Inner city of Graz (mausoleum).

Contributions
to the
2nd workshop "Methods in Ostracodology"

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Geometric Morphometrics and its use in ostracod research: a short guide

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Abstract: Three decades ago, geometric morphometrics transformed the way biometry was done. There was a revolution in concepts and techniques that, nevertheless, was deeply rooted in historical grounds. The traditional multivariate approach merged with methods that enhance visualization of changes in the biological form inspired in the proposals of D'ARCY THOMPSON. Meanwhile a growing body of shape theory lent support to the new methodology. RICHARD BENSON, ROGER KAESLER and RICHARD REYMENT, well-known ostracodologists, soon realised the significance of, and made relevant contributions to, the new approach. Geometric morphometrics, however, never became a standard for daily ostracodologist's work. And that even when ostracods exhibit plenty of features (hard carapace, high diversity, worldwide distribution, rich fossil record, etc) that makes them ideal organisms for geometric morphometric analysis. This paper, which has been prepared within the framework of the course "Methods in Ostracodology-2" (Graz, 2011), aims to present a practical sketch, a short walk through the main issues in Geometric Morphometrics. But, beyond introducing some widely used methods (e.g., Procrustes analysis, relative warps, outline-based methods), we provide the reader with many general references that might help anyone to go deep into this methodology, and a nearly exhaustive list of references specifically dealing with ostracods from a geometric morphometrics perspective.

Key Words: Geometric Morphometrics; Ostracods; Landmarks; Outlines; Shape analysis.

1. Introduction

In 2008, Prof. W. PILLER and Dr. M. GROSS organised the first "Methods in Ostracodology" (MIO) Workshop to which we both were kindly invited as lecturers. At that time one of our talks started with the following quote by ROGER L. KAESLER (1983): "*We have told each other so often and with such force and such eloquence of the uses to which the study of ostracodes has been applied that we have overlooked one startling fact: almost no one uses ostracodes for anything.*"

That statement is nearly 30 years old, but it still reflects quite accurately the state-of-the-art in ostracodology. Disturbing as it is, we always considered KAESLER'S account a call for action, a request for us, ostracodologists, to do better science and to better communicate our achievements to scientists in other fields, so that many people will end up using ostracods for as many scientific purposes as we soundly claim. That was the motivation behind our landing into the field of morphometrics applied to ostracodology that occurred in the late nineties (BALTANÁS & GEIGER 1998). Definitely we were neither pioneers – as some others paved our way before – nor mavericks – as other colleagues have independently dealt with ostracod morphometrics too – but had a well-defined commitment from the very beginning: persuasion through example. By practically showing how powerful and informative some morphometric techniques are, that was our idea, ostracodologists will include such methods into their scientific "toolbox", expand their areas of research and, most important, produce much and better quantitative scientific information in the field. In order to fulfil that aim we published some papers (a list is provided in the "Bibliography" section), addressed talks at GSA (Seattle, 2003), EOM-V (Cuenca, 2003), ISO-15 (Berlin, 2005) and EOM-VI (Frankfurt, 2007), participated in the first MIO Workshop (Graz, 2008), and produced some free software for morphometric analysis (Morphomatica v.1.6; LINHART et al. 2007). Do we succeed? Not at all, as can be deduced from the sporadic occurrence of morphometric analysis in ostracod literature. Should we give up? Absolutely not because (a) as image technology improves and computer software becomes increasingly available, the use of morphometric techniques is more accessible and easily applicable for everyone; (b) ostracodologists are now better trained for using a quantitative/numerical approach in their research; and (c) … we assumed things take time.

Accordingly we want to present again, hopefully in a more convincing manner, arguments in favour of the regular use of morphometric techniques in ostracod research. Given the large amount of literature describing the basics and methods of Geometric Morphometrics, and the many studies that have applied them to a number of issues in a wide variety of organisms, this paper does not pretend to be a comprehensive account of morphometrics but a useful summary for the beginner, especially the ostracodologists who pretends to apply this techniques in his/her daily scientific work. That means that simplicity and generality will be favoured along the text, and that many complex details that populate this methodological approach will be lost. Nevertheless, plenty of references will be provided for all those wishing to dive deeper into the waters of morphometrics.

Of course, special attention will be provided to papers that already applied those methods to the study of ostracods. Although we tried to keep the whole story as balanced as possible, there might be some bias towards non-marine ostracods because of our own experience. We apologize for that.

The paper is organised in three sections. First section aims to introduce the basics of morphometric analysis, including core concepts, motivations, and main approaches (i.e., Traditional vs. Geometric Morphometrics), underlining the adequacy and potential of those methods for ostracodology. Second section deals with a summary of main methods in the realm of Geometric Morphometrics, including references to those papers in the ostracod literature that have dealt with each technique. Third section is a brief reminder that multivariate numerical methods are still playing a leading role in morphometric analysis. Once shape descriptors have been gatherer using different methods, we still need to analyse their similarity and variability, and to test for statistically significant differences. Finally, there is an appendix with a non-exhaustive, but hopefully useful, account of available software for performing morphometric analysis and websites where to find relevant information on the topic.

2. Not in the eye of the beholder

Ostracod species recognition is mainly done on the basis of morphological features (e.g., MEISCH 2000) [for some stimulating thoughts on morphology see WEBSTER & GOODWIN (1996), MCGHEE (1999) and KELLER (2002)]. Certainly, other sources of information, like molecular analysis, slowly add to the traditional morphological approach either for dealing with phylogeny (YAMAGUCHI & ENDO 2003), ecology (ROSSI & MENOZZI 1990; WYSOCKA et al. 2000; ROSSI et al. 2004), biogeography (Rossi et al. 2010) or evolution (SCHÖN et al. 1998; TINN & OAKLEY, 2008; ADOLFSSON et al. 2010). The molecular approach has also contributed significantly to ostracod systematics by suggesting that currently recognised morphospecies can actually comprise extensive species complexes (BODE et al. 2010). Still, most of current ostracod taxonomy is predominantly morphology-based, and morphology is used extensively by ostracodologists in their non-taxonomic daily scientific work too.

Within such scenario one might presume that morphological features used by ostracodologists are abundant, reliable and universally accepted and recognised. Indeed, there are a lot of morphological traits in ostracod soft-parts (like use to happen with most arthropods), and the ostracod carapace is rich in morphological features too (hinge, pore canals, inner lamella, ornamentation, muscle scars, …; MOORE & PITRAT 1961; VAN MORKHOVEN 1962). True as that general statement is, we must also recognise that the number of operational morphological characters is frequently limited. For instance, palaeontologists working with ostracods have no soft-parts to study (except for some exceptional cases, e.g., SIVETER et al. 2003; WILLIAMS et al. 2008; SIVETER et al. 2010); and ostracodologists dealing with non-marine ostracods face poorly ornamented, less informative carapaces as compared to those in the marine realm. Even when both hard and soft parts are readily available and a huge number of morphological traits can be inspected, ostracodologists must severely limit the number of specimens to be studied because dissecting, mounting and examining an ostracod is a time-consuming process that cannot be afforded when addressing patterns that involve large numbers of individuals (like in population ecology or palaeoecological studies). Under such circumstances ostracodologists focus on carapace features, mainly overall aspect, and move away from the strict morphological approach to fall into the typological view, one that perceives phenotypic variation as a nuisance (Debat & David 2001), overstates the importance of subjective experience in the "art" of taxonomic identification, enhances idiosyncratic taxonomic propositions and precludes a truly evolutionary "populational thinking" (Rieppel 2006, but see Love 2003 for arguments that bring both viewpoints together). That scenario promotes observations like: "*There are no good characters in either valve or soft part morphology to characterize this subfamily, which must be considered a collection of species without special features*" (Meisch 2000: 278, with regard to the Eucypridinae); and "*In taxonomic descriptions of different species, different characters are diagnosed, while others may not even be mentioned. It appears therefore that either authors disagree on what characters are taxonomically important for the Cypridacea, or that such characters have not been established by ostracod taxonomists.*" (De Deckker 1979: 9, about the Cypridoidea).

Practice is one way to improve the whole situation, but as much as training enhances our awareness of the peculiarities and subtleties of ostracod (external) morphology, it does not guarantee unbiased and accurate information. Truth is not necessarily in the eye of the beholder. However, some methodologies can add consistency, objectivity and repeatability to our observations for the study of ostracods (for a more detailed discussion about objectivity in taxonomy see Danielopol et al., 2011). Morphometrics is one of such approaches.

