



The overrated use of the morphological cryptic species concept: An example with *Nyctelia* darkbeetles (Coleoptera: Tenebrionidae) using geometric morphometrics

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ABSTRACT

The cryptic concept refers to two or more species that have been classified as a single nominal species due to the fact that they are apparently morphologically indistinguishable. In recent years, the development of advanced morphometric tools has allowed researchers to differentiate subtle variations that cannot be distinguished by mere “eyeballing” as was carried out in traditional cladistics. These robust statistical techniques alongside with molecular analyzes have enabled the distinction of formerly “cryptic” species, thus highlighting the associated problems and overuse of this concept. In this article we used geometric morphometric (GM) methods to test if it was possible to distinguish two species of tenebrionid beetles (*Nyctelia multicristata* and *Nyctelia confusa*) that has been traditionally classified as cryptic. The goal of this study was twofold: on one hand it was expected to draw the attention of taxonomy researchers to be careful when using the concept of cryptic species. On the other hand, it was tested if the GM toolkit allows the differentiation of a species usually categorized as cryptic. Our results allowed the distinction between *N. multicristata* and *N. confusa*, hence rejecting the “cryptic” denomination. Based on our results it is possible to suggest that possibly many times species are classified as cryptic due inappropriate methodological designs and comparisons.

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1. Introduction

The biological species concept is possibly one of the most popular species concepts currently used in zoology (Mayr, 2000; Noor, 2002). It proposes that species are interbreeding populations (at least potentially) isolated from other groups. Thus, this concept implies that new species arise when reproductive barriers are generated (Mayr, 2000). Due to the fact that different mechanisms of reproductive isolation exist among taxa, the biological species concept does not provide a unanimous measure to define species (Agapow et al., 2004). Meticulous taxon-specific approaches and knowledge are consequently required to discriminate between species. Based on the above definition several studies have applied molecular and morphological methods to get insight about the

affinities between species by applying the concept of “cryptic” species (Bickford et al., 2007; Feulner et al., 2006; Henry et al., 2014; Molbo et al., 2003; Seifert, 2009). The notion of cryptic refers to two or more species that have been classified as a single nominal species since they are at least apparently morphologically indistinguishable (Bickford et al., 2007; Colborn et al., 2001; Rocha-Olivares et al., 2001; Saez and Lozano, 2005). Some authors have proposed that species designated as ‘cryptic’ might have recently diverged, therefore being distinguishable only by means of refined molecular analyses (Paterson, 1991; Stebbins, 1950); nonetheless, there is no consensus regarding this latter attribute as an essential feature of cryptic species. Various common but possibly incorrect assumptions about cryptic species saturate the available literature (Knowlton, 1986; Saez and Lozano, 2005). One of the most common suppositions is that most cryptic species result from speciation phenomena that are so recent that morphological traits or any other traditional diagnostic characters have not yet evolved (Howard and Berlocher, 1998; Paterson, 1991; Winker, 2005).

In recent years, the development of a morphological quantitative toolkit known as geometric morphometrics (GM) have allowed

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to differentiate small variations that used to be difficult to distinguished by mere sight. These robust statistical techniques together with more sophisticated molecular analyses have enabled the differentiation of previous “cryptic” species, thus highlighting the associated problems and abuse of this concept (Baylac et al., 2003; Marsteller et al., 2009; Seifert et al., 2014; Villemant et al., 2007).

