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Title:

Size and shape interspecific divergence patterns partly reflect phylogeny in an *Onthophagus* species-complex (Coleoptera: Scarabaeidae).

Authors

Anna L. M. MACAGNO, Astrid PIZZO, Antonio ROLANDO, Claudia PALESTRINI

Institution

Università degli Studi di Torino, Dipartimento di Biologia Animale e dell'Uomo, Via Accademia Albertina 13, 10123 Torino, Italy

Short running title

Size and shape divergence in an *Onthophagus* species-complex.

Corresponding author

Astrid Pizzo, Dipartimento di Biologia Animale e dell'Uomo, Università

degli Studi di Torino, via Accademia Albertina 13, 10123 Torino, Italy.

Phone number: +39 011 6704535

Fax: +39 011 6704508

E-mail: astrid.pizzo@unito.it

Abstract

Polyphenism has been suggested as an accelerator for morphological evolution and speciation. In the dung beetles of the genus *Onthophagus*, horn expression is polyphenic, large males developing horns whereas smaller males expressing greatly reduced or no horns. Horn static allometries seem to diverge rapidly among extant taxa, a process which might trigger changes in the male genital morphology, thus possibly promoting speciation as a by-product. It could therefore be hypothesized that inter-specific distances in allometries and, possibly, in other morphological traits mirror phylogenetic distances.

In this study we first assessed the phylogenetic relationships among three closely related taxa belonging to the so called '*Onthophagus fracticornis-similis-opacicolis*' species-complex by sequencing the mitochondrial gene *cox 1*. Biomolecular results indicated three independent lineages, the closest relationships being found between *O. similis* and *O. opacicolis*. Then we assessed the extent to which divergence pattern of horn static allometries and size and shape divergence patterns of one genital (paramere) and two non-genital (head and epipharynx) structures mirrored the phylogenetic relationships. Interspecific divergence patterns of horn static allometries, paramere and head shape were found to be congruent with the evolutionary relationships inferred from biomolecular data. Nevertheless, paramere size and epipharynx shape showed patterns not consistent with the phylogeny. Furthermore, the relative size of non-genital structures showed little inter-specific divergence compared to their shapes. Our results suggest that size and shape inter-specific divergence mirror phylogeny only in part; they also indicate that distinct morphological traits may differ in their tendency to evolve in concert, and that size and shape of the same trait can evolve independently across species.

Keywords

Bayesian inference – mt-COI - geometric morphometrics - horn polyphenism - male genitalia - maximum likelihood- morphological evolution - mouthpart - neighbour joining- static allometry

Introduction

In recent years, *Onthophagus* beetles have become a model system for the integration of development of morphology, evolution and ecology (e.g. Nijhout & Emlen, 1998; Emlen *et al.*, 2005; Emlen *et al.*, 2007; Parzer & Moczek, 2008; Moczek, 2009a,b). There are several reasons for this rising interest, although one of the main ones is certainly their environmentally-mediated and condition-dependent male horn expression, an extreme case of phenotypic plasticity (Emlen, 1994; Moczek & Emlen, 1999; Moczek, 2006; Moczek, Cruickshank & Shelby, 2006). In all species studied so far, male horns are expressed as a result of an explosive proliferation of specific epidermal regions during the pre-pupal and pupal stages, predominantly triggered by larval feeding conditions: individuals exceeding a critical larval mass moult into large, horned (major) adults, while larvae with access to limited feeding resources eclose at smaller sizes and express very reduced horns or none at all (minor males) (Moczek & Emlen, 1999; Moczek & Emlen, 2000; Moczek, 2009b). In adult *Onthophagus*, evidence of horn polyphenic expression can be detected by means of a horn length – body size sigmoidal scaling relationship, i.e., static allometries based on measurements of con-specific individuals at the same life stage. Since horn expression is related to body growth, which is influenced in turn by larval nutrition, such horn static allometries indirectly depict horn reaction norms (Emlen & Nijhout, 2000), i.e., the full set of horn phenotypic responses to larval nutrition (West-Eberhard, 2003). However, the regulation of horn expression appears extremely evolutionary-labile, to the extent that static horn allometries diverge rapidly between closely related species (Moczek & Nijhout, 2003; Emlen *et al.*, 2007), and even between native and introduced population of the same species (Moczek & Nijhout, 2003). Therefore, individuals appear to differ genetically in the way horn precursors respond to environmental stimuli, and both competitor density-mediated selection for optimal investments into horn expression and drift can act on these genetic differences, producing population- and species-specific allometries (Moczek & Nijhout, 2003; Moczek *et al.*, 2002; Moczek, 2009a,b). Furthermore, some authors (Moczek & Nijhout, 2004; Parzer & Moczek, 2008) proposed an antagonistic co-evolution between horns and male copulatory structures acting through resource allocation trade-offs, highlighting a possible coupling between the diversification of horn static allometries 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).

Onthophagus beetles have undergone a dramatic evolutionary radiation: they are the largest genus of beetles, and one of the most species-rich genera of life on Earth (Emlen *et al.*, 2005). Overall, more than 2000 species have already been described in the genus which originated only 23–33 million years ago (Hanski & Cambefort, 1991; Davis, Scholtz & Philips, 2002; Emlen *et al.*, 2005). The above-mentioned

peculiar evolutionary aspects have most likely contributed to this rapid radiation, and the existence of many species-complexes and evidences of ongoing speciation processes further demonstrate the recent diversification of the genus. Closely related *Onthophagus* sister species (see for example Pizzo *et al.*, 2006a,b) and species-complexes are ideal models to investigate the micro-evolutionary dynamics promoting speciation and excellent material for qualitative/quantitative analyses that could provide additional clues to explain the evolution of morphology in the genus. In this paper, we focused on the morphological variation in the species-complex *Onthophagus (Palaeonthophagus) 'fracticornis-similis-opacicornis'* [viz. *O. similis* (Scriba 1790); *O. opacicornis* (Reitter 1892); *O. fracticornis* (Preyssler 1790)], wherein *O. fracticornis* is considered better differentiated from the other two taxonomical units, which are in turn more closely related (Palestrini, 1981; Martín-Piera & Boto, 1999; Martín-Piera & López-Colón, 2000; Angus, 2008). The systematic rank of *O. similis* and *O. opacicornis* (whether to the species or subspecies level, reviewed in Martín-Piera & Boto, 1999) is still under discussion.

