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**Combination of geometric morphometric and genetic approaches applied to a debated taxonomical issue: the status of *Onthophagus massai* (Coleoptera, Scarabaeidae) as an endemic species vicarious to *Onthophagus fracticornis* in Sicily.**

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## Abstract

The present study deals with the phenomenon of insular speciation and discusses, as a case study, the debated taxonomical issue of the status of *Onthophagus massai* (Coleoptera, Scarabaeidae) as an endemic species vicarious to *O. fracticornis* in Sicily. The authors investigated the differentiation patterns between an insular population belonging to the supposed species *O. massai* (collected in its *locus typicus*, Piano Battaglia) and three Italian *O. fracticornis* populations (collected along a N-S latitudinal gradient). These patterns have been described and analysed using multiple approaches: the qualitative inspection of the microsculpture of elytral surfaces, considered a diagnostic character for *O. massai* identification; the comparison of horn static allometries, known to be a good indicator of divergence processes between closely related species or isolated populations of the same species; the comparison of the patterns of shape and size difference of the head, epipharynx and genitalia attained with a combination of traditional and geometric (landmark and semilandmark) morphometric methods, and, finally, the estimation of the genetic relationships between Sicilian and continental populations obtained by analysing COI mitochondrial gene sequences. The integration of the results of these approaches indicates that there is not sufficient evidence to vindicate the species status for *O. massai*, which should more likely be considered a small-sized version of *O. fracticornis* (a possible case of insular dwarfism). However, the complex pattern of shape, size and genetic variation observed between the populations analysed hinted at the possibility that a diversification process is ongoing, but not only between insular and continental populations; each population showed a tendency to evolve as an evolutionary independent unit.

## Introduction

Islands cover a low percentage of the global land area and even if, in general, they show less specific biodiversity with respect to continental areas, new species are continuously described and a great part of them are endemics (Croft et al., 2006; Cucchi et al., 2006; Grill et al., 2007; Lohman et al 2010; Perez-Gelabert, 2008). Due to their restricted habitat and vulnerability, endemic species can easily become endangered or undergo extinction, and islands are among the ecoregions which have the highest percentage of extinct and extant endemic taxa; for this reason, the scientific community has been called upon to make a special effort in estimating, describing and preserving island biodiversity (Dapporto and Dennis, 2008).

On islands, scientists have often documented the acquisition of adaptive features - e.g. a tendency towards flightlessness or flight reduction, lifecycle acceleration and body size changes - which may take place rapidly and could accelerate insular speciation. These phenomena, primarily described for mammals, are not rare in birds (Grant and Grant, 1996; Trewick, 1997) and are also described for insects (Vernon, 1981, Salomon, 2001). In some cases, especially when islands are very close to, or have been in physical connection with, the continent at some stage, the endemic fauna inhabiting the island could be the results of a secondary isolation, accompanied by the extinction of continental populations.

In this study we consider the case of *Onthophagus massai* (Coleoptera, Scarabaeidae), a supposed species endemic to Sicily belonging to the so-called “*fracticornis-opacicollis-similis*” species complex (Martín-Piera and Boto, 1999); *Onthophagus massai* has been described as a separate species, vicarious with respect to *O. fracticornis*, by Baraud in 1975 (Baraud, 1975, 1992), on the basis of the study of material from Piano Battaglia, a locality in the Madonie natural park, but its specific status is controversial (Palestrini, 1981).

In his works, Baraud (1975, 1992) considered the microsculpture of the elytral interstriae as a diagnostic character to differentiate *O. massai* from *O. fracticornis*: *O. fracticornis* should show a prominent granulation and a total absence of punctuation, whereas *O. massai* has small presetal granules and marked perisetal punctuation (Baraud, 1992, Falahee and Angus, 2010). Additionally, the two species should be different in the shape of the parameres of the aedeagus. Palestrini (1981) refuted the species status for *O. massai*, ascertaining it could be considered a small-sized variant of *O. fracticornis*. The slight differences in the apical region of parameres which, following Baraud, are characteristic of this taxon, according to Palestrini (1981) may be found occasionally in other, small individuals from Italian, Greek and Anatolian populations. Moreover, Palestrini (1981) did not find any differences in the endophallus sclerites (i.e. the “lamella copulatrice”), nor in female genitalia between Sicilian and continental populations.

