variables for distinguishing between these sibling species, although again none of them could discriminate between the three species at the same time. RW2 discriminated between *Littorina saxatilis* and the others, while RW5 discriminate between *L. compressa* and the others. Therefore, the thin plate spline representation of these variables does not afford a comprehensive diagnostic shape variable for all the species simultaneously (results not shown).

We also used a canonical discriminant analysis using the 31 derived shell variables (CS, U1 and U2, RW1–28) in females to compare the shell shape and size of the three species simultaneously. Starting with all the variables, we used the stepwise method in order to find those which significantly contributed to the discrimination (16 out of 31; not shown). The discriminant index successfully distinguished between the three species (Wilks' $\lambda = 0.566$; $\chi^2 = 173.434$, df = 15, $P < 0.001$). The first canonical discriminant function explained 54.2% of the morphometric variance (canonical correlation = 0.690) and the second canonical discriminant function explained 45.8% (canonical correlation = 0.659). With this method, we found that 80.6% of the original cases were cross-validated correctly into their species groups (85.6% for *L. arcana*, 78.1% for *L. saxatilis* and 74.5% for *L. compressa*).

In addition, we did a second set of discriminant analyses for the different pairs of species in sympatry, to see if the diagnosis of sibling species is improved when only sympatric populations are considered. In this analysis the percent of 'grouped' cases correctly classified by cross-validation for each of the pairs of species within locality averaged 96% (range between 85.7% and 100%; Table 3). These results obtained demonstrate that, comparing pairs of species that live in sympatry, a correct diagnosis is practically ensured. As we have mentioned above, similar analyses were used to diagnose *L. saxatilis* and *L. arcana* males

Table 2. Results of the two-way ANOVA on derived shell variables, centroid size (CS), two uniform (U1 and U2) and the eight main nonuniform estimates (RW1 to RW8) of shell shape for $\alpha = 0$.

Measure	Sp	Locality	Sp × Locality	Error
CS	61.5^{ns}	251.2^{*}	15.9^{*}	3.8
U1	0.9^{ns}	3.5^{*}	0.2^{*}	0.001
U2	0.1^{ns}	0.001^{ns}	0.001^{ns}	0.001
RW1	1.7^{ns}	1.5^{ns}	0.4^{*}	0.1
RW2	1.3^{*}	0.9^{*}	0.1^{ns}	0.1
RW3	0.7^{ns}	0.7^{ns}	0.1^{ns}	0.1
RW4	0.001^{ns}	0.2^{ns}	0.1^{*}	0.001
RW5	0.4^{*}	0.001^{ns}	0.001^{ns}	0.001
RW6	0.1^{ns}	0.1^{ns}	0.1^{*}	0.001
RW7	0.2^{ns}	0.001^{ns}	0.01^{*}	0.01
RW8	0.01^{ns}	0.001^{ns}	0.001^{*}	0.01

This included the factor *taxon* (fixed; *Littorina arcana*, *L. saxatilis* and *L. compressa*), the factor *locality* (random; Watwick, Westdale and Pettico Wick) and the interaction *taxon* × *locality*. Mean squares (×100) and their one-way ANOVA significance are shown for the factor *taxon*. ns is nonsignificant. $^{*}P < 0.05$.

Table 3.

where they were sympatric. Such application of the method allows us to use such diagnosis as *a priori* classification of male shells for a new round of discriminant analyses.

For the male only data set the discriminant index successfully distinguished all the three species (Wilks' $\lambda = 0.711$; $\chi^2 = 69.47$, df = 9, $P < 0.001$). The first canonical discriminant function explained 81.7% of the morphometric variance (canonical correlation = 0.804) and the second canonical discriminant function explained 18.3% (canonical correlation = 0.538). With this method we found that an average of 76.3% of the original cases were cross-validated correctly into their putative species groups (75.6% for *L. arcana*, 67.2% for *L. saxatilis* and 85.3% for *L. compressa*). The efficiency of this discrimination was only slightly lower than in the female data set (above). The diagnosis of sibling species was also improved when only sympatric populations were considered (Table 3). In this analysis the percent of 'grouped' cases correctly classified by cross-validation for each of the pairs of species within locality averaged 96% (range 92.6–100%; Table 3). These results show a nearly identical correct diagnosis in males and females.

