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Are Horn Morphological Patterns Able to Differentiate the Two Closely Related Species *Copris klugi* Harold and *Copris sierrensis* Matthews?

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Abstract

Several thousand species of beetles evolved impressive, exaggerated horns or horn-like structures. The horn phenotypic patterns and the developmental mechanisms are well documented especially in the Scarabaeidae, the family most predominated by species with horns. The regulation of horn expression appears to be extremely evolutionary labile to the extent that horn allometric patterns have been seen to rapidly diverge between closely related species. For this reason, it has been suggested that horn morphological pattern may be able to differentiate closely related and sibling species even when other traits fail. In this study, we used horn morphological pattern (shape and allometric variation) as a "tool" to evaluate the differentiation of two closely related scarab species, *Copris klugi* Harold and *Copris sierrensis* Matthews whose full species status has long been debated due to their high similarity. Combining traditional and geometric morphometric methods, we evidenced that male head horn phenotypic pattern is able to clearly differentiate *C. klugi* from *C. sierrensis*, supporting the hypothesis that they are two true species.

Introduction

In recent years, dung beetles became a model system for studies aiming to integrate developmental biology, systematics, evolution and ecology (e.g. Nijhout & Emlen 1998, Emlen et al 2005, 2007, Parzer & Moczek 2008, Moczek 2009, Macagno et al 2009, Kijimoto et al 2012). There are several reasons for this rising interest, although one of the main ones is certainly their peculiar mechanisms of horn expression (Emlen 1994, Moczek & Emlen 1999, Moczek et al 2006). Among beetles in fact, thousands of species develop horns, rigid outgrowths of the exoskeleton whose size is often extraordinary disproportionate with respect to body size, especially in males. The Scarabaeidae is the family most characterized by horned species.

The studies carried on Scarabaeidae, and in particular on the genus *Onthophagus* (Emlen & Nijhout 1999, Emlen et al 2005, Moczek 2011, Pizzo et al 2006a, b, 2012, Macagno et al 2009,

2011, Kijimoto et al 2012), have shown that horns in scarab beetles commonly scale with body size either (a) in a linear fashion, with horn size increasing relatively faster than body size or (b) in a threshold-dependent, sigmoidal fashion. In the latter case, males smaller than a certain critical body size develop no or only rudimentary horns, whereas males larger than the threshold size express fully developed horns (Moczek & Emlen 1999, Kijimoto et al 2009, Moczek 2011). The development of linear vs. sigmoid horn allometric pattern is thought to require fundamentally different ontogenetic regulatory mechanisms (Emlen & Nijhout 2000, Nijhout & Grunert 2002, Kijimoto et al 2012). The regulation of horn expression appears to be extremely evolutionary labile to the extent that horn allometric patterns have been seen to diverge between closely related species (Moczek & Nijhout 2003, Emlen et al 2007, Macagno et al 2009). It could therefore be hypothesized that interspecific differences in horn allometric pattern might mirror phylogenetic distances, and for this reason can be used as a "character" for species differentiation (Macagno et al 2009).

In this paper, we studied the horn allometric pattern in two species of a genus less considered in researches regarding the models of horn expression: the genus *Copris* Müller, belonging to a group of Scarabaeidae that originated in the Paleotropical belt of the Old World and then reached North America, the Mexican Transition Zone, and in one case, even South America (Zunino & Zullini 2003, McCleve & Kohlmann 2005, López-Guerrero et al 2009).

We used horn morphological pattern as an indicator of divergence in two closely related scarab species: we considered as case study *Copris klugi* Harold and *Copris sierrensis* Matthews whose full species status has long been debated due to the high similarity of the two taxa (Matthews 1961, Marchisio 1983, Zunino 1983, 1987, Delgado & Kohlmann 2001, López-Guerrero et al 2009). As a comparison and reference, we used *Copris* armatus Harold, a related taxon belonging to the same species complex of *C. klugi* and *C. sierrensis*.