Coope (2001) reported the identification in fossil materials from the Last Interglacial in England, at Trafalgar Square, of at least 49 specimens of *O. massai*, on the basis of their small size, elytral puncturation and tenebrosity. A recent study based on karyotype analysis (Falahee and Angus, 2010) evidenced significant differences between Sicilian and continental samples of *O. fracticornis* (in RCL for autosome 1, 5, 8 and 9, in CI for autosome 1, 5, 7-9, and in centromere position for X and Y chromosomes). These authors suggested that this comparison revealed a degree of difference vindicating the placing of *O. massai* as a separate species.

The species complex to which *O. massai* should belong includes at least three different species well recognisable in allopatry (*O. fracticornis*, *O. opacicollis*, *O. similis*) (Angus, 2008; Macagno et al., in press), and it might hide other taxa (Martín-Piera and Boto, 1999; Falahee and Angus, 2010, Pizzo et al., unpublished data). This is not a rare condition in this group, because *Onthophagus* beetles have undergone a dramatic evolutionary radiation making them the largest genus of beetles, and one of the most species-rich genera of life on Earth (Hanski and Cambefort, 1991; Davis, Scholtz and Philips, 2002; Emlen et al., 2005). Their environmentally-mediated and condition-dependent male horn expression, an extreme case of phenotypic plasticity (Emlen, 1994; Moczek and Emlen, 1999; Moczek, 2006; Moczek, Cruickshank and Shelby, 2006), is probably one of the key reasons for this rapid radiation; the existence of many species-complexes and the evidence 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 (Macagno et al., in press) are ideal models to investigate the micro-evolutionary dynamics promoting speciation and represent excellent material for qualitative/quantitative analyses that could provide additional clues to explain the evolution of morphology in the genus.

With the aim of resolving the species status of *O. massai* as vicarious to *O. fracticornis* in Sicily, and underlining the complexity of the phenomenon of insular differentiation, we investigated the pattern of diversification between Sicilian and other Italian populations of *O. fracticornis* with a combination of different approaches, by assessing **1**) the value of qualitative differences in the microsculpture of elytral interstriae as a diagnostic character for *O. massai* identification; **2**) the pattern of horn static allometries: as horn allometries are known to diverge rapidly among extant taxa, even between closely related species or isolated populations of the same species (Moczek et al., 2002; Moczek and Nijhout, 2003), different allometries are often optimal indicators of a divergence process.

Moreover, previous studies on *Onthophagus* beetles have hinted at the occurrence of a certain degree of developmental correlation between male horns and genitalia (Moczek and Nijhout, 2004; Parzer and Moczek, 2008) and between male horns and head shape (Macagno et al., 2009;

Pizzo *et al.*, 2006a,b). For this reason, we also explored **3**) the pattern of shape and size difference between head, epipharynx and male genitalia. To inspect the morphological differentiation between Sicilian and continental populations, we used a combination of traditional and geometric (landmark and semilandmark) morphometric methods, the latter being able to detect subtle shape differences and also to quantify the two aspects of morphological variations, shape and size, as uncorrelated traits (Zelditch *et al.*, 2004). We therefore 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 - were congruent with the pattern of horn expression. Additionally, we inspected the divergence patterns of the epipharynx - one of the mouth parts - which is considered not to be subject to costs associated with horn development (Pizzo *et al.*, 2009), and whose morphology could therefore be more free to evolve independently from other traits, possibly under selective pressures for optimal feeding functions (Verdú and Galante, 2004). As mitochondrial markers can provide fine details on the degree of genetic differentiation that may have resulted from historical processes leading to speciation events, we investigated **4**) the genetic relationships between Sicilian and continental populations by comparing cytochrome oxidase subunit 1 (COI) mitochondrial gene sequences.

## Materials and Methods

### *Sample*

The sample is composed of individuals from 3 populations of *O. fracticornis* collected in Italy in three localities along a N-S latitudinal gradient (North Western Alps, Central Apennine, and Southern Apennine) and by individuals collected in Sicily, in the *locus typicus* of the supposed species *O. massai*, Piano Battaglia. The number and the geographic origin of specimens used in each morphometric analysis are reported in Table 1.