Morphometry is not morphology but a school of morphologic analysis. It is the quantitative description, analysis and interpretation of shape and shape variation in biology (Rohlf 1990a). Although the term "morphometrics" seems to be rather modern (according Reyment 2010, it was coined by Robert E. Blackith in 1957), the discipline has deep roots in biology starting with the early biometricians Francis Galton and Karl Pearson (for a detailed historical account see Benson 1982a; Bookstein 1993, 1996, 1999; and Reyment 1996, 2010). Biometry focuses in covariances among sets of measurements of the biological form without regard to any geometric organization. Quite interestingly, most methodological developments within the biometric approach, including many univariate, bivariate and multivariate analysis, were far more successful in fields other than Morphology. All those methods, called Traditional or Conventional Morphometrics, have been extensively applied to the study of ostracods, and some of them are useful examples of the aims and limitations of that approach.

Traditional morphometric methods are based on the statistical analysis of a number of point-to-point measurements (distances, ratios or angles) presumed to reflect morphological characteristics of the organisms under study. An example of this approach is the bivariate plot of carapace length and height measurements (see Fig. 1) that have been frequently used for characterizing ontogenetic stages, sexual dimorphism and differences between ostracod species (e.g., Shaver 1953; Whatley & Stephens 1977; Heitkamp 1979; Smith & Martens 2000; Baltanás et al. 2004b; Smith & Kamiya 2003; Hunt 2007a).

As carapace length and height provide the simplest estimate of an ostracod overall shape, an increasing number of meristic measurements should allow a better characterization of its form. Following that argument, Tétart (1982) addressed the identification of different morphs among a series of populations of the non-marine species *Eucypris virens* (Jurine, 1820). Eighteen point-to-point distances were measured on 684 specimens from 27 localities, and a discriminant analysis was performed on the resulting data matrix to conclude that morphological variation in the shell is discontinuous (Tétart 1982).

Similarly, Kaesler & Lohmann (1976) measured ten morphological characters in the carapace of 103 specimens of *Krithe producta* Brady, 1880 and performed a principal component analysis to address the relationship between phenotypic variation and habitat depth.

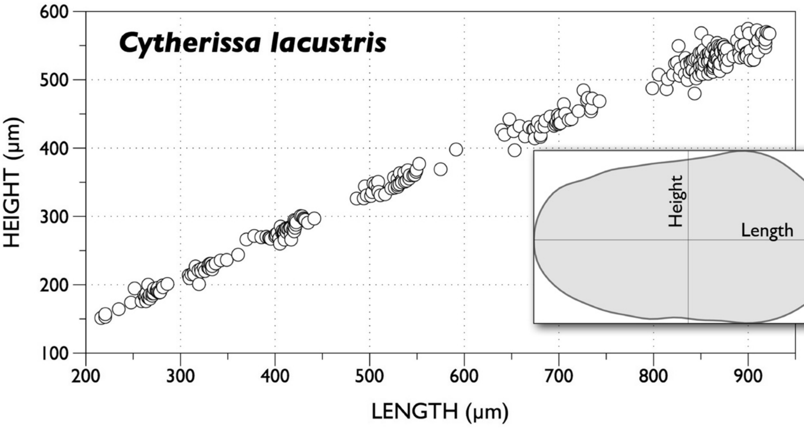

Fig. 1: Bivariate plot of carapace length vs. height for a number of specimens of *Cytherissa lacustris* retrieved from a sediment core from lake Sebino (Italy). Growing stages (A-5 to adults) can be easily identified and their phenotypic variation estimated.

More recently, Roberts et al. (2002) performed a correlation analysis between seven morphometric variables (valve area, perimeter, major axis length, minor axis length, height, elongation and roundness) and Sr/Ca-inferred salinity in Holocene samples of *Limnocythere inopinata* (Baird, 1843). Although ostracod carapace is most frequently used in quantitative morphological analysis, the same approach has been applied to soft-parts too (Maddocks 1988).

Nevertheless, and despite its large potential and extended use, traditional morphometrics also has some important limitations (Bookstein 1982; Manness & Kaesler 1987; Lestrel 1997):

a) it contains a large subjective element, with measurements (distances, angles or ratios) selected on the base of *ad hoc* criteria;
b) it does not preserve information of shape; i.e., it is not possible to recover the original form from the angles, distances or ratios that were measured on it;
c) all the variables used represent a very small percentage of the information displayed in the biological form; and
d) it has difficulties to cope with size and shape as different, although related, morphological features.

Accordingly, what we need is a less subjective method, capable of comparing shape independently of size and detecting subtle differences in shape, that preserves information of shape, and deals with shape as a whole (Fig. 2). Such method was anticipated by D.W. Thompson (1917) at the beginning of the XX Century as a graphical technique that analyses changes in the biological form as distortions of Cartesian coordinates systems.

But despite how appealing Thompson's proposal was, it remained purely graphical and so, of very limited use. Still, there is a very interesting application of Thompson's approach to ostracods, it is that of Kesling (1951) who cleverly tried the method to analyse growth in the freshwater ostracod *Cypridopsis vidua* (O.F. Müller, 1776). Although Kesling (1951: 107) was able to detect some heterogonic growth in the dorsal-posterior part of the carapace, he concluded that "*the graphic method gives false results in some instances*" and that "*caution is required in the evaluation of D'Arcy Thompson's method*".

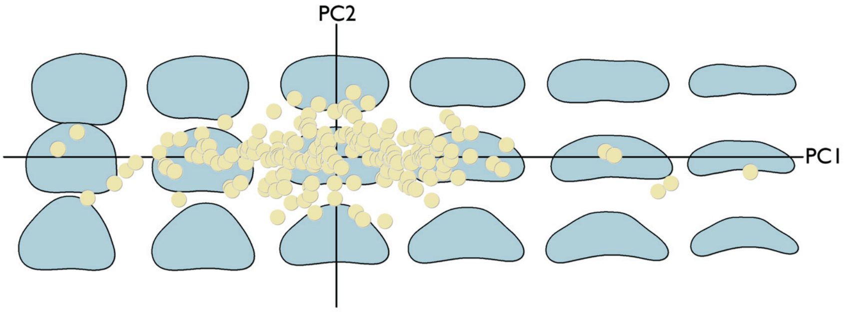

Fig. 2: Modern morphometric techniques preserves information of shape all along the analysis, thus allowing the recovery of the original shapes at any time or, like in this plot, the reconstruction of hypothetical shapes that correspond to exact locations in the morphospace. The figure, modified from Sánchez-González et al. (2004), displays shape variability in the Cypridoidea. Actual species (dots) are arranged according their outline shape (size removed), whereas outlines in the background show the hypothetical shapes in the corresponding areas of the morphospace.

However, things changed dramatically in the late 70s and along the 80s when both forms of biometric modelling, the multivariate that emphasizes the geometry of the covariance matrix of a set of arbitrarily chosen measurements and the one that focus on the direct visualization of changes in biological form, merged into what have been named Geometric Morphometrics and qualified as a revolution in morphometrics (Rohlf & Marcus 1993).

3. The Great Leap

The key elements of the new approach were the concept of shape and the way to capture information on shape so that it bears exhaustive geometric information that can be retrieved at any time throughout the study. Shape is redefined as the geometric properties of a configuration of points that are invariant to changes in translation, rotation and scale [for this and other definitions we strongly recommend the glossary compiled by Slice et al. (1996) which is kept updated at the Stony Brook website].

Now, imagine there is a photograph lying on the table and a glimpse is enough so that you can identify the object in it (Fig. 3).

Fig. 3: Can you recognize the object in this photograph?

Undoubtedly, it is the Eiffel Tower! However obvious it might be, you should recognize that any unbiased observer might be astonished with your statement given that the actual Eiffel tower (a) stands in the upright position (rotation), (b) is much bigger than this (scale) and (c) is placed far away, actually in Paris (location). Nevertheless, most people will endorse your verdict “It is the Eiffel tower, indeed”. That happens because when looking at the photograph our minds focus on shape ignoring all other attributes of the object in the photograph. In other words, our value judgement is based on shape attributes (the geometric relationships in a specific configuration of points) that are constant (= invariant) despite any change in scale, rotation or translation.

That definition of shape has deep concerns. First of all it emphasizes shape as a whole. Contrary to former approaches, shape is no longer a collection of arbitrary measurements that provide an indication on some shape/size related features but a unitary property of physical objects. However, if shape concerns “a configuration of points”, we need to identify which points do belong to such geometric configuration. Well, those points are the so-called landmarks. A landmark is any specific point on a (biological) form located according to some rule and claimed to be homologous from case to case (SLICE et al. 1996; Fig. 4).