Copris klugi is distributed in the Transverse Volcanic System and in the Sierra Madre del Sur, between altitudes ranging from 1500 to 2000 m asl. The distribution of this Mexican species is wide, from Aguascalientes in the west to Puebla and Veracruz in the east and Oaxaca in the south. With some rare exceptions, this species is altitudinally replaced by *C. armatus* in the Transverse Volcanic System. *Copris* armatus reaches the highest altitudes in America. It is distributed in the Transverse Volcanic System, from Jalisco in the west to Veracruz in the east, and on the mountains that are adjacent to the Mexican Plateau, such as the Sierra de Pachuca, at altitudes between 1830 m asl (more frequently 2000 m asl) and 3000 m asl (López-Guerrero et al 2009). *Copris sierrensis* is found in the Sierra Madre Occidental ranging from Nayarit and Jalisco in the south to Chihuahua in the north, between altitudes of 1380 and 2600 m asl.

To inspect horn phenotypic pattern variations (allometric and shape variations) between the species, we used a combination of traditional and geometric morphometric methods.

Material and Methods

Sample

We examined specimens field collected in horse and cattle dung in different neighboring sites in Michoacán (*C. klugi*—35 males, 39 females) and Durango (*C. sierrensis*—21 males, 17 females) by M. Zunino and V. Halffter during June–July 1979; they are preserved in the entomological collection of the Dipartimento di Scienze della Vita e Biologia dei Sistemi of the University of Turin. *Copris armatus* specimens (27 males, 25 females) are from a M. Dellacasa's personal collection and have been field collected by the owner in Zirhauén, Michoacán.

Morphometric analyses

Specimens were cleaned in 100°C distilled water for 10 min and then dissected for morphometric analyses. Heads and pronota were separately fixed on horizontally leveled plasticine supports by completely plunging their convex ventral side. 2D images of each structure were taken using a Leica Z16Apo stereoscopic dissecting scope (Leica Microsystems AG, Wetzlar, Germany). After image calibration, linear measurements of pronotum width (PW) and horn lengths (female and male head horn length: FHH and MMH; first pronotum horn length: PH1; second pronotum horn length: PH2) were taken with the software LAS v 2.5.0 (Leica Application Suite) (Fig 1). Some studies (Tomkins et al 2005, Moczek 2006) have suggested that differences in the pattern of horn allometries can arise from the method used to measure the beetles horns. In general, measurements that reflect the changing shapes (i.e. those able to follow curves) of the structures in question are preferable. On the dorsal view, we measured pronotum width (PW) which was used as a proxy for the overall body size (Emlen 1994, Moczek 1998, Parzer & Moczek 2008, Pizzo et al 2012).

We first inspected the trend of natural variation in pronotum width and horn length measurements, producing frequency histograms. The number of classes for size frequency distribution histograms for each sex was chosen using Sturges' rule (k=1+log2n), which has been found to be relatively effective for sample sizes that are smaller than 200 specimens (Gouws et al 2011). We tested for approximation to a normal distribution by running a Kolmogorov–Smirnov test (Sugiura et al 2007). We then graphed the log-log scaling relationship between horn lengths and body size (horn expression pattern). In *C. klugi*, the numerically better represented species in our sample, the scaling relationships were determined also by fitting to the log-scaled data both a simple linear (y=y 0+bx) and a four-parameter nonlinear regression in the form of the four-parameter hill equation:

$$Y = y_0 + a\ x^b / c^b + x^b$$

where x is the pronotum width (used as a proxy for body size), y is the horn length, y_0 specifies minimum horn length, a defines the horn length range in the sample, b is a slope coefficient and c is the body size at the point of inflection of the sigmoid curve. To distinguish which model was the most appropriate to describe a particular scaling relationship, we first compared r^2 values generated by the two regression types.

Geometric morphometrics

Landmark configurations were digitized by using TpsDig 2.16 (Rohlf 2010) (Figs 3 and 4): 5 landmarks on the lateral view of the male and female head horn and 12 landmarks on the frontal view of the male and female pronotum. Landmarks were chosen for their relative ease of identification and ability to capture general shapes. Superimposition of landmark configurations and subsequent principal component analyses of shape variables (PCA) were performed using MorphoJ (Klingenberg 2008).