The GM allows studying shape, defined as the remaining geometric properties after removing the effects of scale, rotation and translation of an object (Adams and Funk, 1997; Rohlf et al., 1996; Rohlf and Slice, 1990). One of the techniques among these methods to assess shape is finding uneven components of shape change (*Thin Plate Spline*), which would represent all the movements of landmarks, that is, local variations but not linear ones, indicating in this way the changes that occurred in specific points of the shape. Therefore, the GM would provide a better biological interpretation with a graphic tool to visualize and quantify the morphological variation in different ecologic and evolutionary contexts (Adams and Rohlf, 2000; Alibert et al., 2001; Benítez et al., 2014). These improvements in the field of morphology have contributed to deepen the knowledge on taxonomic and ecologic studies, on the definition of species considering geographic dispersion and also on sexual differentiation studies (Alibert et al., 2001; Bertin et al., 2002; Fairbairn, 1997; Lemic et al., 2014). However, in spite of the strong potential of these tools, there has been moderate interest on morphologic variation studies (Adams et al., 2004; Brown et al., 1992; Thomas et al., 1998), mainly due to the lack of statistical accuracy with the conventional methods of morphometry at the intraspecific level. Despite this lack of accuracy, several authors have worked on the use of GM in different scientific fields (Adams et al., 2004, 2013; Bookstein, 1991; Klingenberg, 2013; O’Higgins, 2000; Rohlf and Marcus, 1993; Rohlf and Slice, 1990; Slice, 2007; Toro-Ibacache et al., 2010).

In this sense, *Nyctelia multicristata* Blanchard (1846) and *Nyctelia confusa* Zúñiga-Reinoso & Jerez (2012) are two cryptic species with a distribution restricted to the south end of Chile and Argentina (Zúñiga-Reinoso and Jerez, 2012). Both species would have a disjunct distribution, being present in both sides of the Andes mountain range. So, *N. confusa* is found in the occidental slope of the mountain range (Torres del Paine National Park and its surroundings) and *N. multicristata* is distributed in the eastern slope of the Andes mountain range, in the steppe-like areas, reaching the west coast of the Strait of Magellan (Zúñiga-Reinoso and Jerez, 2012). Then, according to Roig-Juñent and Flores (2001), these species are characteristic and endemic components of the Southern Patagonia. These species are the most abundant in the Patagonian steppe, and like the other species of the genus *Nyctelia*, they exhibit diurnal habits and they are easily found ranging or feeding on both live and dead vegetation in sunny days (Peña, 1963). On the other hand, due to their ectothermic condition, they hide during the night and at peak radiation time (Cloudsley-Thompson, 2001; Flores, 1997).

Zúñiga-Reinoso and Jerez (2012) have recently clarified in part a historical confusion between both morphs, since both entities are morphologically very similar, so that it was necessary to clarify the taxonomic validity of each one of them, being revalidated as independent species by means of a classic taxonomic analysis. From the morphologic standpoint, both species differ in some structures of the elytral edge, legs and genitalia, besides exhibiting a difference in their shape, since *N. multicristata* has a groove in the elytral suture (Blanchard, 1853; Solier, 1851; Zúñiga-Reinoso and Jerez, 2012). However, both species vary in size, leg color and elytral design along the longitudinal climatic gradient that prevails in their corresponding distributions, causing an overlap of some morphologic features