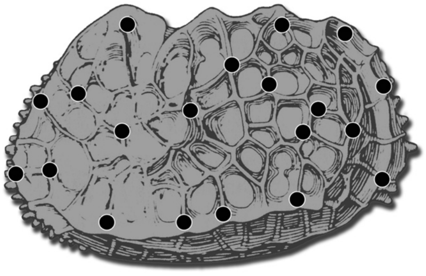

Fig. 4: Pore conuli canals identified as landmarks in the surface of *Bradleya albatrossia* BENSON, 1972, a marine Recent ostracod from the China (modified from BENSON 1982b).

Landmarks on a specimen can be anatomical structures (Type I landmarks; e.g., an eye tubercle or a pore canal), occupy similar geometric locations (Type II landmarks; e.g., the sharpest curvature at the rear part of an ostracod valve) or be defined with respect to some other landmark (Type III landmarks; e.g., the point on the valve outline further away from the muscle scar; BOOKSTEIN 1991). Different types of landmarks differ in the quality of the information they encode, especially with regard to homology. If coordinates recorded over a large number of specimens correspond to landmarks known to be homologous, i.e., known to reflect the position of structures with the same origin, the analysis of changes among landmark configurations will be more biologically meaningful.

Once the meaning of shape and how to record shape information using landmarks are defined, we still need to identify methods of data analysis that allow us to properly measure shape and shape changes.

Several of such methods have been described elsewhere (see Strauss & Bookstein 1982; Rohlf & Bookstein 1990; Bookstein 1991), but we shall briefly present here those that have been successfully applied to ostracods, superimposition based methods and relative warps analysis. However, ostracod valves do not always provide enough landmarks, or landmarks easy to record, to apply those landmark-based methods. For such situations a different approach, also summarised below, must be used: outline-based methods (see also Danielopol et al. 2011).

3.1. Superimposition Methods

Superimposition methods rely on a straight argument: the more similar two shapes are the better they should fit when superimposed one on another. Simple as it is, there are some operational difficulties when superimposing two shapes because any set of landmarks raw coordinates obtained from a biological object (like those on an ostracod valve) does not include information concerning shape only but size, orientation and location too (Fig. 5).

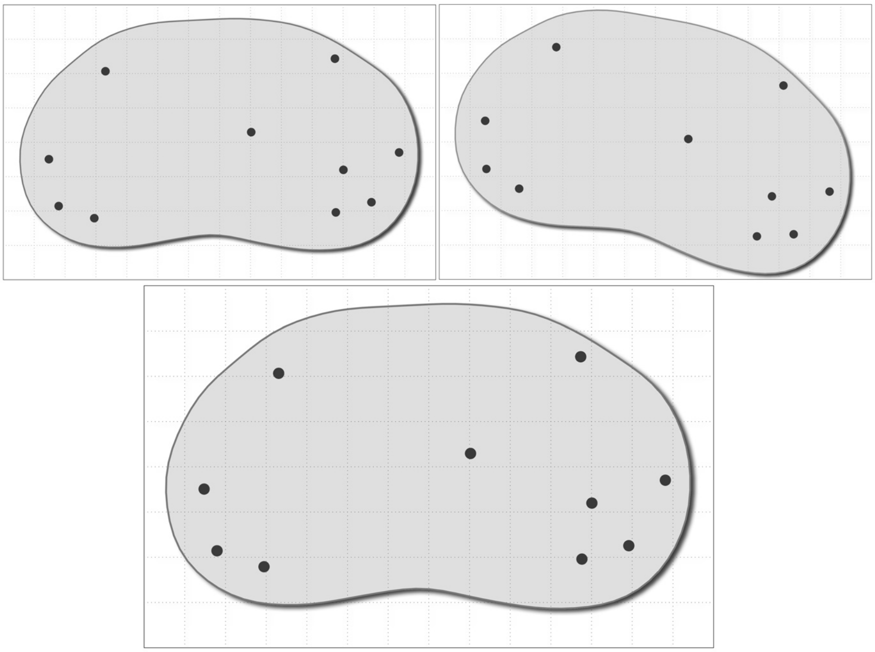

Fig. 5: All plots are from the same specimen (same shape); however, raw landmark coordinates will differ due to differences in rotation, location and size.

Accordingly, the first thing we must do is to properly remove non-shape information in order to make comparisons between specimens agreeable. Procrustes superimposition is an efficient way of doing that (for technical texts on Procrustes methods see GOWER 1975; KENDALL 1984; BOOKSTEIN 1986; ROHLF & SLICE 1990; GOODALL 1991; DRYDEN & MARDIA 1998; ROHLF 1999) [The method is named after Procrustes, a figure from Greek mythology that reputedly made his victims fit his bed by stretching or cutting off parts].

To put it simple, the aim of Procrustes superimposition is to minimize the sum of squared distances between landmarks in two configurations (sets of raw landmark coordinates). To accomplish that goal, one need to translate, rotate and scale one configuration to fit the other as good as possible. Of course, algebraic expressions exist to allow us compute the corresponding (rotation, location and scaling) parameters fast and easily (see the software section below). Sometimes, the scale parameter is not estimated through the least-squares method (full Procrustes analysis) but configurations are scaled to a common size instead (e.g., the ostracod carapace length)(partial Procrustes analysis). Once both configurations of landmarks have been superimposed (fitting each other as good as possible), the squared root of the sum of squared coordinate differences after superimposition is a measure of shape differences between configurations called “Procrustes distance”.

The procedure described above tackling the comparison of two shapes only is called “Ordinary Procrustes Analysis” (OPA). However, we are frequently interested in “Generalised Procrustes Analysis” (GPA), which involves the comparison of more than two shapes. A plausible way to do so is fitting all members of a sample to their overall mean, because the average shape seems to be a good reference for the whole set. Unfortunately, any “mean configuration” will be meaningful for shape comparisons just in case it summarises shape information only (i.e., if differences in landmark coordinates due to rotation, location or size has been removed already). The problem arises because those non-shape attributes cannot be removed without fitting the configurations to a reference. The circularity in the process is avoided by using an iterative process in which any specimen is initially selected to stand for the mean, all the configurations in the sample are fit to that reference, and a new mean is computed as the average location of individual landmarks (GOWER 1975; Fig. 6).

One of the strengths of Superimposition methods based on landmarks concerns the geometry of a space in which “Procrustes distance” is the metric of distances between points representing shapes. KENDALL (1984, 1985) has extensively studied the properties of that space (generally referred to as “Kendall’s shape space”) and provided sound theoretical basis for the analysis and interpretation of results obtained using Procrustes distances [SLICE (2001) expanded that study to the GPA]. One key result is that “Kendall’s shape space” (and the GPA space too) is non-Euclidean. Unfortunately any standard multivariate method (PCA, Discriminant Analysis, …) requires the raw data to be in a Euclidean space; but ROHLF (1999) and SLICE (2001) provide ways to solve the problem by projecting Procrustes coordinates into a linear (Euclidean) space tangent to Kendall's shape space.

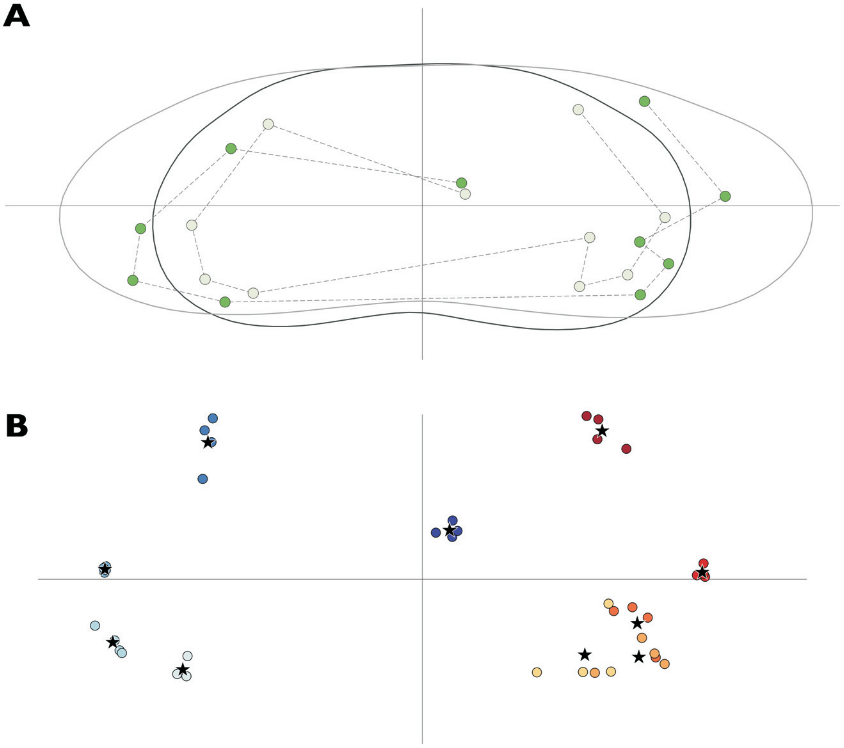

Fig. 6: An example of how Superimposition methods operate. (A) two hypothetical ostracod outlines and their corresponding sets of ten homologous landmarks. (B) Output of a Procrustean fit performed on a sample with four specimens (those plotted above plus two more). Open circles are Procrustean coordinates after scaling, rotation and translation of raw data (different colours are for sets of homologous landmarks across specimens); stars are for coordinates of the mean configuration.