Results

Pronotum width (PW) of males showed a normal distribution for all species; both head (MHH) and pronotal (PH1 and PH2) horn length distribution failed Kolmogorov–Smirnov tests: the visual inspection of the frequency histograms for these traits advocated a bimodal distribution. Female head horn length (FHH) and PW of females were normally distributed for each species (Fig 2).

Visual inspection of the scaling relationships between horn lengths and body size indicated that a sigmoidal model of growth was a good description for male horn length–body size data for each species, both for head and pronotal horns. In particular, in *C. klugi*, we fitted linear and sigmoidal regressions and we showed very high r^2 values for sigmoidal regressions (0.94 for MHH, 0.90 for PH1 and 0.84 for PH2).

On the contrary, log-log scaling relationships between head horn length and body size in females showed a linear model of growth. In *C. klugi*, fitting both linear and sigmoidal regressions we found higher r^2 values for linear regression (0.78 for FHH).

All horn expression patterns, both in males and in females, showed a clear separation between *C. klugi* + *C. sierrensis* and *C. armatus*. As a general rule, males of *C. armatus* showed larger pronota with respect to *C. klugi*+*C. sierrensis*, but a very similar range of horn lengths. This means that males of *C. armatus* have proportionally shorter head and pronotal horns. The horn expression patterns of *C. klugi* and *C. sierrensis* are well distinguishable for the cephalic horn, but less for pronotal horns, where *C. klugi* and *C. sierrensis* specimens often overlap.

In females, *C. armatus* showed larger pronota and larger horns, whereas *C. klugi* and *C. sierrensis*, again, largely overlap.

The cephalic horn and the pronotum shape pattern showed, both in males and in females, a clear shape differentiation of *C. armatus* from *C. klugi*+*C. sierrensis*, which, on the contrary, largely overlap.

Geometric morphometrics showed that the range of morphological variation in the morphospace (Figs 3 and 4) is very similar in the three species. Regarding the shape of the cephalic horn, *C. klugi* minor males and females are more similar between them than in *C. armatus* and *C. sierrensis*: in both of these species minor males seem to have a more transitional shape between majors and females. Moreover, *C. armatus* seems to have a differentiated morphology with respect to the other two species, which in turn have the shapes of the cephalic horn largely overlapping.

Regarding the shapes of the pronotal horns, the principal components analysis on the pronotum shape variables showed a marked difference between male and female pronota. Minor males, in all species considered, have a pronotal horn morphology much more similar to majors and, unlike the cephalic horn, largely differentiated from females. Even though more evident, especially for major males, *C. armatus* appears as the most morphologically differentiated species, whereas, again, *C. klugi* and *C. sierrensis* pronotal shapes largely overlap.

Discussion

Nonlinear horn expression patterns are frequently found in male scarab beetles—i.e. in *Onthophagus* (Emlen 2001, Emlen et al 2005, Macagno et al 2009, Pizzo et al 2012), Xylotrupes (Rowl & 2003) and Calchosoma (Kawano 1995), revealing a widespread threshold-dependent mechanism of horn expression with a developmental switch point separating major and minor morphs within males. These patterns are accompanied by clear bimodality in horn length frequency distributions (but not always in body size). The three *Copris* species analysed in this paper follow this pattern.

Since males are typically the more adorned sex, the majority of studies have focused solely on secondary sexual trait evolution in males (Kijimoto et al 2012). The evolution of such traits in females has generated much less interest and has only recently begun to be explored. When Emlen et al (2005) mapped the presence/absence of horns in females and major and minor males onto a phylogeny in the genus *Onthophagus*, they found that in those rare cases in which

females bear horns, they had the same basic male shape and showed linear allometry (Emlen et al 2005, Moczek 2009).