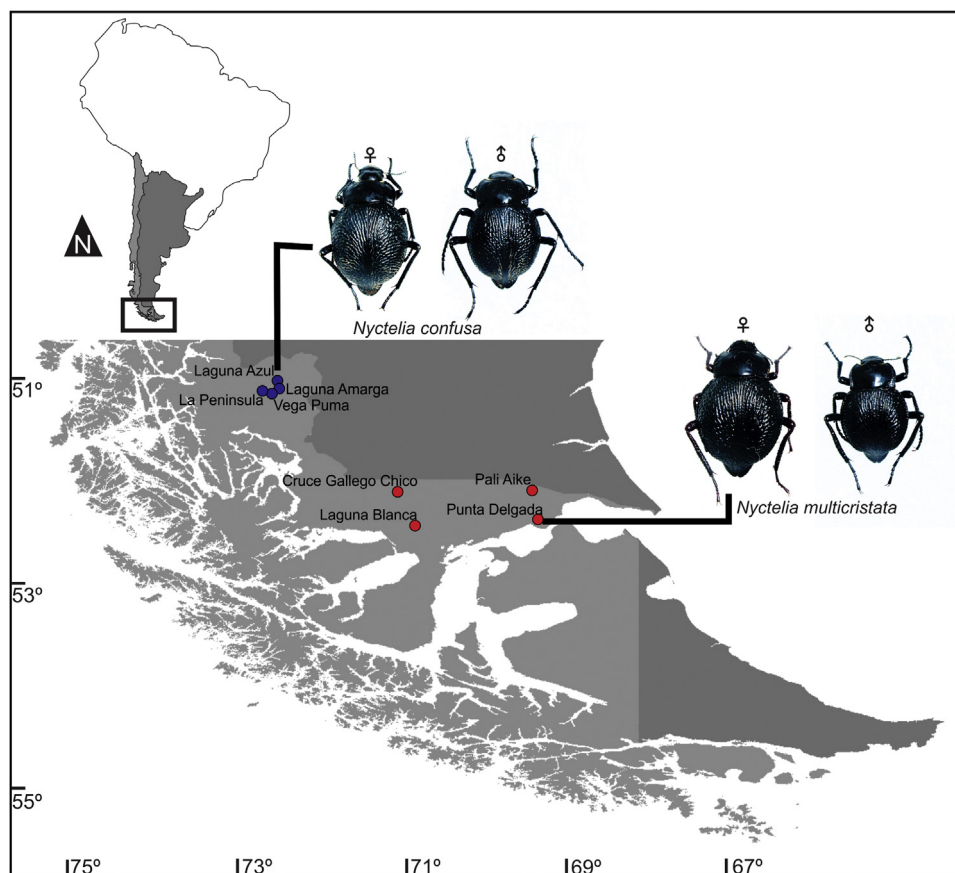


Fig. 1. Geographical locations sampled in the Chilean Patagonia, indicating sampling sites of *N. multicristata* and *N. confusa*.

in these species, hence keeping them under the concept of “cryptic species” (Zúñiga-Reinoso and Jerez, 2012).

For these reasons, this study intends to confirm and draw the attention of researchers on taxonomy to be careful when using the concept of cryptic species, taking the use of geometric morphometrics of the body shape as an example to definitely determine that *N. multicristata* and *N. confusa* (Coleoptera: Tenebrionidae) are two valid entities.

2. Materials and methods

2.1. Biological material used

A total number of 104 adult specimens of the species *N. multicristata* y *N. confusa* were analyzed. These specimens are from various locations of both sides of the Andes in the Magallanes region, Chile (Fig. 1). The samples of *N. multicristata* include 44 specimens from Pali Aike National Park (19 males and 14 females), Camino a Gallego Chico (2 males and 2 females), Laguna Blanca (1 male and 2 females) and Punta Delgada (1 male and 3 females). The samples of the species *N. confusa* include 60 specimens, all of them taken from Torres del Paine National Park in the following locations: La Península (19 males and 19 females), Laguna Azul (9 males and 8 females), Laguna Amarga (3 males and 1 female) and Vega Puma (3 females) (Fig. 1). The specimens used in this study have been taken from entomological collections of the Chilean National Museum of Natural History (MNNC), private collection of Carlos Rojas (CPCR) and the Zoology Museum of the Universidad de Concepción (UCCC).

2.2. Data collection and shape analysis

All the specimens were photographed in ventral view to locate the landmarks, and only 45 specimens were photographed in posterior view to analyze contour. The photographs were taken with a digital camera Nikon Coolpix L1, at an approximate distance of 5 cm from the object, with the digital macro activated and 6 mega-pixels of image resolution.