Morphological analysis of ostracods played a significant role in the development of superimposition methods due to the giant figure of Richard H. Benson (Smithsonian Institution) who contributed significantly to this field (Benson 1967, 1975, 1976, 1981, 1982a, 1982b, 1983; Benson et al. 1982; Siegel & Benson 1982) [we strongly recommend you his paper "The Ontogeny of an Ostracodologist" written shortly before his death (Benson 2003)]. One of the main flaws of the generalised approach using mean configurations is that when differences between specimens are mainly located in one or few landmarks, the method spread such differences across all landmarks (the so-called “Pinocchio effect”; Chapman 1990). To overcome that problem, Siegel & Benson (1982) proposed a robust method (Resistant-Fit Theta-Rho-Analysis, RFTRA), a non-parametric analogue of the least-squares regression, that utilizes repeated medians (a detailed description of the method can be found in Rohlf & Slice 1990). For a recent application of Procrustes analysis to ostracods see Hunt (2007b).

3.2. Relative Warps Analysis

Relative Warps Analysis (RWA) is a method adapted for use in morphometrics by Bookstein (1989, 1991) that corresponds to a Principal Component analysis of the covariance matrix of the principal warp scores, which are different scales of a thin-plate spline transformation of landmarks. The thin-plate spline is a smooth interpolation function directly inspired in the approach sketched by D’Arcy Thompson (1917, 1942) for the analysis of changes in the biological form as distortions of Cartesian coordinates systems and is now a standard for visualizing shape change as a deformation (Zelditch et al. 2004). Bookstein (1989) explains the method using an idealised steel plate as metaphor, with one shape, described by its corresponding (x,y) landmarks placed in the plate, forced to fit another shape (in another plate) not by using displacements within the XY-plane but in the third dimension (Z). That fitting process will cause the plate to tilt, rotate and bend. For real metal plates, the larger the bending energy required to fit one shape onto the other the more different shapes will be. Similarly, thin-plate spline method computes a bending energy matrix that encodes the local aspects of shape differences with the capability of identifying both affine and non-affine transformations (Rohlf 1993). The principal warps mentioned above are the eigenvectors of that bending energy matrix; and their projection onto the X,Y-plane of the data result in the so-called partial warps (the components of the non-uniform deformation). The (orthogonal) partial warps define a shape-space in which all specimens in the sample are represented by a point. Thus, relative warps, the principal components of that partial warps, describe shape variation in a sample (Fig. 7). Every specimen of a sample is compared to the mean configuration and any shape changes along any axis of interest are visualised as deviations from the mean configuration. For a more detailed account of the method see Bookstein (1989, 1991), Rohlf (1993), Zelditch et al. (2004) and Slice (2005).

With regard to ostracod studies, RWA and thin-plate splines have been extensively used by Richard A. Reyment (Abe et al. 1988; Reyment et al. 1988; Reyment & Bookstein 1993; Reyment & Abe 1995; Reyment 1993, 1997), who also produced some brilliant historical accounts of the field of morphometrics (Reyment 1996a, 2010), and actively supported the morphometric approach among ostracodologists (Reyment 1995, 1996b).

His early association with R.E. Blackith (Blackith & Reyment 1971; Reyment et al. 1984) stimulated his interest in shape variation and the use of multivariate techniques in which he became a master (e.g., Reyment & Jöreskog 1993). Nevertheless, and despite his efforts, thin-plate splines have been applied to ostracod studies occasionally (Elewa 2003, 2004).

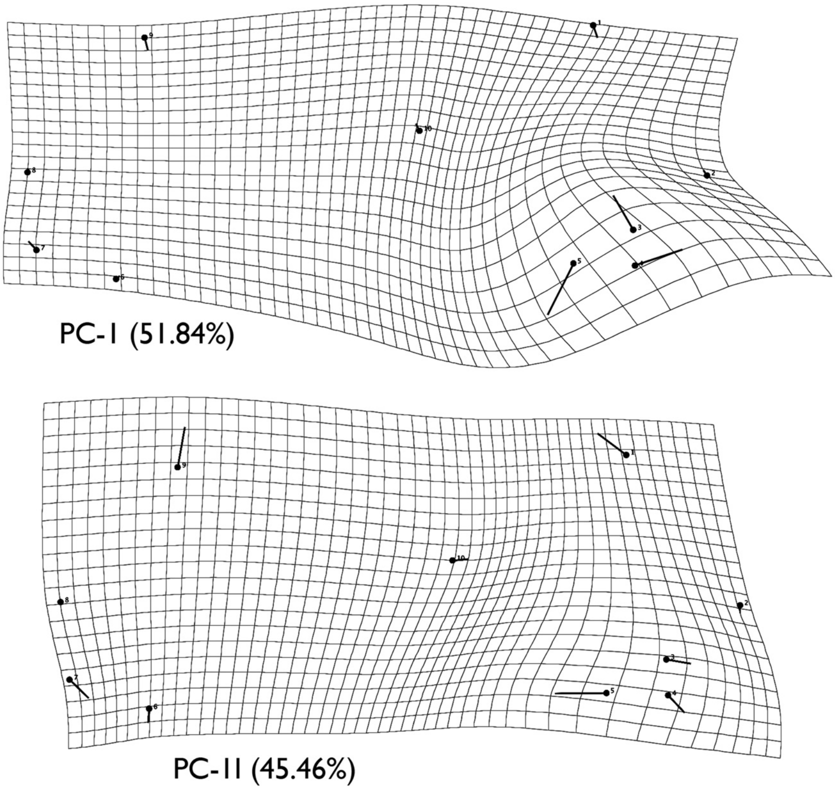

Fig. 7: Relative warp analysis (without weighting for bending energy and including the uniform component of shape variation) of the four hypothetical landmark configurations in figure 6. First and second principal components account for more that 97 % of total shape variability. Transformation grids help to visualize areas of greater deformation.

3.3. Outline Analysis

A main advantage of landmark-based methods is that they are firmly supported by statistical shape theory (e.g., Gower 1975; Kendall 1984, 1985; Small 1996; Dryden & Mardia 1998). Unfortunately, not all interesting biological objects have enough landmarks (sometimes they have no Type I landmarks at all) to make morphometric analysis using landmark-based methods feasible. In such cases, the shape can be captured by the coordinates of a sequence of points along the outline of the object (Rohlf

1990b; Fig. 8). Obviously, a list of outline coordinates is not an optimum way for describing or comparing shapes because of the large amount of redundant information in it (coordinates of points along the outline are not independent observations; the closer they are, the more correlated). A straightforward solution is to fit a function to the sequence of points and use the function parameters as descriptors of shape that can be then submitted to multivariate analysis. That is what outline analysis is all about.