Individuals for morphometric analyses were cleaned in 100°C distilled water for 10 min and dissected. Genitalia (parameres) were extracted and cleared in boiling 5% Potassium hydroxide (KOH) for 5 min. For the lateral view they were positioned on wet cotton wool, taking care to align their edges on the same horizontal plane. Heads, pronota, elytra and parameres(dorsal view), were fixed separately on horizontally-levelled plasticine supports. Epipharynges were treated following the protocol described in Pizzo *et al.* (2009), mounted on microscope slides and covered with coverslips.

Two dimensional images of each structure were taken using a Leica Z16Apo stereoscopic dissecting scope (Leica Microsystems AG, Wetzlar, Germany) at magnifications of 57.5x (epipharynx, dorsal side; phalloteca, lateral side), 50x (paramere, dorsal side), 20x (pronotum,

dorsal side), 40x (elytra, lateral side), 200x (elytra, detail of the dorsal surface), 31.3x (*O. fracticornis* horn, lateral side), 39.4x (*O. similis* and *O. opacicollis* head, dorsal side), 25x (*O. fracticornis* head, dorsal side). After calibration, linear measurements of pronotum width (used as a proxy for body size: Eberhard and 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).

Molecular analyses were carried out on a subsample of 14 specimens, as shown in Table 2. .  
*Analyses*

### 1. Qualitative analyses of the elytral microsculpture

Photographs of elytral surfaces were made at two different magnifications in two alternating views (lateral, at 40x, and dorsal, at 200x magnification) to inspect the pattern of presetal granulation and perisetal punctuation between populations, looking for the diagnostic differences between *O. fracticornis* and *O. massai*.

### 2. Horn static allometry

An earlier study on *O. fracticornis* (Macagno *et al.*, 2009) found that a sigmoidal model was a good fit for male horn length-body size data. For each taxa, the horn static allometry was therefore determined by fitting to the horn length-body size data a 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 and Nijhout 2003; Moczek, Brühl and Krell, 2004; Macagno *et al.*, 2009). Parameter values of the regression were obtained via iterations using Sigma Plot<sup>TM</sup> (Systat Software Inc.) curve-fitting procedures.

The differences between the four sigmoid curves were inspected by first testing inter-specific differences in mean pronotum width (observed values) with a one-way ANOVA and LSD post-hoc tests, and in horn length (observed values) with a Kruskal-Wallis non-parametric ANOVA. Additionally, to test difference between curves, we generated a best fit sigmoid regression curve based on a reference population (a population obtained combining all samples) using Sigma Plot. Using this curve, we computed the expected horn length for each individual given its body size, and then the difference between observed and expected horn length (= residual horn length). We used T-tests to compare residuals across populations. To know which parameter, if any, accounted for these differences, we used parameter estimates as derived from Sigma Plot (means plus standard



errors), and used Welch T-test with sequential Bonferroni corrections to compare each parameter across populations.

### 3. Morphometric analyses

#### *Shape variation*

A landmark- (for the heads, the epipharynxes and the dorsal side of parameres) and semilandmark- (for the lateral view of parameres) based on a geometric morphometric approach (Bookstein, 1991; Rohlf and Marcus, 1993; Adams, Slice and Rohlf, 2004) was used to characterise the shape of the structures analysed. All landmarks and semilandmarks were digitised by the same person using TpsDig 2.10 (Rohlf, 2010) on the left side of the structures, with the aim of removing any bias possibly caused by bilateral asymmetry. For each structure, the landmark or semilandmark 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 the sample size was larger than the number of landmark coordinates). To digitise semilandmarks on the lateral view of parameres (Fig. 1e) with a satisfying degree of accuracy, we manipulated the images with MakeFan6 (Sheets, 2003) software, superimposing a regularly spaced grid on the curve between landmarks 1 and 4 (*comb* option).

Landmark configurations of each structure were superimposed with a full Procrustes fit (Rohlf and Slice, 1990; Goodall, 1991: for semilandmark superimposition we used the slide method as “chord= min bending energy” and 3 iterations), 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 (Kendall, 1981, 1984; Rohlf, 1996) were then projected into a linear tangent space by orthogonal projection (Dryden and Mardia, 1998). To inspect patterns of shape variation in the sample, we performed a Principal Components Analysis (PCA) on shape coordinates by using TpsRelw (Rohlf, 2008). Visualisation of the resultant sample distribution in morphospace was made with SPSS 18.0. Using the same software, we performed canonical variate analyses (CVA) on shape variables of each structure separately. The reliability of the discrimination was assessed by leave-one-out cross-validation (e.g. Lachenbruch 1967).