In this study we first reconstructed the phylogenetic relationships among the species of the *Onthophagus fracticornis-similis-opacicornis* complex with a molecular approach (*cox1* sequencing). Then we assessed the extent to which morphological divergence patterns mirrored the phylogenetic relationships at three levels. (1) Horn static allometries diverge rapidly among extant taxa, a process which might trigger changes in the male genital morphology, thus possibly promoting speciation as a by-product (Moczek and Nijhout, 2004; Parzer and Moczek, 2008). We therefore tested the prediction that horn static allometries diverged mirroring the phylogenetic information, by examining horn expression patterns in the three taxa. (2) Previous studies on *Onthophagus* beetles had hinted at the occurrence of a certain degree of developmental correlation between male horns and genitalia (Moczek & Nijhout, 2004; Parzer & Moczek, 2008) and between male horns and head shape (Macagno *et al.*, 2009; Pizzo *et al.*, 2006a,b). Therefore, we assessed the extent to which size and shape divergence patterns of head and paramere -the part of the aedeagus directly involved in coupling with specific female structures during the copula - were congruent with the phylogeny and the inter-specific divergence pattern of horn expression. (2) Lastly, we contrasted these divergence patterns with that of the epipharynx -one of the mouthparts - which is considered not to be subject to costs associated with horn development (Pizzo *et al.*, 2009), and whose morphology could therefore be freer to evolve independently from other traits, possibly under selective pressures for optimal feeding functions (Verdú & Galante, 2004).

The magnitude of intra-specific (i.e., between male morphs) differences of each trait taken into account were also evaluated to highlight how strongly those traits were affected by male polyphenism.

To inspect the morphological variations in the species complex, we used a combination of traditional and geometric morphometric methods, that not only have the ability to detect even subtle

shape differences, but also allow to quantify morphological variations of the trait under analysis treating its shape and size as uncorrelated features (Zelditch *et al.*, 2004).

Materials and Methods

Specimens

Although the systematics of this species complex (i.e., the status of *O. similis* and *O. opacicollis*) had not been fully resolved, each of the taxa has been referred to as ‘species’ for simplicity throughout the paper.

In the summer of 2006, 2007 and 2008, male specimens of *O. fracticornis*, *O. similis*, and *O. opacicollis* were field-collected respectively in pastures of Val Ferret (N-W Italian Alps, ≈1600 m a.s.l.), Teilhède area (Auvergne, France, ≈600 m a.s.l.), and Pisa area (Tuscany, Italy, ≈5 m a.s.l.). We also collected few individuals of each species from other European populations; due to inadequate sampling, they were used to supplement biomolecular analysis only (see Table 1a).

All individuals were unambiguously assigned to one of the three species using standard keys based on external morphological characters (Paulian & Baraud, 1982).

Biomolecular analyses

DNA of each specimen was extracted using Quiagen Dneasy columns from heads ground up with a Tissue Lyser (Quiagen). A fragment of the mitochondrial gene cytochrome oxidase subunit 1 (*cox1*) was amplified and sequenced using the primers *Pat* (5'tccaatgcactaatctgcatatta) and *Jerry* (5'caacatttatttgatttttgg) (Simon *et al.*, 1994).

Sequencing was performed on both strands using a CEQ8000 automated sequencer (Beckman Coulter). Sequences were assembled, edited and aligned with Geneious Pro 4.7.6 software (Rozen & Skaletsky, 2000). Sequences of closely related *Palaeonthophagus* species, courteously provided by Dirk Ahrens, were added to the alignment as outgroup.

Distance-based and maximum parsimony trees were generated using the MEGA 2.1 software (Kumar *et al.*, 2001). Pairwise distances between haplotypes were obtained under the assumptions of the Jukes–Cantor model, and the tree was constructed by the neighbour joining (NJ) method (Saitou & Nei,

1987). Maximum parsimony (MP) phylogenetic tree was based on close-neighbour-interchange (CNI). Robustness of the inferred trees was tested by bootstrapping (Felsenstein, 1985) with 1000 replications.

A phylogenetic tree inferred through a Bayesian approach was constructed by the Markov chain Monte Carlo method as implemented in the MrBayes 3.1.12 package (Ronquist & Huelsenbeck, 2003), using a General Time Reversible (GTR) (Tavare, 1986) model with gamma-distributed rate variation across sites and a proportion of invariable sites. The main analysis was run until the average standard deviation of split frequencies fell below 0.01. Of the sampled parameter values, 25% were summarised to output a cladogram with the posterior probabilities for each split and a phylogram with mean branch lengths. A 50% majority rule consensus tree was then generated and visualised using TreeView 1.6.6 (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Morphometric analyses

In the laboratory, individuals for morphometric analyses were cleaned in 100°C distilled water for 10 min and then dissected. Heads and pronota were separately fixed on horizontally-levelled plasticine supports by completely plunging their convex ventral side. Genitalia (parameres) were extracted, cleared in boiling KOH 5% for 5 min and positioned on wet cotton wool taking care to align their edges on the same horizontal plane. Epipharynxes were treated following the protocol described in Pizzo *et al.* (2009) mounted on microscope slides and covered with coverslips.

2D images of each structure were taken using a Leica Z16Apo stereoscopic dissecting scope (Leica Microsystems AG, Wetzlar, Germany) at magnifications of 57.5x (epipharynx, frontal side; phalloteca, lateral side), 20x (pronotum, frontal side), 50x (*O. similis* and *O. opacicornis* head, lateral side), 31.3x (*O. fracticornis* head, lateral side), 39.4x (*O. similis* and *O. opacicornis* head, frontal side), 25x (*O. fracticornis* head, frontal side). After calibration, linear measurements of pronotum width (used as a proxy for body size: Eberhard & Gutierrez, 1991) and horn length (taken as described in Macagno *et al.*, 2009) were taken with the software LAS v 2.5.0 (Leica Application Suite).

The number of specimens used in each analysis is reported in Table 1b.

Horn static allometry

An earlier study on *O. fracticornis* (Macagno *et al.*, 2009) assessed that a sigmoidal model was a good fit for male horn length-body size data. In addition, much like other *Onthophagus* species (e.g. Emlen, 1994; Moczek, 1998), major (bearing well-developed horns) and minor morphs (expressing rudimental horns or none at all) coexist in the same population. A visual inspection of the distribution of male horn

length-body size data of *O. opacicollis* and *O. similis* shows an analogous pattern of horn expression. For each taxa, the horn static allometry was therefore determined by fitting to the horn length-body size data a

$$y = y_0 + \frac{a \cdot x^b}{c^b + x^b}$$

four-parameter sigmoidal regression in the form $y = y_0 + \frac{a \cdot x^b}{c^b + x^b}$, where x is pronotum width (used as a proxy for body size), y is 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 body size at the point of inflection of the sigmoid curve (commonly used as an estimate of the average body size threshold at which *Onthophagus* males switch from the hornless to the horned phenotype: Moczek *et al.*, 2002; Moczek & Nijhout 2003; Moczek, Brühl & Krell, 2004; Macagno *et al.*, 2009). Parameter values of the regression were obtained via iterations by using Sigma Plot™ (Systat Software Inc.) curve-fitting procedures. The distinction of the three sigmoid curves was inspected by first testing inter-specific differences in mean pronotum width with a one-way ANOVA and LSD post-hoc tests, and in horn length with a Kruskal-Wallis non-parametric ANOVA. Secondly, *O. fracticornis* (having significantly larger pronotum width) was removed from the dataset and a single sigmoidal reference function in the above-mentioned form was fitted to the horn length-body size distribution of *O. similis* and *O. opacicollis* combined. If interspecific differences in mean residual horn lengths from this reference function (i.e., the difference between observed horn length and the horn length expected for each individual on the basis of its body size) are significant, it can be stated that two distinct regressions describe the sample better than a single one. Such differences were assessed with a T-test. Lastly, to examine the degree to which differences in regression parameters (y_0 , a , b , c) explained allometric differences between the three species, repeated Welch's T-tests on these parameters were carried out (Sokal & Rohlf, 1995; Moczek *et al.*, 2002; Moczek & Nijhout 2003). All significance levels were adjusted for multiple comparisons using Bonferroni corrections.