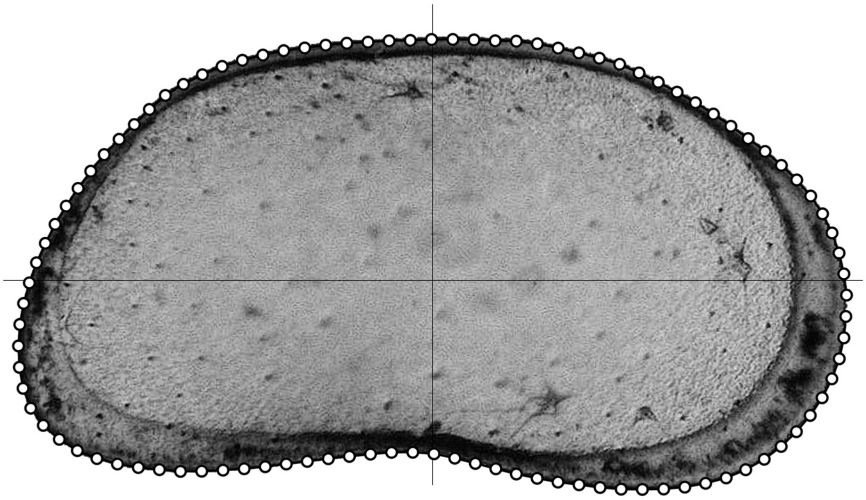

Fig. 8: A sequence of points along the outline can be used for morphometric analysis of ostracod shape when there are few or no landmarks.

There exist several techniques to fit functions to outlines (depending on whether those outlines are open or closed, or if the points along the sequence are equally spaced or not, and so on) but here we shall focus in three: Fourier analysis, eigenshape analysis and B-splines.

3.3.1. Fourier analysis

Fourier analysis, also known as spectral analysis or harmonic analysis, is a large subject area in mathematics that study the way general functions (curves) may be represented by sums of simpler trigonometric functions (the so-called harmonics). Given that any outline is nothing but a curve of varying complexity, one could decompose it into simpler functions using a Fourier transform (Fig. 9); the parameters describing that elementary functions being shape descriptors that can be compared among outlines (LESTREL 1997a). Well before the arrival of modern morphometrics, ROGER L. KAESLER, an outstanding ostracodologist at the University of Kansas, applied conventional Fourier analysis to several ostracod marine species. He first tested the method with a group of assorted ostracod species (*Xestoleberis* Sars, 1866, *Paradoxostoma* Fischer, 1855, *Leptocythere* Sars, 1925; Kaesler & Waters 1972) to apply it later to the systematic revision of 35 Macrocyprididae species (Kaesler & Maddocks 1984). Kaesler also explored other morphometric methods like eigenshape analysis (Schweizer et al. 1986; Maness & Kaesler 1987) and tensor analysis (a landmark-based based method) (Kaesler & Foster 1988). He also dealt with general aspects of shape analysis in ostracods (Foster & Kaesler 1988; Kaesler 1997).

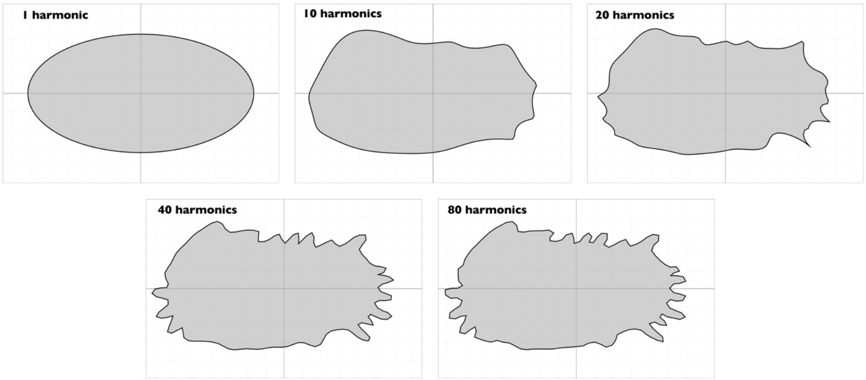

Fig. 9: The complex outline of the marine ostracod *Pterygocythereis ceratoptera* (Bosquet, 1852) is rebuilt using an increasing number of elliptic Fourier harmonics.

Additional examples of early applications of conventional Fourier descriptors to the analysis of ostracod shape are Burke et al. (1987), for the recognition of three closely related fossil *Candona* Baird, 1845 species, and Irizuki & Sasaki (1993), for the analysis of ontogenic changes in species of the marine genera *Baffinicythere* Hazel, 1967 and *Elofsonella* Pokorny, 1955. Ali Bachnou produced a series of papers supporting the use of conventional Fourier series (Bachnou 1999), including the analysis of outlines of Hemicytherinae (Bachnou et al. 1999) and Trachyleberidinae (Bachnou et al. 2000) ostracods, and a Visual Basic program, WANMOPRH, to run that kind analysis (Bachnou 2004).

Despite how useful they are, conventional Fourier descriptors have a series of requirements that limit its application to objects with relatively simple contours: the Fourier function must be single-valued (i.e. the outline cannot crossback into itself), the data must be set up in polar form, angles must be equal between vectors, and integration is required for the estimation of the Fourier coefficients (Lestrel 1997b). To circumvent those problems, Kuhl & Giardina (1982) formally described a parametric technique called Elliptic Fourier analysis (EFA) which has the following advantages (although at the expense of an increase numerical complexity): equal intervals between points in the outline are no longer required, the method can deal with complex curves, and functions are generated using an algebraic approach, not an integral one, thus saving computation time. Right after being proposed, EFA successfully extended to the biological context (Rohlf & Archie 1984; Ferson et al. 1995) and continue to be used extensively since then (e.g., Liu et al. 1996; McLellan & Endler 1998; Arnqvist 1998; Innes & Bates 1999; Loy et al. 2000; Cannon & Manos 2001).

The first application of Elliptic Fourier analysis to ostracods was the study of within- and among-population shape variability in two non-marine ostracod species, *Eucypris virens* and *Limnocythere inopinat*a (Baltanás & Geiger 1998). Afterwards it has been employed to assess morphological phenotypic plasticity within a clone of *Heterocypris barbara* (Gauthier & Brehm, 1928) (Alcorlo et al. 1999), ontogenic shape changes and patterns of shape variability in sexual and asexual populations of *Eucypris virens* (Baltanás et al. 2000, 2002), relationships between biogeography and disparity within the genus *Cryptocandona* Kaufmann, 1900 (Baltanás et al. 2004b), patterns of morphospace occupation in the Cypridoidea (Sánchez-González et al. 2004), links between carapace morphology and longevity in trachyleberidid ostracodes (Liow 2006), developmental trajectories in several non-marine species (Danielopol et al. 2008a) and the relationship between ecological factors and carapace shape in the Cytheroidea (Tanaka 2009).

3.3.2. Eigenshape analysis

Eigenshape analysis is an outline-based method originally developed by G.P. Lohmann (1983), a marine paleoecologist at Woods Hole Oceanographic Institution. The method uses the phi-star [$\Phi^*(l)$] angular function algorithm of Zahn & Roskies (1972) for describing the object's outline. The phi-star is the normalized net angular change in direction at each step around the perimeter (l) of the ostracod carapace shape or, like in a treasure map, the set of instructions (including heading and number of steps) for covering the whole outline; it is an exact description, independent of size, of how a shape differs from a circle (Lohmann 1983). Once phi-star functions are defined for series of points along the outlines in a sample of objects, they are submitted to an eigenanalysis that decomposes the covariance matrix into its eigenvalues and eigenvectors, these latter considered to be empirical shape-functions (and, hence, called eigenshapes; Lohmann 1983, Lohmann & Schweitzer 1990). The first eigenshape is approximately an average of all the shapes in the sample, whereas the second eigenshape represents the principal dimension of shape variation about that average, the third eigenshape represents the next dimension, and so for (Fig. 10).

Although Lohmann (1983) originally suggested to perform the eigenanalysis on the correlation matrix, it is now generally accepted that such procedure has the undesired property of normalizing each object's angularity and the use of the covariance matrix is favoured instead (Rohlf 1986; Schweitzer & Lohmann 1990; MacLeod & Rose 1993). Rohlf (1986) also demonstrated the close connections between this approach and that of Fourier analysis; indeed, the results obtained with eigenshape analysis are identical to those of Fourier analysis of Φ^* followed by a principal components analysis. Finally, a highly recommended paper that provides plenty of details about the computation and interpretation of eigenshape analysis plus a generalisation of that procedure for open, three-dimensional curves is MacLeod (1999).