The specimens were digitized with 19 landmarks (LMs, anatomical homologous points) on every picture, by TpsDig 2.17 (Rohlf, 2013) (Fig. 2). The shape information was extracted with a generalized Procrustes analysis (Rohlf and Slice, 1990), taking into account the object symmetry (Klingenberg et al., 2002). Procrustes superimposition is a procedure that removes the information of rotation, position and orientation, and standardizes each specimen to unit centroid size (Dryden and Mardia, 1998). Because of the symmetry of the structure, reflection is removed by including the original and mirror image of all configurations in the analysis and simultaneously superimposing all of them. The shape variation of the whole dataset was then analysed by performing a Principal Component Analysis (PCA), calculated from the covariance matrix of the symmetric component of shape. This analysis was also used to visualize the morphological changes observed between the average shapes of the specimens of *N. multicristata* and *N. confusa*. The level of statistic differentiation was assessed by computing a Procrustes ANOVA as applied in other studies to analyse object symmetry. The results were reported as sums of squares (SS) and mean squares (MS), which are dimensionless. Just to confirm the statistical power of the differentiation of GM analysis of shape a discriminant analysis and a Hotelling’s T^2 test (1000 permutations) were performed, to assess whether there were significant differences in the body shape between the cryptic species. All analyses were then run using MorphoJ software version 1.05 d (Klingenberg, 2011).

The Elliptic Fourier Transform (EFT) was used to estimate and visualize interspecific shape variation of elytra (i.e., elytral cavity)

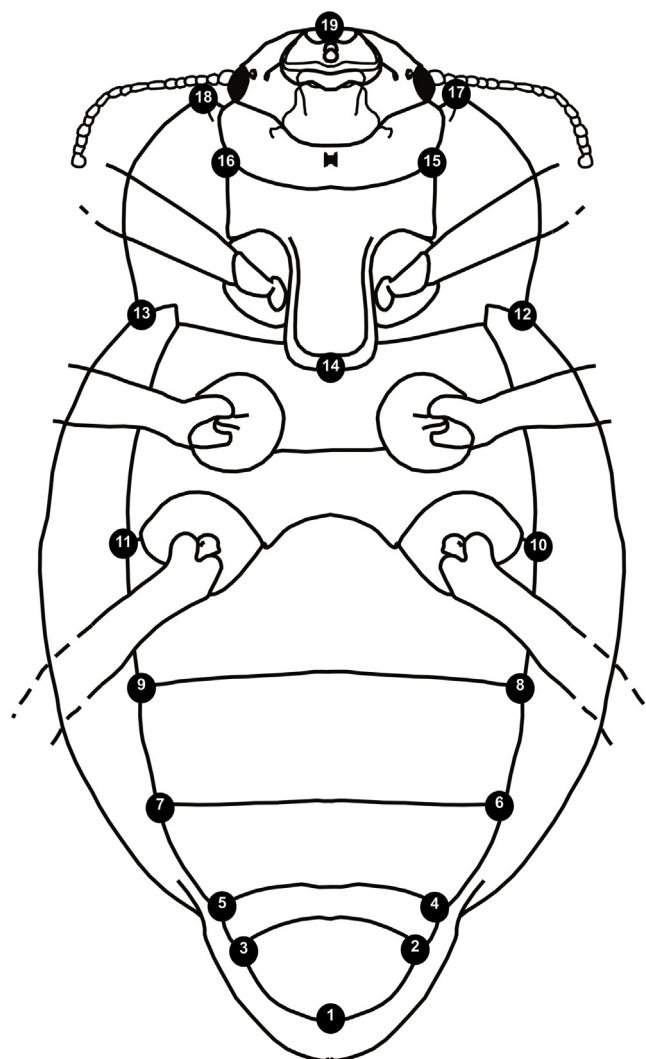


Fig. 2. Representation of the 19 anatomical landmarks of *Nyctelia* species.