Patterns of shape variation of head, epipharynx and paramere

In some *Onthophagus* species, horn expression has been related to both head (Macagno *et al.*, 2009; Pizzo *et al.*, 2006a,b) and aedeagus (Parzer & Moczek, 2008) variation. Major and minor morphs were kept separate for morphometric analyses.

A landmark-based geometric morphometric approach (Bookstein, 1991; Rohlf & Marcus, 1993; Adams, Slice & Rohlf, 2004) was used to characterise the shape of heads, epipharynxes, and parameres, and to inspect their patterns of variation between morphs and supposed species separately from data on their sizes. All landmarks were digitalised by the same person using TpsDig 2.10 (Rohlf, 2006) on the left side of the structures, with the aim of removing any bias possibly caused by bilateral asymmetry. For each structure, the landmark configuration (Fig. 1) was chosen following criteria of homology (Bookstein, 1991), detection ease, and on the basis of the available sample sizes (i.e., taking care that sample size was larger than the number of landmark coordinates).

Landmark configurations of each structures were superimposed with a full Procrustes fit (Rohlf & Slice, 1990; Goodall, 1991), i.e., they were translated to a common origin, scaled to unit centroid size and rotated to best fit using a least-squares criterion. The resulting coordinate configurations in a non-Euclidean (Kendall's) shape space (Rohlf, 1996) were then projected into a linear tangent space by orthogonal projection (Dryden & Mardia, 1998). To inspect patterns of intra- and inter-specific shape variation between pre-defined groups, we first performed a canonical variate analysis (CVA) on Procrustes coordinates. Secondly, we computed Mahalanobis pairwise distances between groups (measures of differences between groups relative to the within-group variation: Klingenberg & Monteiro, 2005) and assessed their significance with permutation tests (10,000 iterations). As a further test of the reliability of inter-specific differences highlighted by the CVA, for each structure pairwise classification functions were derived to assign individual specimens to the pre-defined species based on shape differences; the accuracy of classifications was evaluated through cross-validation, in which a subset of specimens is left out of the "training set" used to form the discriminant function (Polly & Head, 2004); significant departures from random classification were assessed using a randomization test with 1,000 iterations. All previously described analyses as well as the visualization of deformation grids allowing a description of shape variation were performed using MorphoJ (Klingenberg, 2008).

Head, epipharynx, paramere static allometries

The centroid sizes (CS) of each landmark configuration (computed in MorphoJ: Klingenberg 2008) were used as an estimate of epipharynx and paramere size. For the head, images were acquired at different magnifications and CS were therefore made comparable between species by dividing them by the photo magnification.

For each species, a preliminary inspection of scatterplots of log-transformed pronotum width vs log-transformed head, epipharynx, and paramere size did not reveal any significant deviation from linearity. Therefore, linear regression models were used to fit these distributions. Kolmogorov-Smirnov tests were used to check the assumption of normality, and the assumption of linear scaling was further tested by checking the absence of visible trends in the scatterplot of regression standardized predicted values vs residuals (Sokal & Rohlf, 1995). For each structure, the homogeneity of regression coefficients between the three species were assessed including the interaction of log(pronotum width) and species in an analysis of covariance (ANCOVA) (Engqvist, 2005). Given the non-significance of these interactions, interspecific differences in the intercept of regressions were tested with an ANCOVA holding species as a fixed factor. Levene's tests (Levene, 1960), were used to test the assumption of homoscedasticity. All analyses were conducted in SPSS 13.0.

Results

Phylogenetic reconstruction

We aligned 601 bp sequences resulting in a combined matrix with 122 parsimony informative characters; the mean uncorrected p-distance between any two sequences was 0.0813. The closest phylogenetic relationship was between *O. opacicolis* and *O. similis* (p-distance= 0.062) whereas *O. fracticornis* was more differentiated (p-distance= 0.087 from *O. opacicolis* and 0.098 from *O. similis*). Distances of each species respect to the out-group are all in the same range of magnitude (0.1). Intra-specific p-distances are always much lower than inter-specific ones (*O. fracticornis* = 0.019, *O. opacicolis* = 0.005, *O. similis* = 0.006).

MP analysis generated a large number of equally parsimonious trees resulting in a consensus tree with main branches supported with high bootstrap values; for parsimony informative sites, the consistency index (CI) was 0.78, the retention index (RI) 0.96 and the rescaled consistency index (RCI) 0.74; tree length was 155.

The trees generated by the different methods showed the same basic topology, with very slight differences in the resolution of terminal clades; the *fracticornis-similis-opacicolis* sample split in three well supported clades with high bootstrap values (NJ and MP analyses, Fig. 2) or posterior probabilities (Bayesian analysis, Fig. 3).

Horn static allometries

An ANOVA on mean pronotum width and subsequent LSD post-hoc tests showed that *O. fracticornis* was significantly bigger (mean pronotum width: 3.76 ± 0.35 mm) than *O. similis* (3.35 ± 0.28) and *O. opacicolis* (3.41 ± 0.22) ($F_{2,94}=18.4$, $P<0.001$), while the latter two species had comparable size ($P>0.05$). On the other hand, a Kruskal-Wallis non parametric ANOVA failed to detect any difference in horn length across the three species ($\chi^2_2=2.78$, $P=0.25$). However, after removing *O. fracticornis* from the dataset and fitting a horn length-body size sigmoid regression to *O. similis* and *O. opacicolis* combined, a significant difference between mean residuals of the two species ($T_{43}=2.95$, $P<0.01$) highlighted their different horn allometric scaling. Horn length-body size scaling relations are shown in Fig. 4, and sigmoid regression parameters of the three species are reported in Table 2. Pairwise comparisons conducted with Welch's T-tests showed that the three species have diverged significantly one from another with respect to the body size at the point of inflection of the sigmoid curve, while horn length range, minimum horn length and the slope coefficient did not vary significantly across species.