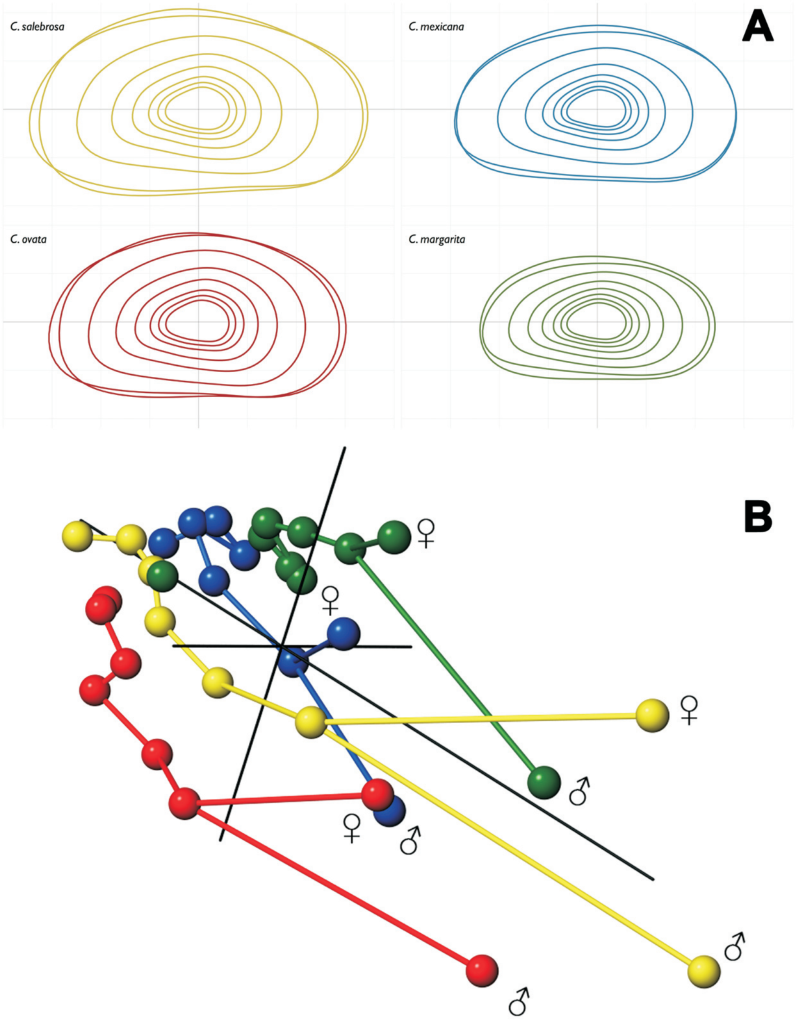

Fig. 10: Shape variation among species of *Cyprideis*. (A) Outlines of the average of each instar stage (including adult males and females). (B) Eigenshape analysis of Φ^* shape functions. Outlines have been standardised for size but instars still arrange along the first eigenshape according their ontogenetic state, showing the allometry of growth in *Cyprideis* species. Data from P.N. Schweitzer (see Schweitzer & Lohmann 1990).

Eigenshape analysis was first applied to the study of shape change in planktonic foraminifers (Lohmann 1983), but focus on ostracods followed immediately after and have remained active since then (Schweitzer et al. 1986; Maness & Kaesler 1987; De Wever et al. 1989; Schweitzer & Lohmann 1990; Majoran 1990, Crasquin-Soleau & De Wever 1993; Majoran et al. 2000; Elewa 2003; Liow 2006).

3.3.3. B-splines analysis

Curves (shape outline) can also be represented by splines that are but sets of polynomial curves of low degree that smoothly fit together. Each elementary curve is defined by a control point, and the list of all geometrically meaningful control points describes the whole curve (Brauneis et al. 2006). "Bezier curves", probably the best-known method in this category, has been seldom used in morphometry (see Loy et al. 2000; Chi et al. 2003 for some applications to the study of biological forms). Nevertheless there is another splines-based technique that works similarly to Bezier curves but requires fewer control points for curve description and is well-established in computer aided geometric design, B-splines method (De Boor 1978; Hoschek & Lasser 1993). Recently, Johann Linhart (University of Salzburg) and his collaborators have adapted that method to the analysis of ostracod outlines (Bayer et al. 2002; Baltanás et al. 2003; Brauneis et al. 2006; Neubauer 2007; Neubauer & Linhart 2008, Danielopol et al. 2008b) and have also produced "Morphomatica", a free software for running all the computations (Linhart et al. 2006). In their approach, ostracod outlines are translated (so that the centroid lies in the origin), oriented (using one of the main axes of inertia which is indeed the major axis of the ellipse described by the first harmonic in an elliptic Fourier analysis), and scaled (using a scaling factor like centroid size or outline area), and the elementary polynomial curves best fitting the outline, with their corresponding control-points, estimated (Fig. 11). The vectors between the control-points of several forms reflect the degree of disparity between them and overall shape similarity can be measured as the quadratic mean of the (Euclidean) distances between corresponding sets of control points. A number of studies have already used and tested this technique that showed up as helpful for shape analysis of poorly ornamented ostracods (Iepure et al. 2007; Minati et al. 2008, Danielopol et al. 2008a, Gross et al. 2008; Stracke et al. 2008a, 2008b). But beyond the already proved capabilities, the most interesting potential of B-splines analysis concerns future developments. Can control-points be considered a kind of pseudo-landmarks so that the full range of landmark-based techniques can be applied? That is an appealing possibility that could boost the use of the B-splines approach; especially, as compared to the use of semilandmarks (Sampson et al. 1996; Bookstein 1997), because B-splines properly summarises ostracod shape with a small number of control-points (sixteen are frequently enough for most non-marine species).

Finally, a word concerning homology is worth here as it has been a key concept when discussing about the advantages and limitations of outline analysis as compared to landmark-based methods. Some critics enhanced the supposed lack of true homology between shape descriptors used in outline methods (e.g., Fourier coefficients) and, consequently, qualified any a posteriori analysis based on them as inadequate (e.g., BOOKSTEIN et al. 1982; BOOKSTEIN 1994). Those statements were relevant to morphometric analysis in many fields but especially in systematics and phylogenetics, and boosted the interest in the concept of homology itself and its consequences for morphometric applications (RAY 1992; ZELDITCH et al. 1995; MACLEOD 1999, 2002) with some even suggesting that evolutionary novelty is best analysed using analyses of outlines or surfaces that do not require a correspondence of structures between members in a sample (POLLY 2008; but see KLINGENBERG 2008).

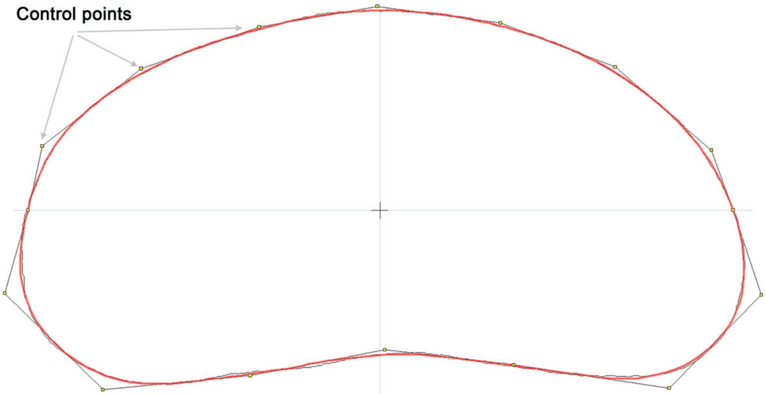

Fig. 11: An ostracod outline is here described with the polynomial curves defined by sixteen control points that were estimated using the B-splines method. Cartesian coordinates of those control points can be further used as shape descriptors.

4. Making sense of numbers

The aim of morphometric methods is to study biological shape and shape variation. That means that once we have collected shape descriptors of the biological objects under scrutiny using any of the techniques summarised above, we still need to analyse those sets of descriptors in order to find regularities and structures within. That is basically done with the same numerical techniques, most of them multivariate, already applied in traditional morphometrics (BLACKITH & REYMENT 1971; REYMENT 1991; MARCUS 1993). Among the plethora of numerical methods available [like Principal Coordinate Analysis (PCO, PCoA or MDS), Nonmetric Multidimensional Scaling (NMDS), Factor Analysis (FA), multivariate regression, Partial least squares (PLS), MANOVA, etc.] we will briefly sketch here three most frequently used, complementary methods, one in each of three main categories of numerical analysis: Principal Component analysis (Ordination methods); Canonical Variate Analysis (Group Discrimination); and Disparity measures (Characterization of morphological diversity). More information on those methods can be found in Blackith & Reyment 1971; Reyment 1991; Reyment & Jöreskog 1993; Marcus 1993; Manly 1994; Legendre & Legendre 1998; Everitt & Dunn 2001; Zelditch et al. 2004; Strauss 2010 and in the highly recommend PalaeoMath 101 columns by Norman MacLeod from the Palaeontological Association Newsletter [http://www.palass.org/modules.php?name=palaeo_math].

