An example of the application of geometric morphometric tools to the morphological diagnosis of two sibling species in *Nassarius* (Mollusca, Prosobranchia)

Un ejemplo de aplicación de la herramienta de morfometría geométrica en el diagnóstico morfológico de dos especies gemelas en *Nassarius* (Mollusca, Prosobranchia)

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ABSTRACT

Geometric morphometric methods have made possible the study of shape and size independently. Here we have used uniform and non-uniform shape variables to study shell shape in two sibling species of *Nassarius* from NW Spain. The study revealed that the main shape difference between *N. nitidus* and *N. reticulatus* is that the former is more globular than the latter, a characteristic that is concordant with visual inspection. This method allows the use of multivariate and univariate statistical analysis on shell shape, as well as facilitating the biological interpretation of the shape variables obtained, and may thus be a fundamental tool for similar studies in taxonomy and evolution.

RESUMEN

Los métodos de geometría morfométrica han hecho posible el estudio de la forma y el tamaño independientemente. Aquí hemos usado las variables de cambio de forma uniforme y no uniforme para estudiar la forma de la concha en dos especies gemelas de *Nassarius* del NO de España. El estudio reveló que la principal diferencia de forma entre *N. nitidus* and *N. reticulatus* es que la primera es más globular que la segunda, una característica que está en concordancia con la impresión visual. Este método permite el uso de análisis estadístico univariable y multivariable sobre la forma de la concha, así como facilitar la interpretación biológica de las variables así obtenidas, y por lo tanto puede resultar una herramienta de gran utilidad para similares estudios de taxonomía y evolución.

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INTRODUCTION

Definition and measuring of shape is not an easy task. Traditional morphometrics used distances and ratios to characterize morphology of the shell in many species (JANSON AND SUNDBERG, 1983; JOHANNESSON, 1986; MILL AND GRAHAME, 1995; CRUZ, ROLÁN-ALVAREZ AND GARCÍA, 2001). However, such an approach has some well-known deficiencies (reviewed in BOOKSTEIN, 1991). First, selection of variables is very arbitrary and it can importantly affect the results obtained. Second, direct distances are typically highly correlated with the overall size of an organism, making it impossible in practice to study size and shape separately. Finally, even if ratios (between informative distances) were used as an attempt to correct the effect of size, such an approach has its own statistical problems (see SOKAL AND ROHLF, 1996:17). Therefore,it was evident that classical methods were rather imperfect to single out size and shape components from general morphology.

It was Sir D'Arcy Wentworth Thompson who first proposed depicting changes in shape by using reference deformations of an organism superimposed on a grid (see BOOKSTEIN, 1991). However, a general approach to solve this measuring quantitatively does not appear until shape could be described by the coordinates of a set of well-defined points or landmarks (BOOKSTEIN, 1991). Such landmarks link the geometry of the organism, the mathematics of deformation and biological inference. Thus, a set of coordinates of homologous points from a group of individuals that have been adequately superimposed, i.e. translated, rotated and scaled to match one another by some reasonable criterion, can be used to compare and contrast their shapes. Then, differences in shape among individuals will be directly characterized by differences in these coordinates. The main aim of this paper is to introduce the taxonomist and marine ecologist to the use of geometric morphometric techniques, as they have supposed a revolution in morphometric

studies (ADAMS, ROHLF AND SLICE, 2004). Other alternatives to classical morphometric analysis s (based on index and ratios) are also known (see MACLEOD, 1999; RICHTSMEIER, DELEON AND LELE, 2002). Recently, the application of geometric morphometric methods to the study of shell morphology in *Littorina saxatilis* has opened the possibility of employing it as standard methodology for morphological studies in molluscs (CARVAJAL-RODRÍGUEZ, CONDE-PADÍN AND ROLÁN-ALVAREZ, 2005). Here, we apply this technique to a well known pair of sibling species of *Nassarius* to characterize their differences in shell shape.