between both species. In this method the contour shape is expressed in periodic signals, using a minimum square criterion, where each signal is adjusted by the sum of trigonometric functions (or harmonics) at various amplitudes and phases (Bookstein, 1997; Rohlf and Archie, 1984; Sheets et al., 2006). Each harmonic represents curves that are aggregated in decreasing order to describe contour, so that the first harmonics describe the general contour shape, while the last ones represent small scale variations (Bookstein, 1997). Each harmonic is broken down into four coefficients: A_n and B_n for X, and C_n and D_n for Y, which define an oval on Cartesian plane. The first harmonic is used to standardize the object size and orientation (Renaud and Michaux, 2003). According to Crampton (1995), this first harmonic and its coefficients would correspond to residuals after standardization and they should not be used in the subsequent statistical analyses. A photograph matrix was set up with the software program tpsUtil version 1.54 and contours were digitalized with the program tpsDig version 2.17 (Rohlf, 2013). The superimposition of shapes involved and the consensus configuration were prepared in directly in the software program MorphoJ et al. (Slice 1998, 2013; Slice 1998, 2013), by rebuilding the shape through the reverse method (Crampton 1995). Eight harmonics resulted from each shape (i.e., 28 coefficients). Since the first harmonic does not show variation in the first three coefficients, these were not considered for subsequent analyses (e.g., Crampton, 1995). The shape variation of the

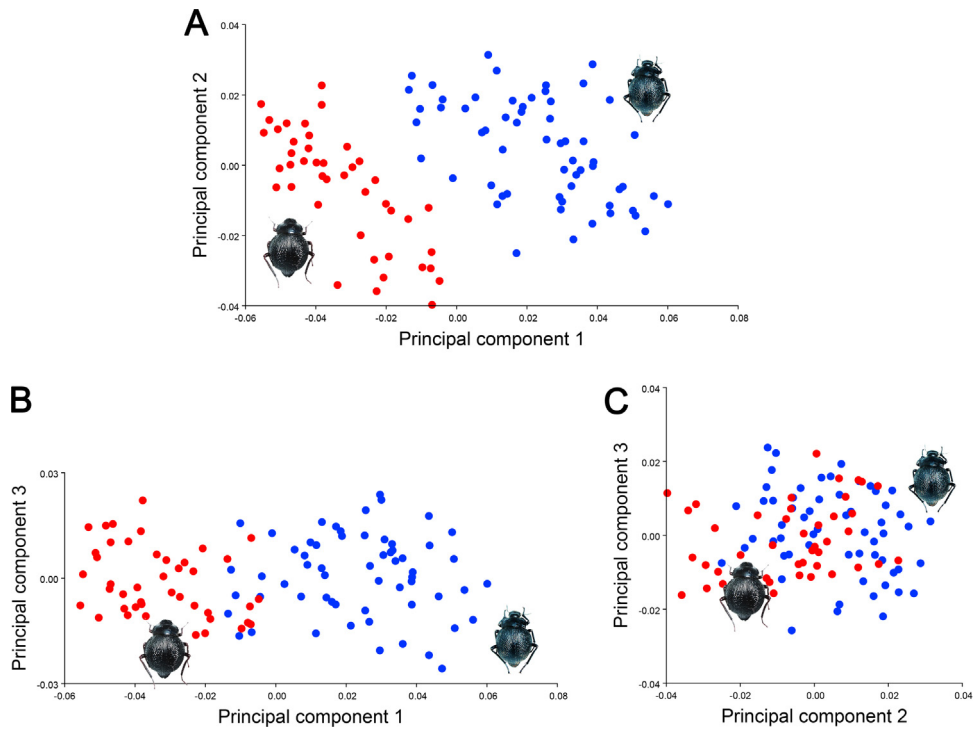


Fig. 3. PCA scatterplot of *N. multicristata* (red) and *N. confusa* (blue) body shape variables. A–C, Plots of the PC scores. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