#### *Size variation and static allometry of head, epipharynx and parameres*

The centroid sizes (CS) of each landmark and semilandmark configuration (computed in Tps Relw, Rohlf, 2008) were used as estimates of head, epipharynx and paramere size. A preliminary inspection of scatterplots of pronotum width vs 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 standardised predicted values vs residuals (Sokal and Rohlf, 1995).

All analyses were conducted in SPSS 18.0.

#### 4. Molecular analyses (COI sequencing)

DNA was extracted using Quiagen Dneasy columns from heads ground up with a Tissue Lyser (Quiagen). A fragment of the mitochondrial gene COI was amplified, purified and sequenced using the primers TL2-N-3014 (alias Pat) and C1-J-2183 (alias Jerry) (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 and Skaletsky, 2000). Sequences of the two closest related species (*O. similis* and *O. opacicolis*, Pizzo *et al.*, in press) and a sequence of *Onthophagus (Palaeonthophagus) vacca*, courteously provided by Dirk Ahrens as an outgroup, were added to the alignment.

Pairwise distances between haplotypes were estimated under the assumptions of the Jukes–Cantor model in MEGA 2.1 (Kumar *et al.*, 2001). Phylogenetic trees were constructed by the neighbour joining (NJ), minimum evolution (ME) and Parsimony (MP) methods in MEGA 2.1. A 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. Maximum likelihood (ML) analyses used the default search parameters in RAxML VI-HPC v2.2.0 (Stamatakis 2006) with 25 replicates. A Bayesian analysis was also performed using MrBayes version 3.2 (Ronquist and Huelsenbeck, 2003). The substitution model for Bayesian analyses was selected using the Akaike Information Criterion test implemented in the software MrModeltest v. 2.3 (Nylander, 2004). The test suggested GTR+G+I as the best fitting model. Four independent Markov chain Monte Carlo runs, with one cold and three incrementally heated chains each, were performed for 10 million generations, sampling trees every 1000th generation. The first 2 million generations were discarded as burn-in, and the remaining trees used to construct the Bayesian consensus tree. We used the TCS 1.06 software of Clement *et al.* (2000) to generate haplotype network (Templeton *et al.*, 1992).

## Results

### 1. Qualitative inspection of the elytral surface

Dorsal and lateral views of elytral surface (Fig. 2) showed the presence of marked presetal granules and the absence of punctuation in the samples from the N-W Alps and Southern Apennines. We also highlighted this microsculptural pattern in some Sicilian specimens (as in the ind. 1, Fig. 2), coexisting with individuals showing evident punctuation and virtually absent presetal

granules, the typical pattern described for *O. massai* (as in the ind. 2, Fig. 2). This last configuration can be unexpectedly found also in the Central Apennines sample (fig. 2) .

## 2. Horn allometry

The ANOVA on mean pronotum width and subsequent LSD post-hoc tests for multiple comparisons showed that, with the exception of the Southern Apennines and the NW Alps having similar size ( $P=0.068$ ), all populations differed significantly with respect to their pronotum width ( $F_{3,171}=26.539$ ,  $P<0.001$ ). On the other hand, a Kruskal-Wallis non-parametric ANOVA failed to detect any difference in horn length across the four populations ( $P=0.162$ ). T-tests performed to compare the residuals derived from the unique sigmoidal regression across samples showed that they were significantly different between the Sicilian and the other three populations, and between the Central Apennines and the NW Alps, but not between the Southern and the Central Apennines nor between the Southern Apennines and the NW Alps: four separated sigmoidal regressions, one for each population (Fig. 3), probably describe the allometric structure of the sample better than a single one. Parameters significantly differing between curves are shown in Table 3.