There has been considerable confusion in the taxonomy of the genus *Nassarius* from Atlantic shores (as an example, see RÖDING, 1798; BUCQUOY, DAUTZENBERG AND DOLLFUS, 1882; LOCARD, 1887; COEN, 1933; NOBRE, 1940; MONTEROSATO, 1912). Although *Nassarius reticulatus* (Linnaeus, 1758) and *N. nitidus* (Jeffreys, 1867) have been considered by some of the former authors as sibling species (JEFFREYS, 1867), the taxonomic consensus up to the early 1990s (CERNOHORSKY, 1984; FRETTER AND GRAHAM, 1984; SABELLI, GIANNUZZI-SAVELLI AND BEDULLI, 1990; POPPE AND GOTO, 1991) was to consider only *N. reticulatus* as a valid species. ROLÁN AND LUQUE (1994) were the first to show that both were valid species, based on the comparison of museum types, as well as on new morphological and behavioural differences detected in laboratory conditions studying two sympatric populations. They found that natural populations of *N. reticulatus* live preferentially along open shores and bays, while *N. nitidus* lives nearly exclusively in bays at NW of Spain. They also found both species living in sympatry in a few localities. SANJUAN, PÉREZ-LOSADA AND ROLÁN (1997) demonstrated the taxonomic status of these species by detecting diagnostic genetic (allozymic) differences in sympatry. A similar result has been observed with mtDNA variation in the same populations (R. Barreiro, com. pers.).

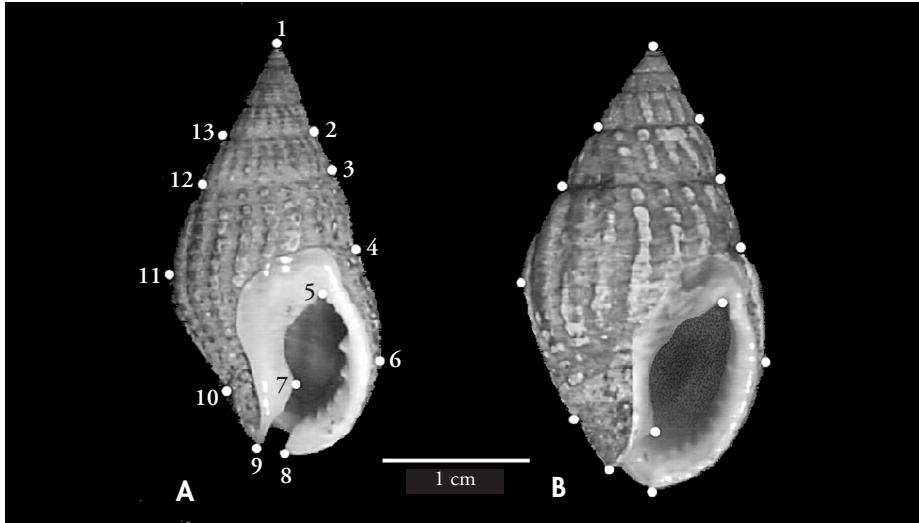Thus, although there are diagnostic differences between these species in

Figure 1. Photo of *N. reticulatus* (A) and *N. nitidus* (B) in the established position for obtaining the shell measurements from the 13 landmarks (LMs) used (see text).
Figura 1. Fotografía de N. reticulatus *(A) y* N. nitidus *(B) en la posicion establecida para obtener las medidas de las 13 coordenadas (LMs) usadas (ver texto).*

individual shell characteristics (shell colour, profile, external lip, columellar callus, etc.; ROLÁN AND LUQUE, 1994), shell shape differences between these species have not been formally demonstrated yet. In fact, the shell shape of both species is apparently rather similar (see Figure 1). Our interest in this study was twofold. First, to characterize shell shape in these two sibling species in sympatry, interpreting them in biological terms if possible. Second, to provide a representative example for taxomists and marine ecologists, that helps to apply this technique to similar cases. Our data demonstrate that this technique can be of general utility as a diagnostic tool to differentiate sibling species just in terms of shell shape (see also JOHNSTON, TABACHNICK AND BOOKSTEIN, 1991; CARVAJAL-RODRÍGUEZ *ET AL.*, 2005).

MATERIAL AND METHODS

Adult specimens of both *N. reticulatus* and *N. nitidus* were obtained from San Simón (Ría de Vigo, Galicia, Spain) in 1992 and 1996. In this locality each species lives in different habitats, but usually buried in sand, sometimes under algae. All specimens within an area of a few square metres were captured following the methods described by ROLÁN AND LUQUE (1994), and taken to the laboratory. Then a subsample of 20 adult snails (larger than 20 mm) for each species (40 in total) was chosen for morphometric studies.