The distribution of female-averaged values for discriminant scorings of function 1 (x-axis) and discriminant scorings of function 2 (y-axis) from different species (H and M morphs included in *L. saxatilis*) and localities are plotted in Figure 4. This graphic representation shows the absolute diagnosis capability of this method when populations rather than individuals are used.

We also investigated the shell size and shape differences between two ecotypes (H and M) of *L. saxatilis*. The multivariate analysis of two factors (ecotype and locality), using all shape components, showed significant differences in shape between ecotypes (Wilks' $\lambda = 0.044$; $F = 21.649$; $P < 0.001$), localities ($\lambda = 0.25$; $F = 3.002$; $P < 0.01$) and the interaction ($\lambda = 0.271$; $F = 2.687$; $P < 0.01$). The factor *ecotype* explained the largest amount of the variation in shape ($\eta^2_{\text{Ecotype}} = 0.956$; $\eta^2_{\text{Locality}} = 0.750$ and $\eta^2_{\text{Interaction}} = 0.729$). Shape components, however, were apparently affected by allometry, as size accounted for 58.8% of the overall variation in shape under multivariate regression ($\lambda = 0.412$; $F = 1.38$; $P = 0.194$). Using a two-way univariate ANOVA for factors *ecotype* and *locality*, only centroid size and two shape variables (U1 and RW1) showed significant differences between ecotypes (results not shown). These differences between ecotypes for U1 and RW1 remained significant in all cases using ANCOVA (when corrected for the covariable centroid size; $P < 0.001$). We did not find differences between localities for any of these two variables. This trend is graphically represented in Figure 5, where a clear differentiation between the two ecotypes is observed for the uniform U1 and RW1 components (H ecotypes present larger U1 and smaller RW1 values than M ecotypes).

The interpretation of the RW1 variation, representing local variation, can be carried out using the interpolating function (thin-plate splines) describing shape change in RWA (Fig. 6). The H ecotype presented the most negative deformations, while the M showed the most positive ones (Fig. 5, 6). The external landmarks (LM1, LM2, LM3, LM4, LM16, LM6, LM17, LM10, LM11, LM12, LM9, LM13, LM14 and LM15) and those that best represent the aperture (LM5, LM6, LM17, LM10 and LM7) were connected by lines for an easier visualization of the meaning of RW1 deformations in each ecotype. RW1 can be mainly described as variation in the relative size and shape of the aperture as well as an approximation to the size of the body half a whorl before the aperture. This figure shows how the H ecotype presents a relatively bigger aperture, and accordingly, a smaller region to accommodate the snail within

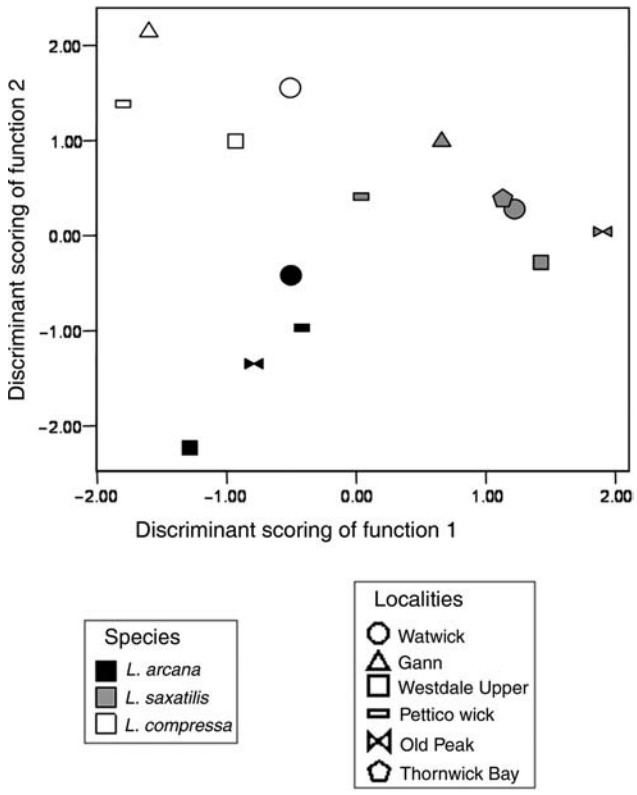

Figure 4. Discriminant function scores for 14 population averages representing three species from six localities using the 31 derived shell variables (CS, U1 and U2, RW1–28).