Pattern of shape variation of head, epipharynx and paramere

Results of the CVA conducted on paramere, epipharynx and head as well as the deformation grids showing shape modifications described by the first two canonical variates are shown in Fig. 5. Patterns of inter-specific shape variation of paramere were consistent with that of the head: *O. fracticornis* was clearly distinct along CV1 from *O. opacicornis* and *O. similis* for having a considerably longer apex of paramere and a more pointed clypeus. On the other hand, *O. opacicornis* and *O. similis* showed a weaker, but nonetheless significant, differentiation pattern of both structures along the CV2: differences regarded mainly the bending of the apex of paramere (directed upward in *O. similis*, downward in *O. opacicornis*) and the shape of the head (*O. opacicornis* having a more compressed clypeus, wider genae, thinner eyes and, overall, a more sinuate profile). While these analyses detected a consistent gap between *O. fracticornis* and *O. similis* - *O. opacicornis*, the variation between the latter two species was more properly described as a continuum in the morphospace. On the contrary, epipharynx had a rather different inter-specific differentiation pattern. Specifically, *O. opacicornis* appeared distinguishable from *O. fracticornis* and *O. similis* on the basis of epipharynx shape, while the latter two species partly overlapped in the CVA plot.

Pair-wise cross-validated percentages of correct classifications of individual specimens in the pre-defined species confirmed that inter-specific differences evidenced by the CVA were significant for each structure: results obtained were significantly different from random classification in all cases ($P < 0.001$). For the head, percentages varied between 100% and 97%, except in the contrast between *O. opacicornis* and *O. similis* (94% of *O. opacicornis* and 77% of *O. similis* were correctly classified); a similar situation was found for the parameres, where the only case in which the percentage of correct classification was lower than 100% was that between *O. opacicornis* and *O. similis* (respectively 90% and 80%). For the epipharynx, percentages of correct classifications were higher than 90%, except in the contrast between *O. fracticornis* and *O. similis* (73% and 79%, respectively).

As for intra-specific comparisons between male morphs, paramere and epipharynx shapes appeared not affected by male horn polyphenism (no shape differences were detected between major and minor males of the same species), while in each species major and minor males were consistently different in head shape (Table 3). Furthermore, intra-specific distances were always lower than inter-specific ones for both epipharynx and paramere. Head shape, however, showed a more complex pattern: the distance between minor and major *O. fracticornis* was about one third of its inter-specific distances respect to *O. opacicornis* - *O. similis*; on the contrary, distances between major and minor morphs within *O. opacicornis* and *O. similis* (which are comparable to intra-specific distances reported for *O. fracticornis*) were approximately of the same magnitude as their inter-specific distances (Table 3).

Head, epipharynx, paramere static allometries

Plots of log-transformed pronotum width vs log-transformed centroid size of paramere, epipharynx, and head of the three species are reported in Fig. 6. Levene's ($P>0.14$ in all instances) and Kolmogorov-Smirnov tests ($P>0.70$ in all instances) ensured that the adjustment of all allometric functions to a linear scaling in the form $y=y_0+ax$ was statistically correct. However, in the three species, the percentage of variation of head and epipharynx size explained by body size was considerably higher than that of the paramere (see adjusted R^2 in Table 4). For each structure, no interspecific differences in the slope of the regression lines (term a in Table 4) were detected, the interactions of $\log(\text{pronotum width}) \times \text{species}$ in the analyses of covariance being constantly not significant ($P>0.65$). However, with full-factorial ANCOVAs, significant differences across the three species were found in the intercept of paramere ($F_{2,49}=127.29$, $P<0.001$) and head allometry ($F_{2,67}=21.87$, $P<0.001$). Specifically, *O. opacicolis* had substantially larger paramere than *O. similis* and *O. fracticornis* (the latter two being of comparable size: $F_{1,34}=2.16$, $P=0.15$), while the head of *O. fracticornis* was slightly larger than the one of *O. similis* and *O. opacicolis*, which instead did not differ from each other ($F_{1,41}=0.99$, $P=0.33$) (Table 4).

Discussion

Phylogenetic reconstruction. *Cox1* sequencing and the relative phylogenetic reconstruction for the *fracticornis-similis-opacicolis* species-complex identified three main independent mitochondrial lineages, each including only specimens belonging to one morphologically defined species. Tree topologies and genetic distances supported the hypothesis that the three taxa were good species. The phylogenetic pattern was characterized by significant differences between *O. fracticornis* and *O. opacicolis* - *O. similis*, along with less marked differences between *O. opacicolis* and *O. similis*, which appeared as sister species. Our results also showed that the three species were monophyletic, without any evidence of introgression among mitochondrial clades, neither of historical hybridisation among them, in keeping with the karyotypic studies carried out by Angus (2008), who strongly suggested that these taxa function as reproductively isolated entities.

In his cytogenetic comparisons, Angus (2008) didn't detect any sign of hybridisation between the two species not only in allopatry but also in Spanish sympatric populations. This evidence of a complete genetic isolation appears in contrast with the findings on allozymes of Martín-Piera and Boto (1999), who concluded that the presence of shared allozyme alleles indicated some introgressive exchange of genetic material between *O. similis* and *O. opacicolis*. Differently, Angus (2008) interpreted allozymatic results as an effect of alteration by natural selection, by considering that genetically related species should respond to the same selective pressures in similar ways. We think that DNA data on Spanish material of *O. similis*

and *O. opacicornis* will deserve to be carefully reconsidered, but this is outside the scope of the present paper.

(1) *Divergence pattern of horn static allometry.* The species complex analysed in this paper showed distinct, species-specific horn static allometries (Fig.4). The body size at the point of inflection of the sigmoid curves (that have been often used as an estimate of the transition between minor and major morphs: Moczek *et al.* 2002; Moczek & Nijhout, 2003; Moczek *et al.*, 2004; Macagno *et al.*, 2009) was smallest in *O. similis*, and increased progressively in *O. opacicornis* and *O. fracticornis*. *O. fracticornis* was further distinguished from *O. opacicornis* and *O. similis* by its larger body size. This pattern was in keeping with the phylogeny reconstructed in this study, and perfectly congruent with genetic distances (*O. opacicornis*-*O. similis* p-distances=0.062; *O. fracticornis*-*O. similis* =0.098; *O. fracticornis*-*O. opacicornis* =0.087).

(2) *Divergence pattern of head and paramere shape and allometry.* CVA analyses indicated that head shape differences between species were significant and their magnitude was in keeping with phylogenetic relationships. Head static allometries provided a partially congruent pattern; they did not diverge between *O. similis* and *O. opacicornis* (in keeping with their close phylogenetic relationship), whereas *O. fracticornis* appeared to have a slightly larger head relatively to body size.

Within each species, major and minor males had different head shapes, supporting the idea that the developmental processes that boost horn expression are able to affect other areas of the head besides the vertex carina, as previously pointed out for other *Onthophagus* species (Pizzo *et al.*, 2006a,b; Macagno *et al.*, 2009). In *O. fracticornis*, the intra-specific (between male morphs) shape distance was about one third of interspecific distances between *O. opacicornis* and *O. similis*; on the contrary, in *O. opacicornis* and *O. similis* intra-specific distances between major and minor morphs (which are comparable to intra-specific distances reported for *O. fracticornis*) were approximately of the same magnitude as their inter-specific distances, in keeping again with their close phylogenetic relationship. It is interesting to remark that the three species, which are well distinguishable with respect to their polyphenic developmental processes (i.e. their horn static allometries), showed a less marked shape and size divergence, even for a trait (the head, in this case) strongly correlated to polyphenism.