The only previous study on the horn allometric pattern in the genus *Copris* was made on *Copris ochus* Motschulsky (Sugiura et al 2007), an endangered Japanese species. *Copris* ochus shows sexual dimorphism for head horn (with males having longer horns than females), and the relationship between horn length and body size is linear in females and nonlinear in males; these phenotypic patterns of male and female horns are highly comparable with that found in *C. klugi*, *C. sierrensis* and *C. armatus*.

It is very interesting to note that when the separation from the nominal subspecies and the elevation to the rank of species of *C. klugi sierrensis* had been proposed (Matthews 1961), the main reason given was about "the proportion between the length of the cephalic horn and femur in males" (reported in López-Guerrero et al 2009). Our finding on the male head horn expression pattern, which allows for a clear differentiation of *C. klugi* from *C. sierrensis*, mirrors this qualitative observation and is in full agreement with it, supporting the hypothesis that *C. klugi* and *C. sierrensis* are two true species. Moreover, it was evidenced that there is a faint sclerotized longitudinal band on the dorsal surface of the male parameres in *C. sierrensis* (Matthews 1961) and that *C. klugi* males are characterized by parameres thinner than in *C. sierrensis* (López-Guerrero et al 2009). In *C. klugi*, the raspula is formed by seven or less subunits of decreasing length, whereas in *C. sierrensis*, it has nine or more subunits of more or less equal length, each of them with several thinly sclerotized spines (López-Guerrero et al 2009). It has been suggested, indeed, that horn expression pattern, which we found to diverge between *C. klugi* and *C. sierrensis*, might trigger changes in the male genital morphology, thus possibly promoting speciation as a by-product. Some authors (Moczek & Nijhout 2004, Parzer & Moczek 2008) proposed an antagonistic coevolution between horns and male copulatory structures acting through resource allocation trade-offs, highlighting a possible coupling between the diversification of horn expression pattern and the differentiation of genitalia. This could be a crucial mechanism linking micro- and macroevolution due to the key role of genital morphology in reproductive isolation in insects (Eberhard 1985).

As head horn size is able to reveal interspecific differences hidden by head horn shape, we showed that size and shape of the same trait can evolve independently from one another among species, and this might indicate that a genetic uncoupling of size and shape can occur in secondary sexual structures, such as horns.

Lastly, it has been stated that *C. sierrensis* is more closely related to *C. armatus*, while *C. klugi* is more closely related to *Copris* megasoma Matthews & Halffter and *Copris mexicanus* Matthews & Halffter (López-Guerrero et al 2009). These observations, based on genitalia, are only partially reflected in our data concerning horn expression pattern: in fact, even though *C.*

sierrensis appears to be most similar to *C. armatus*, *C. sierrensis* and *C. klugi* are the most similar species.

Our work demonstrated that horn expression pattern can be considered as a valid tool to distinguish species in the genus *Copris*, with good discriminatory power, in particular for cephalic horns; it also provides, by reflecting the phylogenetic distances, additional information on the relationships among species within a species complex (Macagno et al 2009).