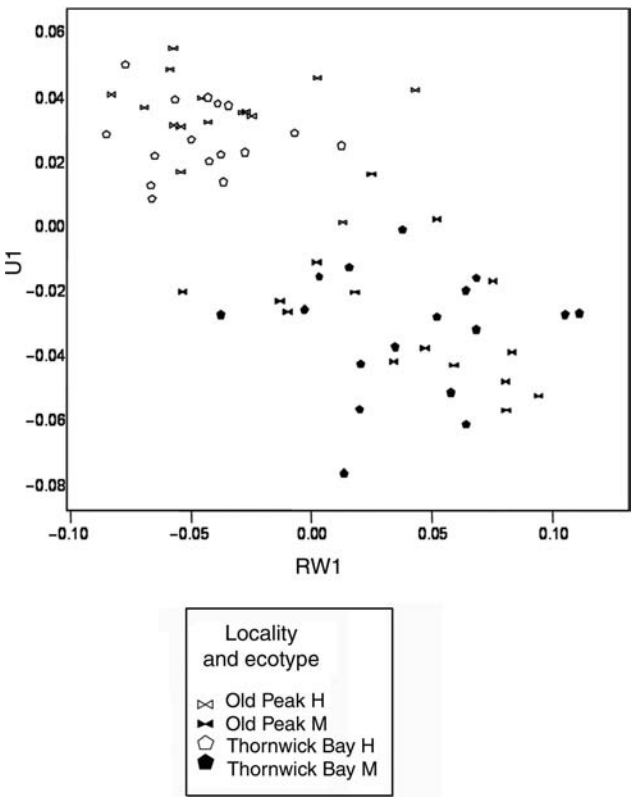

Figure 5. Values for the first uniform component (U1) and the first nonuniform component (RW1) of shell shape, for each ecotype and locality obtained with $\alpha = 0$.

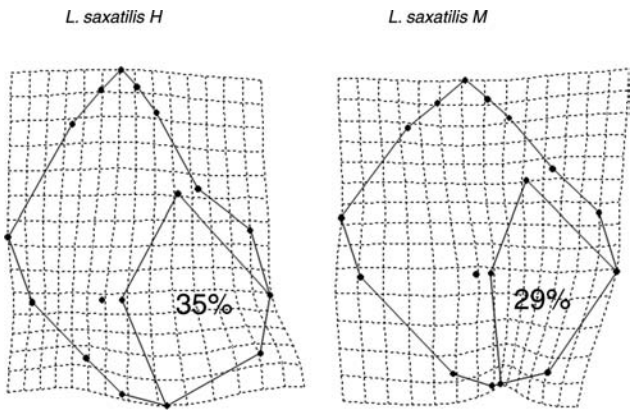

Figure 6. Thin-plate spline representation, from the TPSRELW software (Rohlf, 1998), showing the most extreme positive (M) and negative (H) deformation of the landmarks (within the 95% confidence interval within each group) with respect to the reference configuration (using $\alpha = 0$). Some landmarks 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 H ecotype.

the shell than the M ecotype. This is also in agreement with the differences in U1, which show that the M ecotype is flattened compared with the H ecotype.

DISCUSSION

The geometric morphometric method used here allows considerable differentiation of populations and specimens of the sibling species of British rough periwinkles, based solely on their morphometric variability on shell size and shape. The method is very secure for distinguishing pairs of species that live in sympatry (with average successful diagnosis of 96%). These findings are broadly consistent although more efficient than another study comparing some of these species (e.g. *Littorina saxatilis* and *L. arcana*; Caley *et al*., 1995). In fact, geometric-morphometric methods were also more efficient in finding shape differences between sympatric ecotypes of *L. saxatilis* (Carvajal-Rodríguez *et al*., 2005) compared to a previous study using 13 shell distances (Johannesson *et al*., 1993). We are not aware of a study comparing distance and landmark methods on the same set of shells. Where such an approach has been used (e.g. with cichlid fish; Parsons, Robinson & Hrbek, 2003), geometric methods gave overall more accurate classification into groups. From a practical perspective, the efficiency of the method applied here makes it potentially useful for the design of field or laboratory experiments. It is possible to obtain accurate morphological diagnosis and mark specimens of these two species for use in behavioural experiments or released and studied *in situ* at the field. Application of the method would depend on discriminant functions calculated using the experimental populations actually to be used, as many factors (seasonality, other localities, particular laboratory conditions, for example, were not tested in this study) could affect to the efficiency of the method. An example of such application has been done here for *L. saxatilis* and *L. arcana* males. The diagnosis was apparently as good as with the females that can be diagnosed *a priori* based on anatomical information (Reid, 1996). So as an example, we could use our males (which can be easily sexed and photographed alive) for any behavioural experiment carried out at the laboratory with minimal confounding of these two sibling species in sympatry.