4.1. Principal Component Analysis (PCA)

Consider that you have described specimens in a sample with the corresponding sets of shape descriptors (e.g., Procrustes coordinates, Fourier coefficients, …). Any attempt to explore the structure (similarities, differences or groupings) within the data is fruitless because any plot we draw can only include information from three (out of many) shape variables. Even more, such a plot is not only inaccurate but a distorted representation of reality because it assumes the variables are independent (axes are orthogonal) whereas correlations between shape variables are likely to exist. A way to overcome those difficulties is by redistributing total variance in the raw data onto a set of mutually orthogonal axes (eigenvectors) that are arranged according the amount of variance each of them explain (eigenvalues).

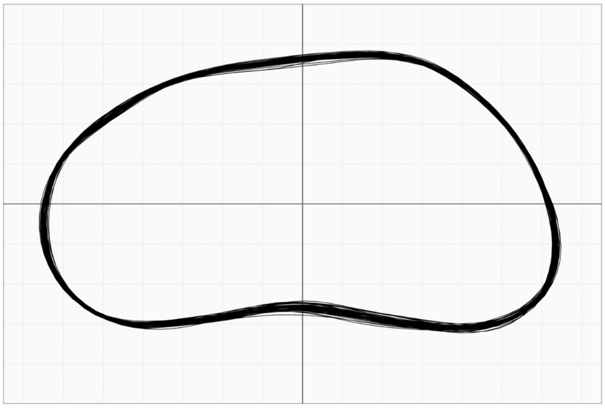

Fig. 12: Outlines of right and left valves of thirty specimens of *Candona neglecta* (adult females) sampled from lake Mondsee (Austria); 15 ostracods from the littoral (12 m deep) and the other 15 from the profundal (65 m deep). All 60 outlines were standardised for size, rotation and location and shape descriptors obtained through EFA and B-splines. Resulting shape descriptors (Fourier coefficients and control-point coordinates) were analysed using different multivariate methods (see below).

That method is called PCA and re-describes the patterns of variation among data. (That is the eigenanalysis approach used for eigenshape analysis; see Fig. 10).

Carapace shapes (2D lateral outline) of 30 specimens of *Candona neglecta* Sars, 1887 (adult females) from two localities in lake Mondsee (Austria; Fig. 12) were documented using two different kinds of shape descriptors: Fourier coefficients (obtained from an EFA) and control-points coordinates (obtained from B-splines). In order to visualize patterns of shape variation, a PCA combines linearly the original variables onto a new set of axes so that few of them already account for a large portion of total variability (Fig. 13).

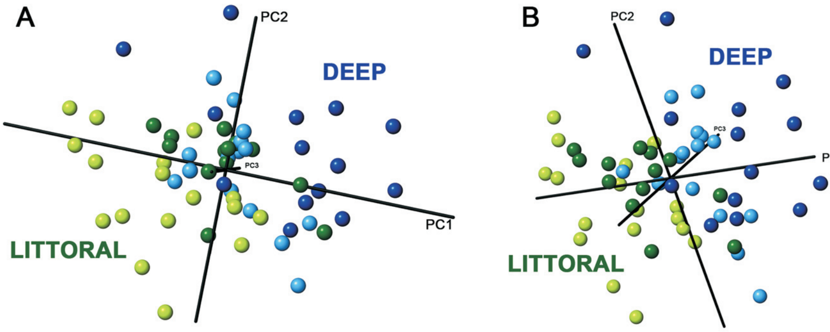

Fig. 13: Plot of the PCA performed on shape descriptors of sixty ostracod outlines from Mondsee (see Fig. 10). Shape descriptors are (A) Fourier coefficients (10 harmonics) obtained with an EFA; and (B) control-points (16) of a B-splines analysis. First three principal components explain 80 % (A) and 60.5 % (B) of total variance in the data. Blue dots: deep sample (light blue = right valves; dark blue = left valves). Green dots: littoral sample (light green = right valves; dark green = left valves).

Specimens of *C. neglecta* do not seem to be randomly distributed within the morphospace, but arranged according the habitat where they live (deep vs. littoral). Are both populations significantly different in shape? That is an interesting question that cannot be solved using PCA because that method is not aimed to finding group differences. Instead we must look at methods specifically designed for discriminating groups, like the Canonical Variate Analysis.

4.2. Canonical Variate Analysis (CVA)

This method (called Discriminant Analysis when only two groups are involved, and CVA otherwise) uses the same basic strategy than PCA does; it rearranges total variance of raw data in a new set of variables (which are linear combinations of the original ones). However, CVA is not aimed to maximise overall variation in the data but variation among groups relative to that within groups. Obviously, groups are known before analysis (and defined by criteria other than shape), but the analysis provide us statistical evidence concerning whether or not groups significantly differ in shape. Figure 14 plots the results of a CVA performed on the same data set already used for the PCA, thus underlining the differences between both approaches.

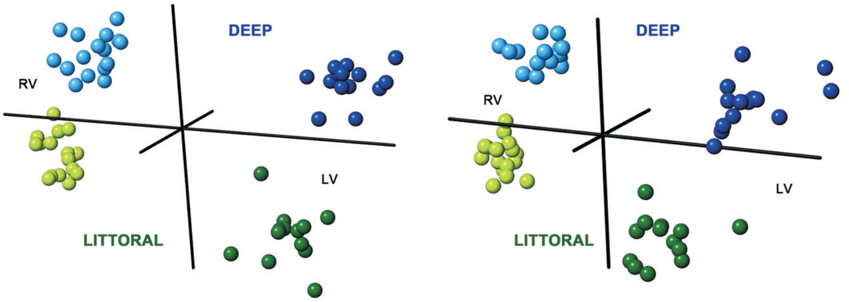

Fig. 14: Plot of the CVA performed on shape descriptors of sixty ostracod outlines from Mondsee (see Fig. 12). Shape descriptors are (A) Fourier coefficients (10 harmonics) obtained with an EFA ($F = 14.04$, $p < 0.0001$); and (B) control-points (16) of a B-splines analysis ($F = 8.51$, $p < 0.0001$). Colour codes as in Fig. 13.

Now, we are closer to solve the question posed above about differences in shape between the littoral and the deep populations. To answer it we should simply submit the CV-scores to an analysis of variance (indeed, a MANOVA or multivariate analysis of variance). MANOVA uses a multivariate-F statistic to measure the heterogeneity among a set of multivariate centroid (groups) with respect to the pooled within-group covariance matrix (Fig. 14). Using either Fourier coefficients (from an EFA) or control-point coordinates (from a B-splines analysis) results are fairly similar. First, shapes of right and left valves differ significantly. This is a non-surprising result because carapaces in most non-marine ostracod are asymmetric (in *C. neglecta* left-valve overlaps the right one). It is nevertheless rewarding to confirm that morphometric methods easily detect such subtle differences. What is, nevertheless, beyond expectations is to find significant differences in shape between populations living at different depths. Because those differences are mainly related to the second axis, which explains a smaller amount of total variance than the first one, it can be inferred that such shape differences are even subtler than those between right and left valves.

4.3. Disparity Measures

Disparity is the amount of morphological diversity within a taxon (Briggs et al. 1992; Foote 1992a, 1996, 1997a). It is assessed by how a group of organisms occupies a morphospace, the multidimensional space that encompasses a taxon's morphological variation, adding a new dimension to understanding biodiversity (Foote 1997a; Ciampaglio, 2002; McClain et al. 2004). There has been significant progress in the quantitative measure of morphological disparity (Briggs et al. 1992; Foote 1992b, 1997a, 1997b; Wills et al. 1994; Ciampaglio et al. 2001, Gerber et al. 2008; Deline 2009) and many taxonomic groups (see Foote 1997 for references), including ostracods (Benson 1975; Benson 1976; Oertli 1976; Whatley & Stephens 1976; Benson 1981; Benson 1982b; Benson et al. 1982; Reyment 1982; Reyment 1985a; Reyment 1985b; Baltanás et al. 2002, 2004b; Sánchez-González et al. 2004) have been studied from the perspective of disparity.

Average pairwise distance, total variance, range of variation, the product of variances or ranges, nearest-neighbor are some of the many proposed disparity measurements (Wills 2001; Ciampaglio et al. 2001); however, there seems to be no single best estimate of disparity as different measures capture different aspects of disparity.