Moczek and Nijhout (2004) and Parzer and Moczek (2008) demonstrated the presence of a developmental trade-off between horns and genitalia in polyphenic *Onthophagus* species, and claimed that evolutionary changes in horn expression may affect genital morphology. In our study, paramere shape confirmed that *O. fracticornis* was well separated from the other two species, which were in turn more closely related to each other, in accordance with horn static allometries and phylogeny. However, within each species, male polyphenism seemed not affect paramere shape (i.e. no differences between major and

minor paramere shape). Therefore, in this species-complex, a developmental trade-off between male horn and genitalia within each species, if present, did not appear to affect paramere shape, but might affect other aspects of paramere morphology (size) or other parts of the aedeagus (the phallobase). As the divergence pattern of paramere shape was in keeping with the phylogenetic relations of the complex, both their significant inter-specific shape divergence and the absence of within-species shape variability appeared in agreement with the lock-and-key hypothesis (Eberhard, 1985; Sota & Kubota, 1998).

Paramere size showed a pattern not consistent with that of shape, with *O. similis* and *O. fracticornis* being equal size, whereas the paramere of *O. opacicollis* was considerably larger. With very low R^2 and slopes, paramere allometries also appeared different from those of the other structures taken into account, in keeping with a trend which is common to most insect genitalia analysed so far (Eberhard *et al.*, 2009). The 'one-size-fits-all' hypothesis proposed by Eberhard *et al.* (1998) attempted to explain the adaptive significance of low allometric slopes in genitalia, proposing that genital size does not change substantially with body size due to the advantage of males having genital sizes that are appropriately adjusted to the most common size of females in the population. These size adjustments might improve male ability to stimulate the female and gaining higher reproductive success due to sexual selection by cryptic female choice (Eberhard *et al.*, 1998; Tatsuta, Mizota & Akimoto, 2001). *Onthophagus* parameres are coupled with sclerotised areas of female pygidium during copulation (Werner & Simmons, 2008), but no studies have checked for the presence of specific receptors in that female anatomical area so far. Conversely, these size adjustments may simply facilitate mechanical fits between male and female genitalia that are needed to transfer sperm (House and Simmons 2003). The low R^2 of paramere static allometries highlighted in this study also point out that genital size does not respond to the same body size-dependent developmental inputs acting on other body traits. As a matter of fact, paramere size appears only weakly correlated with body size, suggesting that it can vary to a certain degree between individuals of the same species (i.e., regardless of their body sizes). Selection on genital size (either stabilizing or directional) should affect genital allometry depending on the strength of the correlation between body and paramere size (Eberhard, Rodriguez & Polihronakis, 2009). Therefore, its effects should be more pronounced in *O. fracticornis* (where this correlation is stronger) than in the other species. Furthermore, since this correlation is virtually absent in *O. opacicollis*, this lineage should be less constrained by selective effects on paramere allometry, and possibly be freer to diverge in absolute paramere size. The comparison between paramere static allometries across species highlighted a remarkably high degree of divergence between *O. opacicollis* and both *O. similis* and *O. fracticornis* – an unexpected result, given that even though relative genital size does occasionally diverge among closely related species (Lux, 1961; Schuh, 1984; Hormiga & Scharff, 2005), the general evolutionary trend in genitalia is to diverge rapidly in shape and not in size (Eberhard *et al.* 1998; Eberhard *et al.* 2009). However, Eberhard (2009) pointed out that in a wide range of animals genitalic size and shape seem to be at least partially uncoupled genetically, and therefore it may not be surprising that

paramere size and shape in this complex could evolve independently. Furthermore, the divergence in size of *O.opacicollis* and *O.similis* might have emphasized their weaker separation at the shape level, providing a reinforcement of morphological divergence of copulatory structures between the two closest lineages in the complex.

(3) *Divergence pattern of epipharynx shape and allometry.* In a previous work, the epipharynx has been pointed out as a useful tool for revealing differentiation between closely related *Onthophagus* species (Pizzo *et al.*, 2009). Being used to filter and select food particles, it plays an extremely specific feeding role, and its morphological variations have been correlated to the type of dung beetles primarily feed on (Verdú & Galante, 2004). Furthermore, the absence of covariation with general head shape and the lack of trade-off with horn development highlighted in a previous study (Pizzo *et al.*, 2009), together with the absence of shape variation between male morphs, indicate that the epipharynx is a developmentally canalised trait, and that therefore it could be free to evolve independently from the other structures taken into account. The allometric relationships between epipharynx and body size did not reveal any significant difference among the three species, possibly indicating the strict evolutionary relationships among the members of the complex and a phylogenetic inertia of the underlying ontogenetic processes, or a shared effect of stabilising selection for optimal size. However, its inter-specific shape variation pattern of the epipharynx was completely uncoupled from that of paramere and head shape and horn allometries, and more similar to that of paramere size. In this case, *O. opacicollis* emerged as the most divergent species, and the considerably lower degree of differentiation between *O. fracticornis* and *O. similis*, which have been identified as the two most differentiated lineages, suggested that its modifications might rely more on adaptive and **selective** than on phylogenetic causes. Specifically, the clear shape modifications of *O. opacicollis* epipharynx hint at the possibility that adaptations to different trophic resource use might have driven its divergence process. The three species have been described to feed on the same types of dung (Martín-Piera & López-Colón, 2000), but to our knowledge no studies have been made to assess whether these species have different preferences (e.g. cattle, horse, carnivore dung), or whether they use dung pads at different times (feeding, for example, on more or less hydrated or decomposed dung). Dung pads are not only feeding but also mating resources for *Onthophagus* beetles (Halffter & Matthews, 1966): such a mechanism of resource-dependent timing or spatial segregation could provide a means of divergence between *O. similis* and *O. opacicollis*. Given the strict association of *Onthophagus* beetles with their feeding resources, the evolution of their mouthparts in relation to feeding strategies deserves to be further investigated, since it could provide further clues to explain the exceptional evolutionary radiation of the genus.

Conclusive remarks.

Size and shape interspecific divergence mirrored phylogeny in the *Onthophagus fracticornis-similis-opacicollis* species-complex only in part. Divergence patterns of horn static allometries, pronotum size, paramere shape and head shape differences mirrored the evolutionary relationships pointed out by phylogenetic analyses of the *cox1* sequences. On the contrary, epipharynx shape and paramere size showed a divergence patterns not consistent with the phylogenetic relationships within the complex.

Overall, our results showed that distinct morphological traits differ in their tendency to mirror phylogenetic relationships and to evolve in concert, possibly depending on differences in the strength of their developmental integration and canalization, their evolvability (horn and paramere static allometry, static allometry) or their dependence on adaptive pressures and selection regimes (i.e. epipharynx shape) (Minelli, 2003, 2009; Moczek 2009a,b). Furthermore, we showed that size and shape of the same trait can evolve independently from one another among species, and this might indicate that a genetical uncoupling of size and shape occurs in both genital and non-genital structures.