We could also characterize the main differences in shell shape between the extreme ecotypes H and M of *L. saxatilis*. Shell morphology, behaviour and life history characteristics are known to differ between these two ecotypes (Hull *et al*., 1996; Pickles & Grahame, 1999; Grahame *et al*., 2006), but traditional methods left unresolved the relative magnitudes of contribution of size as such, and shape. Our use of geometric morphometric methods, however, has revealed not only significant variation in size, but also in both components of shell shape, and this new approach also allows an intuitive and objective interpretation of shell shape variation (see Fig. 6). The differentiation in shell shape affected both uniform and nonuniform components of shell shape, the first uniform (U1) and nonuniform (RW1) components show that the M ecotype has a more globose shell than the H. This may be due to the need for a more globose and robust shell in the M in order to resist crab attacks, which are common in the lower-shore (Johannesson, 1986; Wilding *et al*., 2001; Grahame *et al*., 2006). In addition, the first local component of shell variation showed that they differ in the relative size of their shell aperture. It is known that these two ecotypes are associated with different degrees of wave exposure, H types are associated with more exposed habitats, while M ecotypes are associated with more protected habitats and more frequent crab predation. The differences in RW1 between these two ecotypes match the previous interpretation, (see above) as the larger aperture found in the H specimens is needed to accommodate a large foot necessary to avoid being dislodged by waves. On the other hand, the smaller aperture observed (related to the shell profile) in the M specimens protects the animal from crab predation. Similar morphological variation is also evident in response to environmental gradients among Galician ecotypes of *L. saxatilis*, RB and SU (Carvajal-Rodríguez *et al*., 2005; Conde-Padín *et al*., 2007; Rolán-Alvarez, 2007). In Galician, *L. saxatilis* ecotypes the relative size of the aperture has been experimentally related to the ability of the snails to attach to the substratum (Rolán-Alvarez, Johannesson & Erlandsson, 1997), as well as to the corresponding large differences in muscular foot mass existing between these ecotypes (Rolán-Alvarez, 2007). However, the microexposed and sheltered sites are inverted in these two geographical regions (in Galicia exposed habitats are found at the lower shore site), because of the particular exposure gradients and kind of rocks.

We do not yet know to what extent shape differences between H and M ecotypes are maintained by natural selection in a classical polymorphism, versus being partially or even totally the result of phenotypic plasticity. The adaptive explanations above would apply to either circumstance, or to their combination. We know that these ecotypes show genetic differentiation at a microgeographical scale (Wilding *et al*., 2001; Grahame *et al*., 2006), but little is known in this case about the genetic components that affect the shell traits. However, in the Galician ecotypes the amount of additive genetic variation (incompatible with phenotypic plasticity) estimated in the wild and in laboratory was at least about 60% of the phenotypic variability, and the pattern of ecotype differentiation for size of shell aperture was in agreement with strong natural selection maintaining such polymorphism (reviewed in Rolán-Alvarez, 2007). In summary, in this species, additive genetic polymorphism should be maintained as the most probable explanation for the variation between H and M unless new data contradict this.

In summary, our results suggest that even in species with nonplanktonic dispersal capabilities, which easily can adapt to local conditions, there are some characteristics of the shell morphology that remain unchanged in each species, allowing its diagnosis. This success in diagnosis was refined by geometric morphometric methods, which represent a fundamental improvement both for statistical detection of subtle differences between groups and for interpreting in biological terms the patterns of shell shape variation.

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