## 3. Morphometrics analyses

### *Shape variation*

While PCA conducted on male head shape variables was not able to evidence any differences between populations (results were the same when male morphs were analysed separately or together), female head shape analysis revealed a certain degree of differentiation: the North-Western Alps seemed to be the most differentiated population and shared with those from the Central Apennines one the upper left part of the morphospace; Sicilian specimens were mainly located in the right lower part of the morphospace, while Southern Apennine specimens appeared to connect the two groups (Fig. 4). As the epipharynx is not a sexual dimorphic structure (Pizzo et al, 2009), a PCA of shape variables was carried out taking the two sexes together which showed a high level of overlap between populations; however, it was possible to detect a little differentiation with a pattern similar to that evident for female head shape (Fig. 5). Paramere shape described by landmarks collected on the lateral view showed an evident differentiation of the North-Western Alps population with respect to the other three, which showed some degree of overlap (Fig. 6, left). The dorsal view of parameres (Fig. 6, right) showed different relationships between populations: in the PC plot of the two first principal components, the two most differentiated populations seemed to be those from Sicily and the Central Apennines. The dorsal view of parameres, as already evidenced by Baraud (1992), showed a higher diagnostic power than the lateral view in evidencing differences between populations, as indicated also by the higher percentage of variance explained of the first principal component; however, the pattern highlighted with the analysis of the dorsal view

of parameres did not allow consideration of the Sicilian population as a differentiated species, as its divergence, although extant, was of the same range as the Central Apennine population. Singular values, percentages of variance explained and cumulative variance for all principal components, resulting from each PCA are shown in Table 4. Cross-validated results of CVA on shape variables are summarized in Table 5.

*Size variation and static allometry of head, epipharynx and paramere*

Kruskal-Wallis tests on female and male head centroid size revealed that the Central Apennines had significantly larger head size (females:  $P < 0.01$  in all contrasts; males:  $P < 0.001$  in all contrasts); static allometries of female head had similar slopes between populations ( $P = 0.854$ ), suggesting the same ontogenetic trajectories in all populations, but the intercept value for Central Apennine population was different with respect to the others ( $P < 0.004$ ), indicating the larger head size at the same body size for the individuals of this population with respect to the others. On the contrary, male head static allometry indicated that the allometry of the NW Alps differed significantly from the others in the slope ( $P < 0.001$ ), a result in agreement with the differentiation pattern observed for shape.

Epipharynx size analyses indicated a subdivision of the sample in two groups, NW Alps and Sicily on one side, having lower intercept values in allometries ( $P < 0.001$ ) and centroid size values significantly lower ( $P < 0.001$  in all contrasts), and the Southern and Central Apennines on the other. The non-parametric Kruskal-Wallis test on paramere centroid size (lateral view) revealed significant differences in dimensions between the four populations ( $P < 0.001$ ), with the Southern Apennine population having the largest paramere (lateral view) and the Sicilian one the smallest. Allometric slopes differed, even weakly, between populations ( $P = 0.046$ ) except for the contrast North Western Alps-Central Apennine ( $P = 0.108$ ).  $R^2$  values of the paramere (lateral, view) allometric regressions (N-W Alps  $R^2 = 0.183$ ; Central Apennine  $R^2 = 0.429$ ; Southern Apennine  $R^2 = 0.083$ ; Sicily  $R^2 = 0.072$ ) were very low compared to the  $R^2$  of the other morphological trait allometries, which approached one. The non-parametric Kruskal-Wallis test on paramere centroid size (dorsal view) revealed again significant differences in dimensions between the four populations, but Sicily and NW Alps are dimensionally comparable ( $P = 0.241$ ), and the same is for Central and Southern Apennines ( $P = 0.613$ ). This result fitted with the results of the comparison of the epipharynx allometries. Allometric slopes of paramere (dorsal view) differed between populations except for the contrast North Western Alps-Sicily ( $P = 0.213$ ).  $R^2$  values of the allometric regressions of the dorsal view of paramere were, again, very low, and very similar to those calculated from the allometries of the paramere lateral view (N-W Alps  $R^2 = 0.284$ ; Central Apennine  $R^2 = 0.320$ ; Southern Apennine  $R^2 = 0.081$ ; Sicily  $R^2 = 0.386$ ).