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Figures legends

Figure 1. Landmark configurations chosen for geometric morphometric analyses of head (first row, 9 landmarks) epipharynx (middle row, 9 landmarks) and paramere (last row, 5 landmarks). Left column: *O. fracticornis*; central column: *O. similis*; right column: *O. opacicollis*. For easier visualisation, landmark configurations are reported only on specimens of *O. similis*.

Figure 2. NJ (left) and MP (right) consensus trees of mtDNA *cox1* sequences. Bootstrap values upper then 70% are shown on internal nodes. OF= *O. fracticornis*, OO= *O. opacicollis*, OS= *O. similis*. Specimens belonging to *O. vacca* and *O. nuchicornis* European populations were used as an outgroup (indicated as *Palaeonthophagus spp.* in the trees, courtesy of Dirk Ahrens).

Figure 3. The 50% majority rule consensus phylogenetic tree from the Bayesian MCMC (MrBayes) analysis of mtDNA *cox1* sequences with posterior probabilities $P > 70\%$ for internal node are shown. OF= *O. fracticornis*, OO= *O. opacicollis*, OS= *O. similis*. Specimens belonging to *O. vacca* and *O. nuchicornis* European populations were used as an outgroup (indicated as *Palaeonthophagus spp.* in the trees, courtesy of Dirk Ahrens)

Figure 4. Horn length-body size scaling relations. White triangles: *O. similis*. White circles: *O. opacicollis*. Black circles: *O. fracticornis*. For each species, the horn static allometry was computed by fitting to the horn

length-body size data a four-parameter sigmoidal regression in the form $y = y_o + \frac{a \cdot x^b}{c^b + x^b}$. Parameter values and interspecific comparisons are reported in Table 2.

Figure 5. Intra- and interspecific shape differences of paramere, epipharynx and head. Circles=*O. fracticornis*, triangles=*O. similis*, diamonds=*O. opacicollis*; black symbols=major morphs, white symbols=minor morphs. For each structure, the deformation grids showing shape modifications described by the first two canonical variates are reported.

Figure 6. Paramere, epipharynx and head allometries. White triangles: *O. similis*. White circles: *O. opacicollis*. Black circles: *O. fracticornis*. The centroid size of each landmark configuration (Fig. 1) is used as an estimate of the size of each structure. Inter-specific comparisons of slope and intercept are given in Table 4.

Tables

Table 1. Number of specimens of each species used in this study. **1A.** Geographic origin and number of specimens used in biomolecular analyses. **1B.** Number of specimens used for morphometric analyses.

Table 1A.

Species	Geographic origin	Number of specimens
<i>O. fracticornis</i>	Val Ferret, N-W Alps, Italy	3
	Veneto, N-E Alps, Italy	1
	Molise, Apennine, Central Italy	3
	Romania	2
<i>O. similis</i>	Teillhède, Massif Central, France	4
	Liège, Belgium	1
<i>O. opacicollis</i>	Pisa, Tuscany, Italy	3
	Sicily loc. 1 , Italy	4
	Sicily loc. 2, Italy	4
	Sardinia, Italy	1
	Corsica, France	3
	Central Greece	1

Table 1B.

Species	Static allometries				Shape		
	Horn	Paramere	Epipharynx	Head	Paramere	Epipharynx	Head
<i>O. fracticornis</i>	52	18	28	27	20	30	30
<i>O. similis</i>	29	19	28	28	20	29	30
<i>O. opacicollis</i>	16	16	16	16	20	16	16

Table 2. Interspecific comparisons of parameters of horn length-body size sigmoid allometries. Parameters of sigmoidal regression (a = range of horn length in the sample, b = slope coefficient, c = body size at the point of inflection of the sigmoid curve, y_0 = minimum horn length) are reported \pm their SE. Within each line, values followed by the same letter do not differ significantly between species (significance was assessed at $P < 0.05$ with multiple Welch's T-tests including Bonferroni correction).

Parameter	Species		
	<i>O. fracticornis</i>	<i>O. similis</i>	<i>O. opacicollis</i>
a	1.41 \pm 0.13 a	1.16 \pm 0.22 a	1.42 \pm 0.20 a
b	25.58 \pm 4.77 b	21.39 \pm 7.89 b	44.06 \pm 13.22 b
c	3.88 \pm 0.03 c	3.26 \pm 0.06 d	3.55 \pm 0.03 e
y_0	0.73 \pm 0.05 f	0.53 \pm 0.14 f	0.66 \pm 0.07 f

Table 3. Intra- and interspecific mahalanobis pairwise distances across major and minor males of the three species, resulting from geometric morphometric analyses of paramere, epipharynx and head. Intraspecific contrasts are reported in italics. Significance is marked at $P<0.05$ (*) or $P<0.01$ (**) (permutation tests, 10,000 iterations). Of=*O. fracticornis*; Oo=*O. opacicollis*; Os=*O. similis*;

MA=major males; mi=minor males.

Mahalanobis pairwise distances					
	Of, mi	Oo, MA	Oo, mi	Os, MA	Os, mi
Paramere					
Of, MA	<i>1.5745</i>	8.8206**	9.1615**	7.7818**	8.1795**
Of, mi	--	9.5649**	9.8879**	8.2844**	8.6529**
Oo, MA		--	<i>1.0419</i>	2.3800**	3.5965**
Oo, mi			--	2.6714**	3.7455**
Os, MA				--	<i>1.8200</i>
Epipharynx					
Of, MA	<i>1.7081</i>	4.7845**	5.4642**	2.3632**	3.3043**
Of, mi	--	4.1607**	4.9639**	2.2045**	2.6682**
Oo, MA		--	<i>1.5550</i>	3.9413**	3.9179**
Oo, mi			--	4.4593**	4.1936**
Os, MA				--	<i>1.9848</i>
Head					
Of, MA	<i>2.5221**</i>	7.7702**	7.1126**	6.0894**	7.4449**
Of, mi	--	7.2778**	6.2234**	5.8999**	7.2065**
Oo, MA		--	<i>4.1200**</i>	4.6903**	4.4551**

Oo, mi	--	3.1835**	4.3689**
Os, MA		--	2.8271**

Table 4. Interspecific comparisons of parameters of paramere, epipharynx and head allometries.

Parameters of linear regressions between log-transformed measures of pronotum width and centroid size (CS) of each structure (a = slope, y_0 = intercept, Adj. R^2 = adjusted R^2) are reported \pm their SE. Within each line, values followed by the same letter do not differ significantly from the other species. Significance of interspecific differences ($P < 0.05$) were assessed with analyses of covariance, respectively including (a) or excluding (y_0) the interaction of $\log(\text{pronotum width}) \times \text{species}$.