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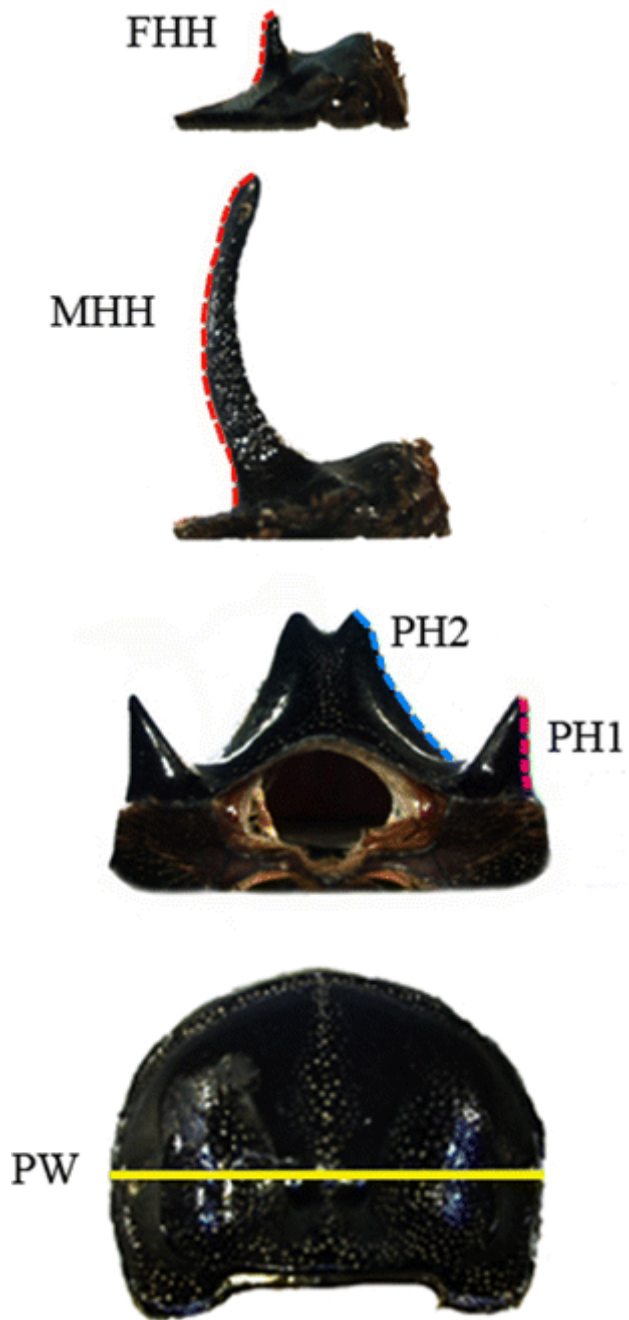Zunino M, Zullini M (2003) Biogeografía. La dimensión espacial de la evolución. Fondo de Cultura Económica, México, p 359