#### 4. Molecular analyses

We aligned 601 bp of COI resulting in a combined matrix with 66 parsimony informative characters. Thirteen haplotypes were identified, grouped coherently with their geographic origin. The mean uncorrected p-distance between any two sequences was 0.049. The two closest populations were NW Alpine and Southern Apennine (p-distance = 0.0277), whereas the most differentiated (p-distance = 0.0374) were Central and Southern Apennine populations; the Sicilian population was as distant from the Southern Apennine population (p-distance = 0.0295) as from the Alpine population (p-distance = 0.0294). Distances of each population with respect to the out-group were all in the same range of magnitude (0.1) and intra-population distances (p-distances < 0.007) were always lower than inter-population distances.

Maximum Parsimony (MP) analysis generated nine 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 0.75, the retention index (RI) 0.84 and the rescaled consistency index (RCI) 0.63; tree length was 161. The sum of the branch Lengths (SBL) for the ME tree was 0.2440441 and for the NJ tree was 0.27080580. The trees generated by these three different methods showed the same topology, with no differences even in the resolution of terminal clades (Fig. 7); in these phylogenetic reconstructions, individuals of each population were clearly grouped in different clades, with the Central Apennine population as the most differentiated. The ML and Bayesian tree showed a different topology, indicating the Sicilian population as the most differentiated. However, the phylograms (Fig. 7 and Fig. 8, right) clearly indicates that the Sicilian population belonged to the *O. fracticornis* lineage, the distance being short with respect to the other Italian populations, if compared with the range of interspecific distance between *O. fracticornis* and the other species of the complex (*O. similis* and *O. opacicollis*).

The analysis made with TCS showed that the maximum number of mutational steps between haplotypes allowing parsimonious connections in the same network with a probability higher than 95% was 10 steps. Haplotype network estimation using parsimony within this probability limit resulted in four separate networks, one for each population. To connect the networks in the same cladogram (as in Fig. 8, left), we had to use at least 16 mutational steps, which is below the accepted 95% probability limit.

#### Discussion

In his works, Baraud (Baraud, 1975, 1992) asserted that *O. massai* is a good species, endemic and vicarious to *O. fracticornis* in Sicily, and that there are some diagnostic characters allowing unambiguous identification of the species, in particular the elytral microsculptural pattern and the

paramere shape. Falahee and Angus (2010) confirmed these arguments, presenting a comparison of the microsculptural details of the elytra of modern and fossil individuals of *O. massai* and *O. fracticornis* (Coope, 2001; Osborne, 1969). They showed the marked puncturation in the interstrial surface in the first taxon and evident presetal granulation in the second, and supported this morphological evidence with karyotypic analyses. Validation of *O. massai* as a good species would indicate that this taxon has substantially altered its geographical distribution as a consequence of dramatic glacial/interglacial climatic changes, as its presence would have been recorded in England in the warm Ipswichian period (about 130.000-110.000 years ago) (Coope, 2001). In this case, its insular endemism should have to be interpreted as the result of isolation in a refugial area during the last ice age, accompanied by the complete extinction of continental and peninsular populations.

From our extensive microscopic inspections of the elytral surfaces, it has unexpectedly emerged that the puncturation pattern typical of *O. massai* is detectable even in Central Apennine individuals, and that *O. massai* specimens are not totally homogeneous for this character. At least three different explanations could follow from this result: a) the elytral microsculptural pattern is not a diagnostic character for *O. massai* identification, and another trait should be found to unambiguously identify this species; b) the microsculptural pattern would be just a phenotypic trait with variable frequencies in different populations of *O. fracticornis* and therefore not a diagnostic character: in this case, Sicilian individuals could be simply insular members of *O. fracticornis* which more frequently exhibit this character, and not a vicarious species; c) *O. massai* is a real species but it would not be endemic and would coexist in microsympatry with *O. fracticornis* in continental localities, such as the Central Apennines. In trying to validate these hypotheses, we proceeded with a thorough inspection of morphological, allometric and genetic patterns between continental and insular populations.