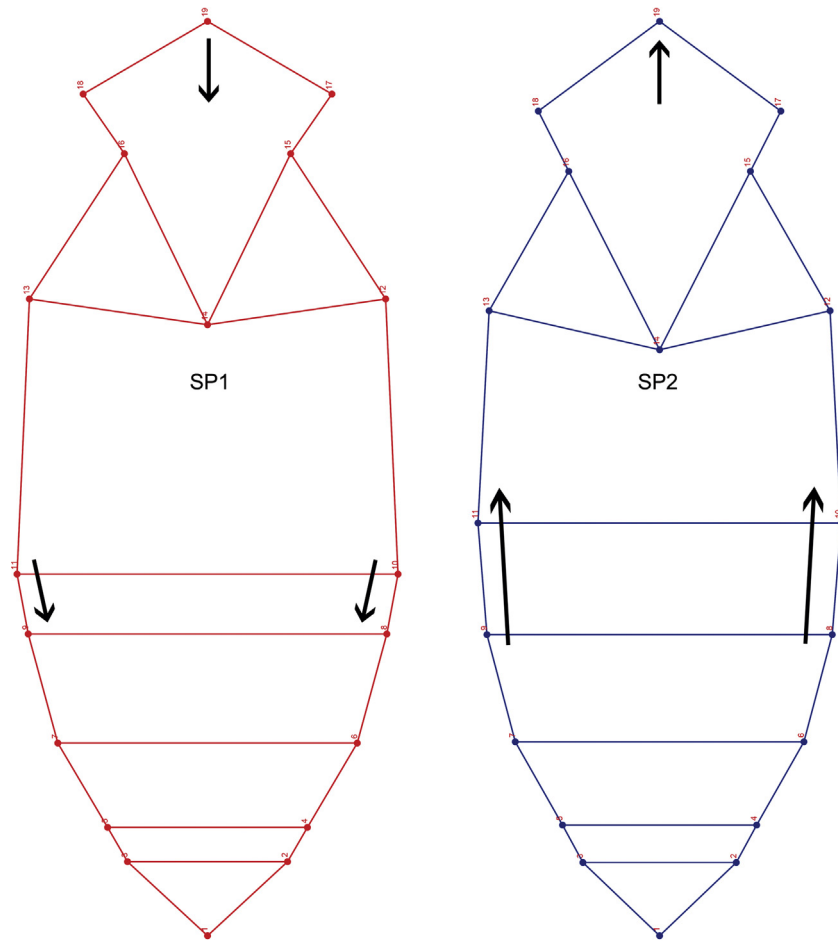


Fig. 4. Wireframe representation of the body shape variation of *N. multicristata* (red) and *N. confusa* (blue). The diagrams show the shape that corresponds to the average shape with the principal body shape differentiation represented with arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