Just to illustrate the rationale behind disparity measures we bring here one of the most frequently used, Foote's overall morphological disparity (MD) (Foote 1993):

$$MD = \Sigma_i d_i^2 / (N - 1);$$

where d_i^2 is the squared Euclidean distance between object i from the group centroid and N is the total number of observations (= species number) (see Fig. 15).

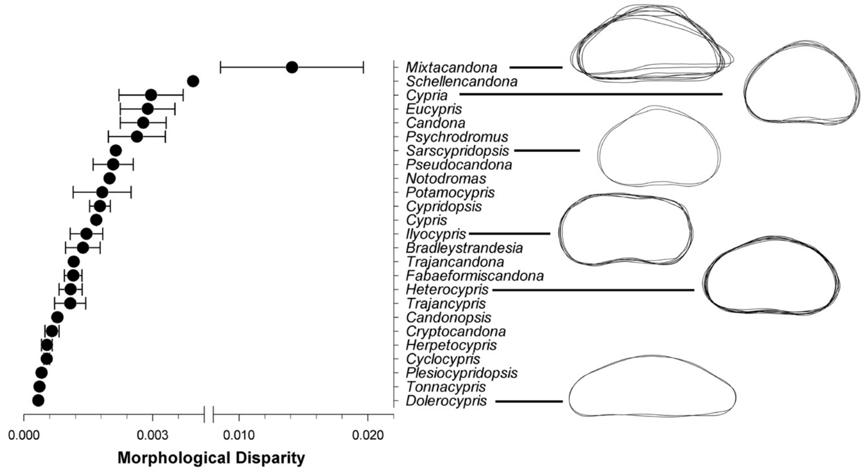

Fig. 15: Estimates of morphological disparity within cypridoidean genera (only European species included). Genera with one species only are not included as they have null disparity. Morphological disparity is measured as the mean squared Euclidean distance (within the morphospace defined by a PCA performed over EFA coefficients) from all members in a group (a genus) to the group centroid. Sets of outlines for some representative genera (*Mixtacandona*, *Cypria*, *Sarscypridopsis*, *Ilyocypris* and *Dolerocypris*) are displayed (from Sánchez-González et al. 2004).

Obviously, MD is measuring the volume of the morphospace that is occupied by members of a group. The more dissimilar the group members are (concerning shape), the farther they will be within the morphospace and that will result in larger MD values. There are two main advantages in using MD as index of morphological disparity; first, it is standardised for size and, second, it can be partitioned among its several component subgroups so that the contribution of each subgroup to total disparity, the so-called partial disparity (PD), can be easily computed (Foote 1993):

$$PD_j = \Sigma_i d_{ij}^2 / (N - 1); \text{ where } MD = \Sigma_j Pd_j$$

Partial disparity is also not dependent on subgroup size or variability within the subgroup but on the location of its members with respect to the overall centroid. Subgroups with a low number of members can contribute significantly to the group-MD if they have shapes that greatly differ from the group mean shape. Subgroups peripherally distributed in the morphospace have larger partial disparities than those placed close to the group centroid. It is like having hundreds of circles in a painting; a single square will add more morphological disparity to the whole group that any number of new circles we could add.

5. Conclusion

Morphometrics is no panacea, but a good complement in the toolkit of any ostracodologist. It can provide accurate, objective numerical information on ostracod carapace at the population level so that variation due to environmental cues or genetic drift can be assessed. It also helps the morphology-based species recognition process, and can be used in systematics and phylogenetics as well. Instruments for recording landmark and/or outline data are widely available nowadays, as is software for analysing those data. There is plenty of literature that provides information on the “know-how” of morphometric analysis, concerning both the technicalities and the theory behind. Ostracods already proved to be excellent material for morphometric analysis. No sophisticated (=expensive) image recording devices are needed because ostracod size is large enough for a standard microscope. The population/statistical approach is also not a problem because ostracod samples frequently include tens to hundreds of specimens. The group is diverse in species, geographically widely distributed and rich in fossil record. So, what else do we need? Apparently nothing but ostracodologist’s will to move forward.

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Appendix: Software for Morphometric analysis

The Morphometric Website at SUNY Stony Brook contains lots of information useful to anyone interested in Morphometry (including news, bibliographic references, software, announcements, etc). It is actively maintained since nearly two decades by F. JAMES ROHLF. [http://life.bio.sunysb.edu/morph/]

TPS range (by F. JAMES ROHLF) and excellent series of free compiled programs (Windows) aimed for morphometric analysis, from data acquisition (tpsDig) to superimposition methods (tpsSuper) and Thin-plate splines (tpsSplin, tpsRelw, tpsTree, …). Downloadable from the morphometrics website at SUNY Stony Brook.
[http://life.bio.sunysb.edu/morph/]

R . – R is a language and environment for statistical computing and graphics suitable for both basic users and developers, and can run on most operating systems (Windows, Linux, or Apple OS). R is freely distributed over the Internet and gathers the achievements of the "R core development team" and numerous contributors of online available packages. It is highly flexible and evolvable; suitable for beginners and for the advanced user that can develop and modify his/her own or programmed functions. Finally, there is a book explaining how to use R to perform morphometrics (CLAUDE 2008).

Morphomatica – A user-friendly computer program designed and written for the morphometric analysis of ostracod outlines using the B-splines approach. It was developed during 2001–2007 by J. LINHART, W. BRAUNEIS and W. NEUBAUER (University of Salzburg) with the biological background of DAN. L. DANIELOPOL (Austrian Academy of Sciences; see BRAUNEIS et al. 2008).
[http://palstrat.uni-graz.at/methods%20in%20ostracodology/Morphomatica.zip]

MorphoJ (KLINGENBERG 2008) – MorphoJ is an integrated program package for doing geometric morphometrics for both 2D and 3D data. MorphoJ is available for free under the Apache License, Version 2.0.
[http://www.flywings.org.uk/MorphoJ_page.htm]

Morpheus et al. (Slice 2008) – Multiplatform software for morphometric analysis. Dennis Slice is indeed developing a new version of this software that will be released under the name “new Morpheus et al.”.
[http://www.morphometrics.org/morpheus.html]

morphologika 2.5 (O'Higgins & Jones 2006) – Morphologika version 2.5 (November 2007) is a set of integrated tools for examining size and shape variation among objects described by configurations of landmark coordinates. These tools provide for 2 and 3 dimensional visualisation of shape and shape differences. “morphologika” is no longer maintained or developed. It is now replaced by the EVAN (European Virtual Anthropology Network) toolkit.

PAST (PAleontological STatistics) – A general statistics free software package (Windows) including some modules for basic morphometric analysis (Hammer et al. 2001; Hammer 2010).
[http://folk.uio.no/ohammer/past/]

IMP (by H. David Sheets) – IMP (Integrated Morphometrics Software) is a set of compiled software tools with graphic user interfaces that carry out a wide variety of tasks related to the display and analysis of 2-D landmark-based geometric morphometric data in the Windows environment. It can be download from the morphometrics website at SUNY Stony Brook.
[http://life.bio.sunysb.edu/morph/]

SHAPE (v. 1.3) – SHAPE is a free software package for evaluating contour shapes based on elliptic Fourier descriptors. SHAPE contains four programs named ChainCoder, Chc2Nef, PrinComp and PrinPrint for processing digital images, obtaining EFDs, performing principal component analysis and visualizing shape variations explained by the principal components, respectively (Iwata & Ukai 2002).
[http://lbm.ab.a.u-tokyo.ac.jp/~iwata/shape/]

PRIMER 6 (**P**lymouth **R**outines **I**n **M**ultivariate **E**cological **R**esearch) – This commercial software was designed by K.R. Clarke & R.N. Gorley (Plymouth Marine Laboratory & PRIMER-E Ltd, Plymouth, UK) and consists primarily of a wide range of univariate, graphical and multivariate routines. It is biologically-oriented software that includes a lot of statistical methods.
PRIMER 6 includes now an add-on package, PERMANOVA+ that was produced in collaboration with Marti Anderson (Department of Statistics, University of Auckland, New Zealand) (Anderson et al 2008). It extends the resemblance-based methods of PRIMER to allow the analysis of multivariate (or univariate) data in the context of more complex sampling structures, experimental designs and models.
[http://www.primer-e.com/]

MDA: Morphospace-Disparity Analysis for Matlab, Version 1.2. – MDA is a program that runs under Matlab® with a simple text-user interface. It has been developed to perform disparity analyses on morphospace data. Most morphospace occupation estimators found in the literature are incorporated in this version (Navarro 2003).
[http://biogeosciences.u-bourgogne.fr/images/stories/ressources/mda/MDAvs1.2.03.zip]

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