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, always with the specimens in the same position (with the axis of the shell on the y-axis and the aperture on the same plane as the objective (Fig. 1) (CARVAJAL-RODRÍGUEZ *ET AL.*, 2005). Shell variables were obtained using 12 landmarks (LM) as shown in Figure 1. LM1 represents the apex of the shell; LM2 and LM3 are placed on the right border of the profile of the shell on the suture located at the beginning of both the penultimate and the last whorl,

Table I. Shape variables, percentage of variance explained and mean values and standard deviations within species. ANOVA check if there is a significant difference between species. ANCOVA check if the differences between species for the same variables are maintained in spite of size differences.
Tabla I. Variables de la forma, porcentaje de la varianza explicado y valores medio y desviaciones estandar entre especies. El test ANOVA comprueba si hay diferencias significativas entre especies. El test ANCOVA comprueba si las diferencias entre especies para una variable se mantienen pese a las diferencias de talla.

	U1	U2	RW1	RW2	RW3	RW4	RW5	RW6
Variance Explained	79%	20%	56%	10%	7%	6%	4%	4%
N.reticulatus Mean	-0.015	-0.002	-0.038	-0.001	-0.001	-0.001	-0.002	-0.001
(std)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
N. nitidus Mean	0.015	0.002	0.038	0.001	0.001	-0.001	,002	0.001
(std)	(0.02)	(0.10)	(0.03)	(0.02)	(0.02)	(0.01)	(0.01)	(0.01)
ANOVA	52.3^{***}	1.9^{ns}	126.8^{***}	0.1^{ns}	0.2^{ns}	0.2^{ns}	1.1^{ns}	0.2^{ns}
ANCOVA (size)	25.7^{***}	0.1^{ns}	65.2^{***}	0.1^{ns}	0.1^{ns}	0.1^{ns}	1.5^{ns}	0.1^{ns}
			ns is non significant, *** $p < 0.001$					

respectively; LM13 and LM12 are the same points on the left side, respectively; LM4 is on the lower suture near the end of the last whorl; LM5 is on the upper part of the internal profile of the aperture; LM6 is the most external position on the external part of the outer lip; LM7 is on the most internal left side of the siphonal channel; LM8 and LM9 are placed on the most external point of the siphonal channel aperture on the right and left sides, respectively; LM10 is the confluence of the external siphonal channel with the last whorl; and LM11 is the most external point on the last whorl (the periphery) at the left profile of the shell. Further details of the methods used are explained in CARVAJAL-RODRÍGUEZ *ET AL*. (2005).

For each specimen, centroid size (estimating shell size) and uniform (affine transformations, for example deformations which leave the sides of a square parallel) and non uniform (non-affine) components of shell shape where obtained. The estimation of shell shape components was accomplished by aligning the raw coordinates of the specimens using the procrustes generalized orthogonal method (GLS; see ROHLF AND SLICE, 1990), with the scaling option of $\alpha = 0$ (see further details in CARVAJAL-RODRÍGUEZ *ET AL*., 2005). We will not present here formal definitions or descriptions for these shell variables. Such information is clearly given in the book of ZELDITCH, SWIDERSKI, SHEETS AND FINK (2004). Here we will focus on the biological interpretation of these variables.

Centroid size is thus one of the best measures of size of any object independently of its shape (BOOKSTEIN, 1991). The two uniform variables (U1 and U2) describe variation affecting all landmarks simultaneously, after being corrected for scale, rotation, and superimposed at the same position (ZELDITCH *ET AL*., 2004). An interpretation in geometrical terms of the differences at the uniform components is, however, not so simple (see ZELDITCH, SWIDERSKI, SHEETS AND FINK, 2004). In our experience with snails U1 has been typically associated with compression/dilation deformations (CARVAJAL-RODRÍGUEZ *ET AL*., 2005; this study). On the other side, the local component of variation gives rise to different variables that can explain orthogonal non-uniform components of the shape. When those non-uniform shape components are pre-
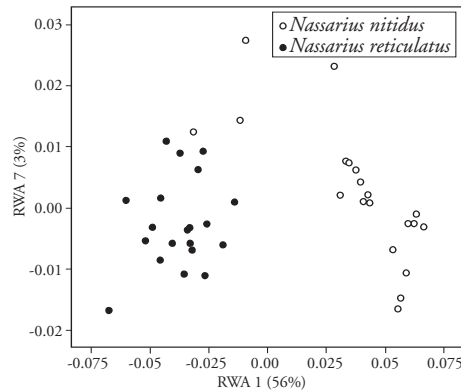
Figure 2. Representation of the two main local shape components (RW1 and RW2) in all specimens studied. The shape component RW1 is nearly diagnostic between the two species in these populations (see Table I).