Fig 1

Measurements on the head pronotum of *Copris klugi* (the same for *Copris sierrensis*). From the *top*: lateral view of the female cephalic horn (*FHH* female head horn length); lateral view of the male cephalic horn (*MHH* male head horn length); lateral view of the pronotal horns (*PH1* external pronotal horn length; *PH2* inner pronotal horn length); dorsal view of the pronotum, with pronotum width (*PW*).
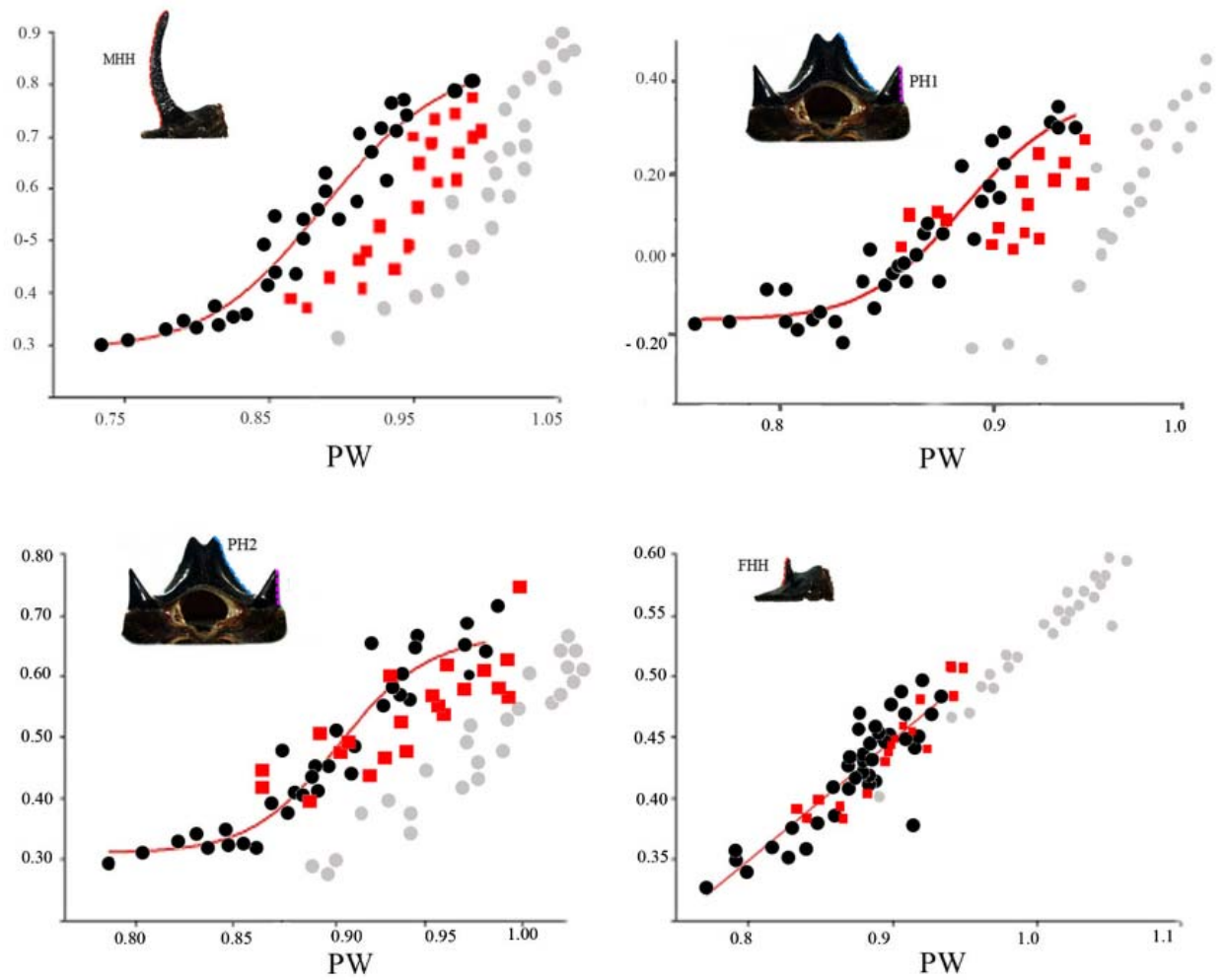
Fig 2

Horn expression patterns: scaling relationships of log-log cephalic (*MHH*, *FHH*) and pronotal (*PH1*, *PH2*) horn lengths versus body size (*PW*). *Black symbols* represent *Copris klugi*, *red symbols* represent *Copris sierrensis* and *grey symbols* represent *Copris armatus*.
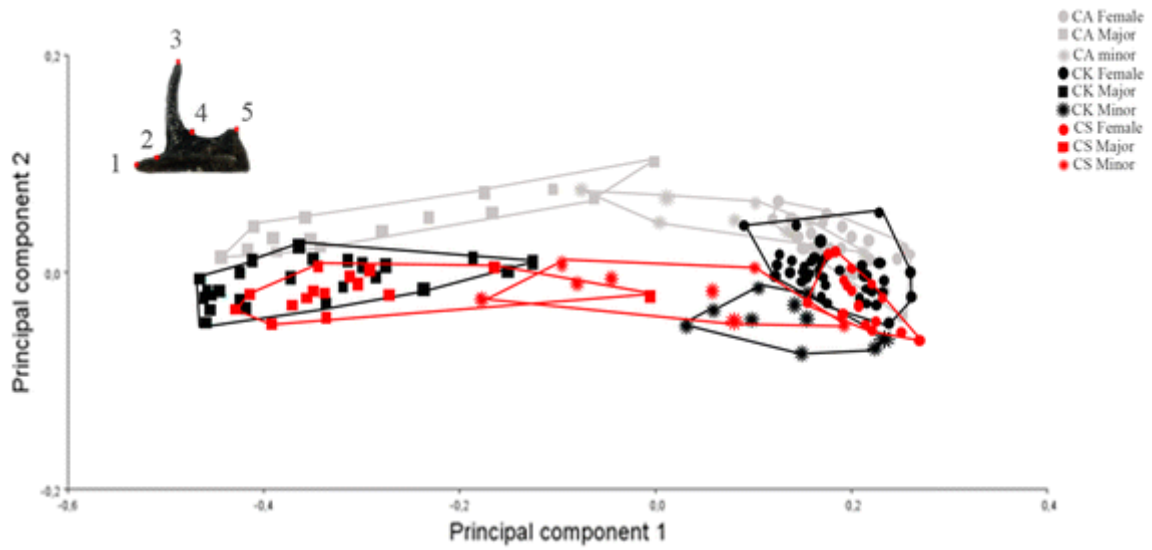
Fig 3