Parameter	Species		
	<i>O. fracticornis</i>	<i>O. similis</i>	<i>O. opacicollis</i>
Paramere			
a	0.25 \pm 0.06 a	0.26 \pm 0.10 a	0.15 \pm 0.08 a
y_0	2.56 \pm 0.03 b	2.56 \pm 0.05 b	2.67 \pm 0.04 c
Adj. R^2	0.49	0.25	0.12
Epipharynx			
a	0.88 \pm 0.06 d	0.90 \pm 0.09 d	0.93 \pm 0.10 d
y_0	2.29 \pm 0.03 e	2.29 \pm 0.05 e	2.26 \pm 0.05 e
Adj. R^2	0.90	0.78	0.86
Head			
a	1.02 \pm 0.04 f	1.01 \pm 0.04 f	1.07 \pm 0.09 f
y_0	1.66 \pm 0.02 g	1.64 \pm 0.02 h	1.61 \pm 0.05 h
Adj. R^2	0.97	0.96	0.89

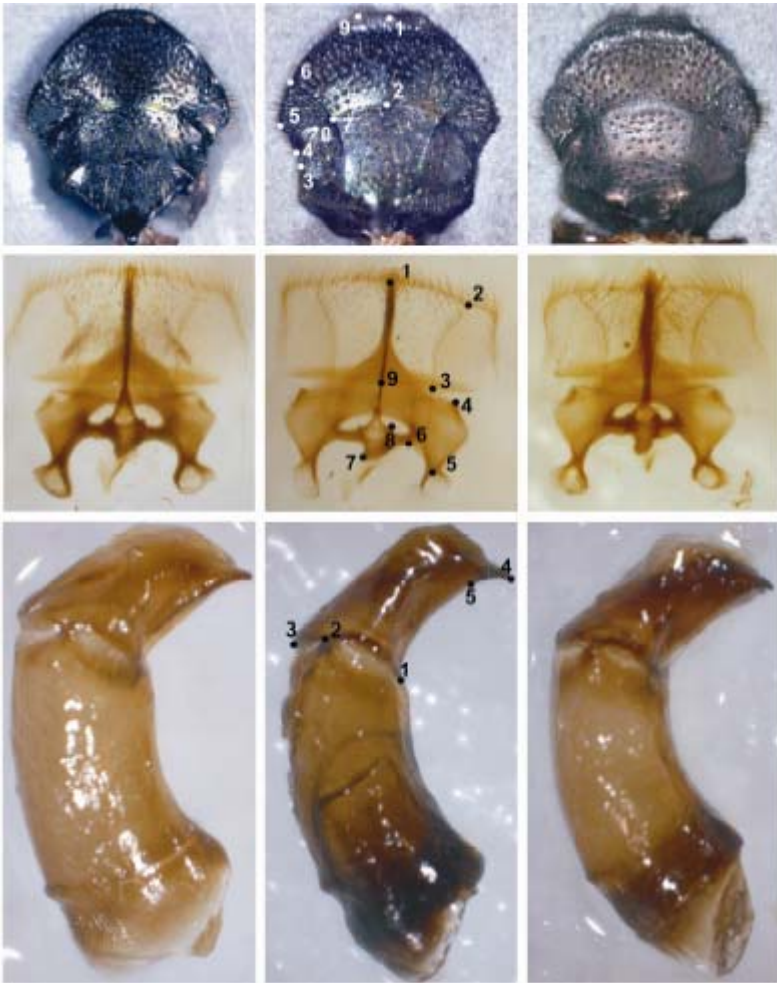


Figure 1

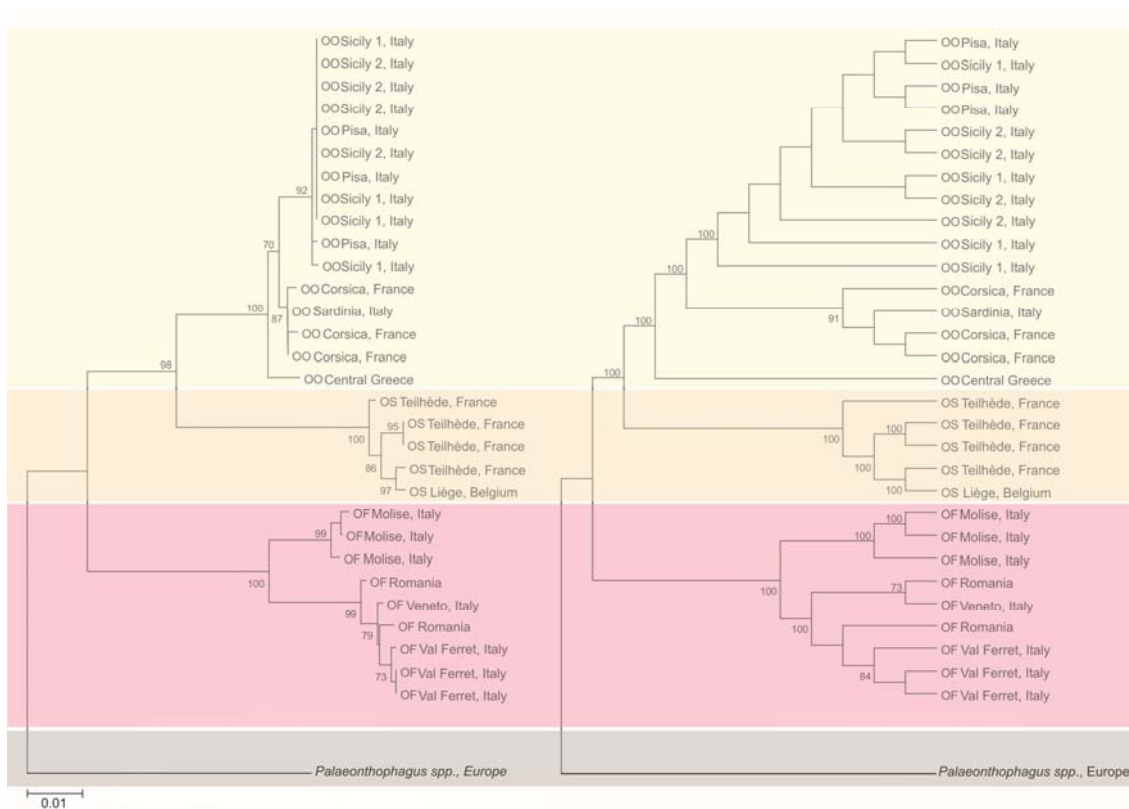


Figure 2

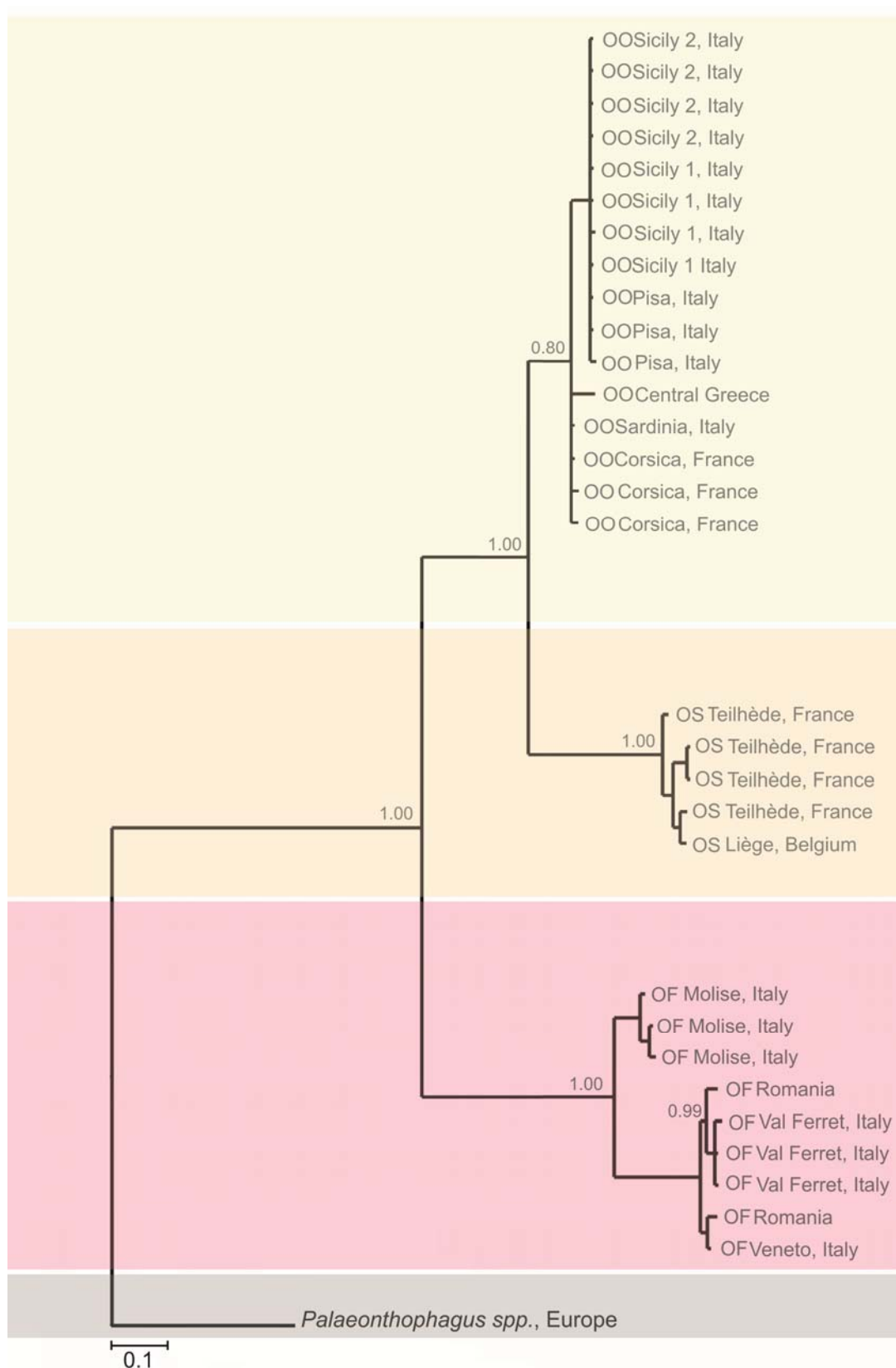


Figure 3

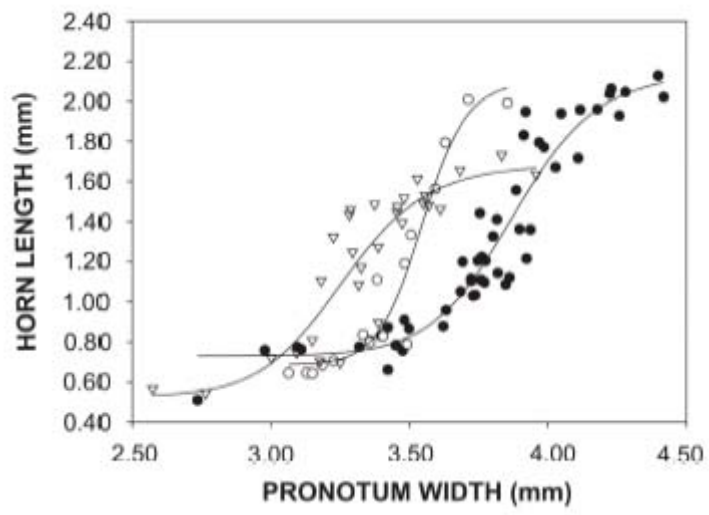


Figure 4

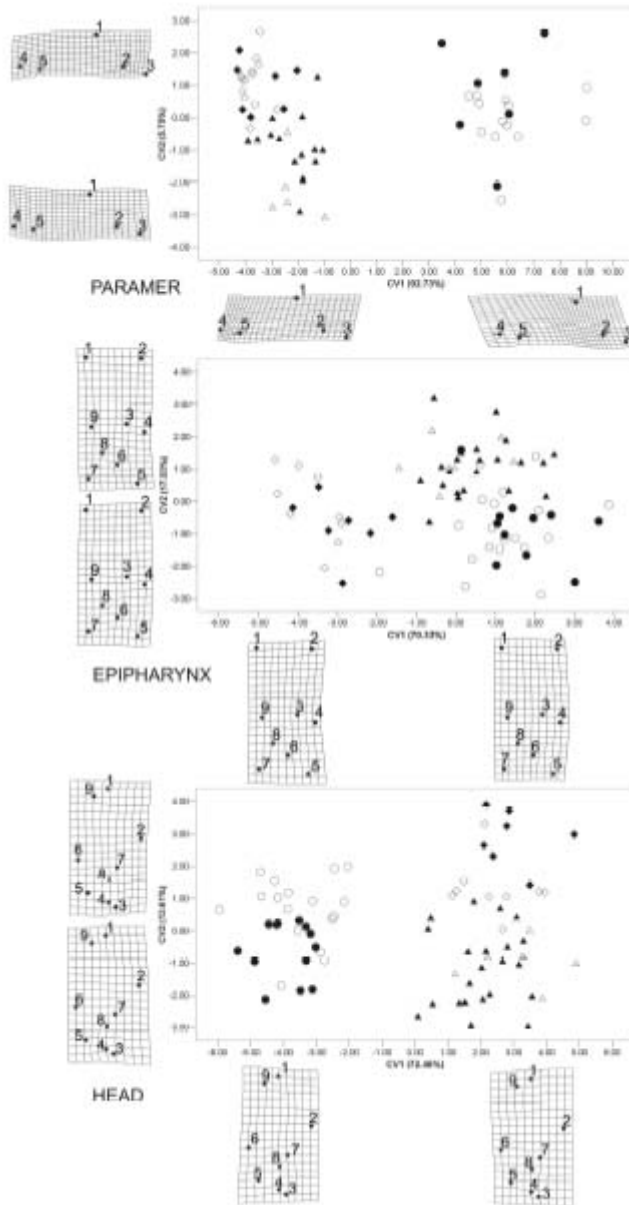


Figure 5