The ANOVA on pronotum width revealed that the NW Alps and Southern Apennine populations had similar body size whereas the Sicilian and Central Apennine populations were the two most differentiated with respect to their general size (the smallest was Sicily, as expected, and the largest was the Central Apennines): these results leads us to presume that it is very unlikely that *O. massai* individuals, considered to be decidedly smaller with respect to *O. fracticornis*, could be present in the Central Apennines sample. However the size is not considered a good character to define a species by. The literature contains many examples showing that the mean body size of an insect species may change along an altitudinal (or latitudinal) gradient, but some species show increasing size with increased altitude while others show the reverse (or a more complicated) trend (Hawkins and deVries, 1996; Chown and Klok, 2003; Krasnov et al., 1996). For the present, an exhaustive information of the altitudinal/latitudinal size variation pattern of *O. fracticornis* is not

available, so we can't interpret our results in this framework. Horn static allometries (Fig. 3, Table 3) reflected the results come out from size comparison, showing that the Central Apennines and Sicily had the two most divergent allometries, both with respect to the body size threshold at which males switch from the hornless to the horned phenotype, and to the horn size range, which was wider for the Central Apennines and narrower for Sicily: the switch point between majors and minors in the Central Apennines occurred at the same body size at which we observed the largest major male in Sicily.

PCA of shape variables showed a pattern coherent among all the morphological structures considered in the analysis: the four populations largely overlapped in the morphospace, even if it was possible to single out each population taking up predominantly specific areas in the morphospace; a biplot of the first two principal components of males and females head shapes showed a similar profile (Fig. 4), with shape diversification between populations more evident in females: the NW Alps appeared to be the most differentiated population, with a very weak overlap with the Central Apennine population, which mainly occupied the upper part of the morphospace; Southern Apennine specimens mainly overlapped with the Sicilian ones. This morphospacial arrangement of populations seems to reflect a clinal variation along a north-south latitudinal gradient. Since the epipharynx is placed in a cavity of the head (anterior pharynx wall), one might have expected variation in its shape to be reflected in that of the head. Epipharynx shape was instead more homogeneous, level of diversification between populations being hardly perceptible; however, a trend of variation similar to that of the head could be summarised (Fig. 5). The greater homogeneity is can be attributable to the strict association of this species with particular feeding resources (Halffter and Matthews 1966, Verdù and Galante, 2004); stabilising selective pressures are likely to act on this structure, so that optimal functionality is maintained in each population and this probably reflects a lack of differences in trophic habits and food selection between the populations along the latitudinal gradient.

Paramere (lateral view) shape variation along the first two principal axes (Fig. 6, left) showed a pattern in which, again, the NW Alps population was clearly identifiable from the others: however, in this case the Central Apennine population appeared as the most differentiated from the NW Alps and the most overlapping with the Sicilian population. Southern Apennine paramere shape was more similar to that of the NW Alps. However, paramere (dorsal view) shape variation along the first two principal axes (Fig. 6, right) shows that the two most different populations, with a similar range of diversification with respect to the average shape, are those from Sicily and Central Apennine; however, some Sicilian individuals overlap with those from NW Alps and Southern Apennine; These results are in agreement with the qualitative observations of Palestini (1981),

who found that the shape of the apical region of parameres of the supposed *O. massai* was similar to that of specimens from other Italian and foreign populations. The two main diagnostic characters for *O. massai* identification (elytral microsculptures and paramere shape, Baraud, 1975, 1992) seem to be unreliable. In cross-validated results of CVA, the NW Alps was the best identifiable population, both for the head and for epipharynx shape, with high percentages of correct classifications; cross-validated CVA results of paramere shape variables indicated that even if the NW Alps was not the population with the best classification percentages, it was rarely confused with other populations.

None of these shape analyses indicated the Sicilian population as the most differentiated, leading us to suppose that *O. massai* is an invalid species. Sicilian individuals almost always overlapped the morphospace of the Southern Apennine individuals, the closest geographic population, except for the paramere shape (lateral view), which was more similar to that of the Central Apennines.

Interpopulational differences, although present, were weak and seemed to be in the range of the differences detectable among populations of the same species.

Individuals from the Central Apennine population can be described as the largest not only in terms of mean body size, but also as they can be distinguished from all other populations for having, proportionally, the largest heads in females, an epipharynx proportionally bigger - even if comparable with those of the Southern Apennine population - and a horn static allometry indicating that the switch point between majors and minors in this population comes at the largest body size value. Surprisingly, the Central Apennines didn't show a copulatory structure proportionally larger than other populations, a primacy set by the Southern Apennine population instead, which largely invests in the development of male genitalia. Paramere allometry suggested that the complete independence of the allometric trajectories between populations, with significantly different slopes, could express the incipient reproductive isolation of the populations. The only allometric result in agreement with information collected from the shape analyses was the male head allometry, in which the NW Alps emerged as the only differentiated population. Epipharynx allometries also showed a perfect superimposition of the allometric trajectories between the NW Alps and Sicily. In conclusion, from the analyses of allometries there is no evidence of a specific divergence in the ontogenetic trajectory of the Sicilian population from the others.