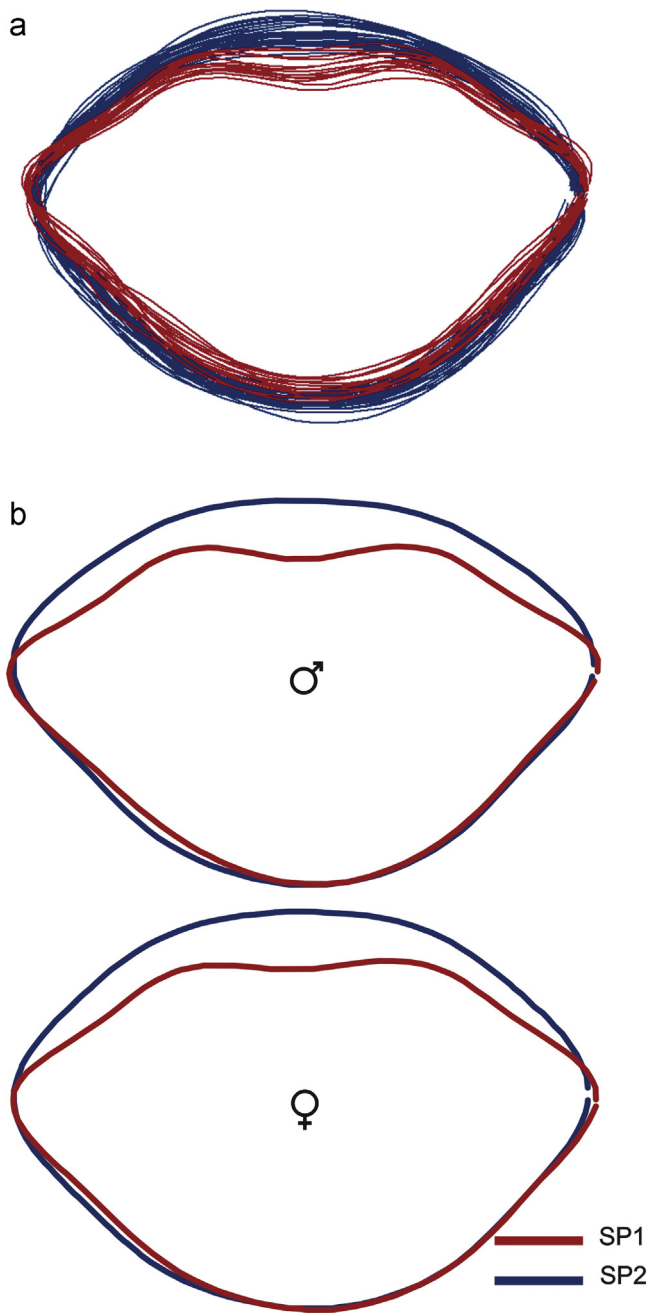


Fig. 5. Elliptic Fourier Transformation of the elytral shape. (a) All individuals of both species: *N. confusa* (blue) and *N. multicristata* (red). (b) Consensus configuration of contours between the two species and sex: *N. confusa* in blue line and *N. multicristata* in red line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

whole dataset was examined by a PCA, when the statistical relationship between the shape variation and the matrix coefficient were analyzed by a MANOVA using the species as a factor. This analysis was performed in the software PAST 1.82b.

3. Results

As the first and most important analysis before any study using GM, the measurement error in the samples was tested by a Procrustes ANOVA showing that MS individual variation due to fluctuating asymmetry exceeded the measurement error.

The PCA of the variation of body shape shows that a large percentage of the variation is contained in relatively few dimensions, with the first three PCs accounting for more than half (78%) of the total variance in the sample. The variation in the point cloud (each point represents an individual and a different shape) was notoriously different for each species being easily differentiated between groups. PC1 shows 57% for interspecific differentiation; however, PC2 shows a intraspecific differentiation that can be defined through sexual dimorphism in the groups (Fig. 3). The wire-frames projected from the average shape of each species, show a clear variation in the movement of landmarks 8–11, corresponding to the first abdominal segment in both species, where distortion vectors in *N. multicristata* show a clear elongation of this structure compared to *N. confusa*. Similarly, the landmarks for head 17–19, show a distinguishable specific variation between both species (Fig. 4).

The discriminant analysis based on the Hotelling's T^2 test showed that the mean body shape of the individuals described as cryptic species (*N. multicristata* and *N. confusa*) was significantly different (T^2 : 966.96; p -value: <0.0001). Additionally, the cross-validated discriminant analysis was able to correctly classify the majority of the individuals. Finally, the Procrustes ANOVA performed to test for differences between species indicated that the shape variation due to this factor is highly significant (Species = F : 76.93; P : <0.0001).

Configurations for EFT are shown in Fig. 4. In this analysis, 95% of variability of elytral shape is explained by the first eight harmonics. In both species, a large part of the elytral shape variation is explained by the first two components (81.2%). The MANOVA shows significant differences between the two species (Wilks's Λ = 0.1268; p < 0.001), well as to compare only the females (Wilks's Λ = 0.0285; p < 0.001) and only the males (Wilks's Λ = 0.1221; p < 0.001) of each species. The main structural differences in elytral shape, are based on the fact that the elytra of *N. multicristata* individuals are more flat and with a deeper slot in the interelytral suture than *N. confusa* individuals. These differences can be observed in the superimposition of consensus configurations (Fig. 5).

5. Discussion

The analyses in this study allowed differentiating the cryptic species *N. multicristata* and *N. confusa* statistically by means of shape rejecting the denomination as a “cryptic”.