Scatter plots of principal components 1 and 2 from head horn shape variables obtained by means of geometric morphometrics. *Black symbols* represent *Copris klugi*, *red symbols* represent *Copris sierrensis* and *grey symbols* represent *Copris armatus*. *Circles* represent females, *squares* represent major males and *asterisks* represent minor males.
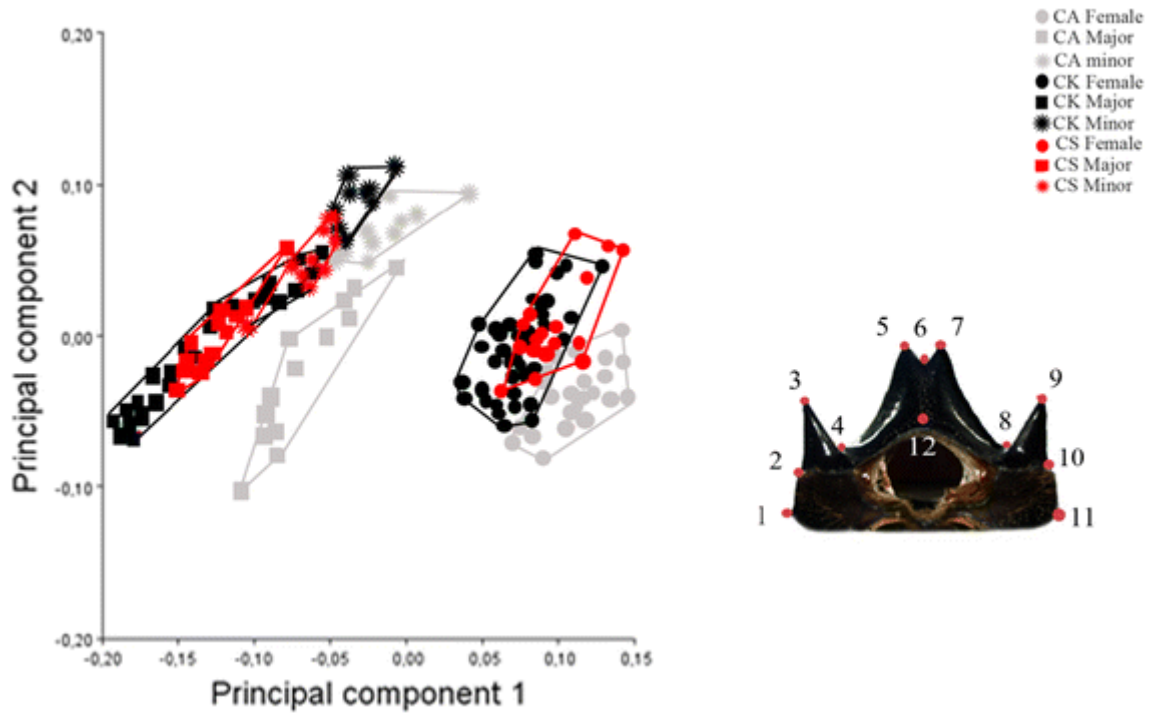
Fig 4

Scatter plots of principal components 1 and 2 from pronotum shape variables obtained by means of geometric morphometrics. *Black symbols* represent *Copris klugi*, *red symbols* represent *Copris sierrensis* and *grey symbols* represent *Copris armatus*. *Circles* represent females, *squares* represent major males and *asterisks* represent minor males.