Figura 2. Representación de los dos componentes locales principales de la forma (RW1 y RW2) en todos los ejemplares estudiados. El aspecto del componente RW1 es casi diagnóstico entre las dos especies en estas poblaciones (ver Tabla I).

sented relative to the overall shape information of the sample, as when using principal component analysis, the relative warps are produced (see ZELDITCH *ET AL*., 2004). The relative warps can be used as classical morphometric variables in statatistical univariate and multivariable analysis and tests (ZELDITCH *ET AL*., 2004). Furthermore, there is a possibility to represent the geometric meaning of the relative warps, using the thin-plate spline representation method (see ZELDITCH *ET AL*., 2004). This gives the researcher the possibility of interpreting an *a priory* abstract shape component in biological terms as we will show in this study.

To get the landmark data in practice, we used digitalized photos of the shells as in Figure 1, and using the software TPSDIG (http://life.bio.sunysb.edu/morph/morphmet/tpsdig2w32.exe) we obtained the coordinates for the different landmarks in the whole data set. Statistical calculations to obtain centroid size and uniform and non-uniform shape components from landmark data were performed with the program TPSRELW, developed by ROHLF (1998) and available at http://life.bio.sunysb.edu/morph/morphmet/tpsrelww32.exe.

Classical parametric tests were computed by the SPSS/PC package version 12.0.1.

RESULTS

Shell variation was decomposed in both size (centroid size: CS) and shape components. The two uniform shape components (U1 and U2) explained 79% and 20%, respectively of the uniform components of shape (after correcting for rotation and size effects). The variables from the local shape component, the relative warps, explained a decreasing amount of the local variation (see the main six RW in Table I). Table I shows the univariate differences between species for those variables. Such analysis, however, assumes that size and shape components are independent (they do not show allometry). In our case, however, size still explained a significant amount of variation in shape (64%) using multivariate regression (Wilks' Lambda = 0.36; F = 7.04, P < 0.001). However, the former differences between species are still detected correcting for size differences using an analysis of covariance (Table I). In

summary, we have detected differences in shape components irrespective of the differences in size between our two species. This is very convenient as differences in size can depend considerably on specimens available in the sample, especially when there is large within species variation in age or size in the field. The only limitation of the method is that we reduce our conclusions to the larger age/size classes in the species.

Finally we quantified the degree of separation between our two groups using shape variables by a step-wise discriminant analysis. We observed significant differences between species for the discriminant function (Canonical correlation = 0.93; Wilks' Lambda = 0.13; ^{2} = 74.4, d.f. = 3; P < 0.001). A cross validation statistical analysis was also accomplished to verify the efficiency of the discrimination. All *N. reticulatus* and almost all (except one) *N. nitidus* specimens were properly classified *a posteriori* (more than 95% of success). Three shape variables contributed to the discriminant function (RW1, RW5 and RW7). In fact, the most efficient variable for discrimination was RW1 (see Figure 2). In Figure 3 the thin plate spline representation allows us to interpret in geometric terms the positive and negative deviations values for RW1 between the two groups. The results graphically support the visual differences in shell morphology that can be observed between two representative specimens of these species. The advantage of RW1 is that we can use a formal statistical variable which summarizes the main differences in shape (after correcting for size and rotation effects) between the two species.