Cryptic species are two or more distinct species that were classified as a single species due to their morphological similarity. It is possible that species usually classified as cryptic due to their “morphological similarity” were perhaps defined in that way based on the restricted morphological structures that were analysed (i.e., some anatomical portions can distinguished between species, while other cannot). Species are cryptic largely due to the anthropocentric perception that relates the lack of evident differences in external appearance with sameness. The development of GM during the last decades has provided useful tools to quantify slight morphological differences between specimens that were not possible to properly measure using traditional morphometrics (Rohlf and Marcus, 1993). Although today the “cryptic species” concept is very clear and it has been used in taxonomy for more than 100 years, Bickford et al. (2007) show in their study that the percentage of papers using the cryptic species concept from 1975 (excluding previous studies) has increased exponentially (>3500 references from the past 50 years) since molecular techniques were developed, making much more effort on morphology itself no longer necessary.

For the evaluated species in this study, the morphological differentiation is mainly based on the fact that the head and abdomen

of the species *N. multicristata* is more compressed (since their first ventrite is narrower). These characteristics had not been detected by Zúñiga-Reinoso and Jerez (2012) to differentiate both species. Besides, the elytra of *N. multicristata* have a groove in the elytral suture and dorsoventrally they are more compressed. This groove, which is not easily noted on plain sight, is detected by the GM analyses and this is consistent with descriptive characters of Solier (1851) and Blanchard (1853) for *N. multicristata* and proposed as the diagnosis for this species by Zúñiga-Reinoso and Jerez (2012).

The geographical distance in the distribution plus the selective pressures could be modeling this divergence in the body-elytral shape of both species; however, these pressures have not involved a major morphological differentiation allowing these two species to be easily distinguishable. This apparent similarity in morphology may be due to recent speciation events and, consequently, to genetic and developmental constraints that limit phenotypic variation (Gould, 2002; Raff, 1996; Smith, 1981) and/or a stabilizing selection on adaptive traits (Charlesworth et al., 1982; Estes and Arnold, 2007; Kirkpatrick, 1982). In this scenario, the shape characteristics of body and elytrum that distinguish these two species would be influencing the sub-elytral cavity. It has been suggested that this structure has an adaptive function in darkbeetles, because it reduces transpiration and allows the abdomen to expand to the storage water during periods of water scarcity in arid and semi-arid ecosystems (Cloudsley-Thompson, 2001). Severe environmental conditions could generate stabilizing selection on morphology (particularly in adaptive characters), constraining the morphological change that is usually related to the speciation process (Bickford et al., 2007). Preliminary phylogenetic analyses suggest that these two species have a common evolutionary history (Zúñiga-Reinoso unpublished data). On the other hand, De Los Santos et al. (2000) suggest that environmental factors, like moisture, have an influence in tenebrionid morphology, so that the abdomen will be larger and elytra will be higher in tenebrionids inhabiting more arid places (i.e. larger elytral cavity). However, this prediction is not fulfilled in this study, because when scaling the size of both species, *N. multicristata*, inhabiting more arid areas, has more compressed body and elytra than *N. confusa* individuals, inhabiting less arid areas. Then, the environmental factors would affect the body size, and the shape in *Nyctelia*. However, shape differentiation could be a reflection of genetic constitution variation (Adams and Funk, 1997) and more recent works have matched molecular and geometric markers that may explain shape differentiation in coleoptera (Garnier et al., 2005).

This study has shown that the classification of *N. multicristata* and *N. confusa* as cryptic species has been probably a result of the little effort applied when classifying. Using two analytical techniques especially developed for morphological studies provided a better accuracy when compared to traditional approaches. It was possible to differentiate almost 100% of the analyzed specimens, thus providing strong evidence against the cryptic species definition. Furthermore, the two species were even visually differentiated along PC1, hence demonstrating again the lack of support for the previously proposed classifications. Likewise, the cross-validated discriminant analysis showed a clear distinction between the two species. This means that these two species have experienced enough speciation to achieve different morphologies, which is consistent with their geographic distribution in the southern extreme of the Andes.

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