DISCUSSION

We have shown that geometric morphometric methods can be used to find significant differences in shape between two sibling species of *Nassarius*. In this case the technique succeeded in discriminating in practice specimens of these two species just in terms of shell shape. This could be fundamental if one is interested in doing manipulative experiments directly in the field with a pair of sibling species, when diagnostic morphological traits are no available. In that case, the two species could be theoretically determined by geometric morphometric methods and marked alive for future recapturing and monitoring. In addition, this method can be a valuable general tool to study and characterize shell and morphological differences in shape, with the possibility of interpreting shape in biological and simple terms. In the populations studied here, the main shape differences can be summarised in one shape variable RW1 (Figure 3). Briefly, the figure shows that *N. nitidus* has a more globular shell shape than *N. reticulatus* (notice that this is a similar interpretation to the uniform differences observed for U1; see Table I). This result is very intuitive as can be observed when visually comparing this two species (Figure 1), showing the good statistical properties of this approach. A similar result has been found when studying different ecotypes of *Littorina saxatilis* (see CARVAJAL-RODRÍGUEZ ET AL., 2005; HOLLANDER, LINDEGARTH AND JOHANNESSON, 2005). CARVAJAL-RODRIGUEZ ET AL. (2005), compared the efficiency of geometric-morphometric methods with the use of classical distances and ratios, and observed that the employment of landmarks considerably increases the chance of finding significant differences in shape between two groups. Furthermore, even if classical principal component analysis made on many distance measurements (obtained from landmark data) can show similar statistical power to detect differences in morphology between two groups, that method can not safely discriminate between size and shape. Moreover, the significant principal components detected are difficult to interpret in biological terms. In summary this technique has the main advantage of being an easy way to interpret in biological terms the differences between our two species.

N. reticulatus
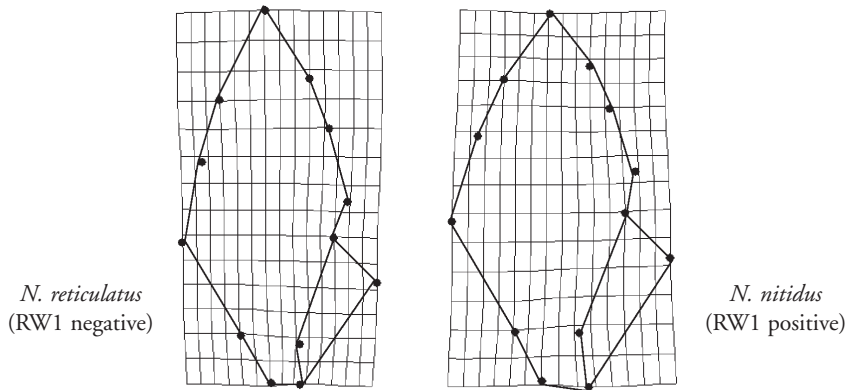(RW1 negative)

N. nitidus
(RW1 positive)

Figure 3. Thin-plate spline representation, from the TPSRELW software (Rohlf, 1998) for showing the most extreme positive and negative deformations of the landmarks with respect to the reference configuration for the main local shape component (RW1). Negative deformations are characteristic of *N. reticulatus* and positive ones of *N. nitidus* (see Figure 2).
Figura 3. Representación de "gradillas de deformación" del programa TPSRELW (Rohlf, 1998) mostrando la interpretación de las desviaciones más positivas y negativas del primer componente local de forma (RW1) respecto a la configuración de referencia. Las deformaciones negativas son características de N. reticulatus *y las positivas de* N. nitidus *(ver Figura 2).*

Some caution is needed, however. As expected from geometrical and mathematical theory, all shape components should be analysed or interpreted together (ZELDITCH *ET AL.*, 2004). This statement has some practical foundation as well, because as with principal component analysis, the percentage of local variation explained by RWs is negatively correlated with the number of such relative warp. For example, in our data set the RW1 explained more than 50 % of the overall local variation in shape, and this percentage decreased to 10% for RW2 or even to 4% for RW6. A common sense argument would suppose that any shape difference between our species would be biologically more relevant and statistically safer if it were based on those relative warps that explain the largest amount of shape variability.

The alternative position would be to avoid the use of any single relative warp just because of theoretical considerations (ZELDITCH *ET AL.*, 2004). Such limitation, if followed strictly would suppose the loss of the main advantage of this new method and would limit its future utility.

We believe that individual variables of shell shape can be extraordinarily useful to understand in biological terms how species are responding in evolutionary terms to environmental changes. The researcher should check in practice the real statistical importance of its geometric variables as well as check experimentally the possible errors of the technique (see CARVAJAL-RODRÍGUEZ ET AL., 2005) before attempting to try a more detailed, and more interesting, biological interpretation of shape differences.

We would like this work to contribute to the application of this new methodology to classical taxonomic and ecological studies in marine and terrestrial molluscs.

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