Phylogenetic reconstruction showed the same general topology evidenced in a recent study on the phylogenetic relationships within the complex (Macagno et al., in press), where *O. fracticornis* has been indicated as the most differentiated species within the *fracticornis-opacicollis-similis* complex and *O. similis* and *O. opacicollis* were grouped as sister species. The weak bootstrap value (0.85, Fig. 7) and posterior probability (0.65) found at this bifurcation in our



analyses likely depend from the fact that this two species are represented by a single individual; in Macagno et al., in press, this same phylogenetic relationship, obtained from a higher number of individuals from each species, is better supported. Each population of *O. fracticornis* was perfectly monophyletic. In ME, MP and NJ phylogenetic reconstruction (Fig. 7), the resulting tree topologies showed the Central Apennines as the most differentiated population, showing its sister relationships with respect to the other populations supported by very high bootstrap values. Otherwise, ML and Bayesian analysis (Fig. 8, right) suggested a greater differentiation of the Sicilian population. The lineages within *O. fracticornis* could be considered as potential speciation events, but they are not supported as species (low genetic distances with respect to the other species of the complex, partial inconsistency of morphometric data with respect to molecular data); at most, they could be considered as sub-species, or just simply genetically distinct units within *O. fracticornis*. Overall, these results indicate that the Sicilian population is not sufficiently and unambiguously differentiated from the populations belonging to *O. fracticornis* from both genetic and phenotypic point of view, and it therefore cannot be considered a good species (*O. massai*). This result partly contrasts with previous karyotypic analyses (Falahee and Angus, 2010), where the authors found differences in RCLs of four of the nine pairs of autosomes between *O. fracticornis* and the supposed *O. massai*. When chromosomal and genic data show two distinct patterns of variation, the lower level of genic variation between populations may be due to homoselection in a constant microenvironment (Nevo et al., 1984; Nevo and Shaw, 1972), whereas extensive chromosomal differences might coincide with an incipient reproductive isolation, maybe due to the physiographic barrier of the Strait of Messina. Extensive reorganisation of euchromatic chromosomal segments can produce genetic isolation via hybrid sterility (Patton and Sherwood, 1983), thus it follows that we cannot at present exclude an ongoing speciation process, occurring without concomitant genetic modification (Selander et al., 1974, Sage et al., 1986).

Network analyses indicated each population as an independent network (Fig. 8, left); the connection of networks is possible only when significantly lowering the probability of parsimonious connections below 95%, suggesting that they appear to be “genetic islands”, with little genetic flow between them; this particular pattern of isolation could be indicative of a low dispersal capacity and a high degree of habitat specialisation (this oligophagous species seems to be restricted to pastures in calcareous areas in the Alps and Apennines, and many authors have emphasised records in mountainous regions (Petrovitz 1956, Avila and Pascual 1988, Baum 1989, Gangloff 1991, Sowig, 1995). However, this pattern can arise from neutral processes, particularly where there is long-term historical isolation (Moritz 2002; Tregenza, 2002). The importance of quaternary glaciations in moulding the population structure is, indeed, well established (Avice and

Walker 1998; Hewitt 2000); however, because it was impossible to perform a true phylogeographic analysis on separate networks, nothing can be hypothesised on the specific dynamics of habitat fragmentation, isolation and recolonisation of the Italian region made in the past by *O. fracticornis*, except that, maybe, the Apennine range has been one of the first geographic barriers separating a western and an eastern lineage of the species. The presence of evident shape differences in the NW Alps population only, not accompanied by strong genetic diversification, could also suggest an ancient isolation of this population in a habitat with strong selective pressures, maybe an alpine *nunatak* (an “insular mountain” maintaining a suitable habitat for a species surrounded by ice during the glacial period). It has been acknowledged that mitochondrial DNA markers are more susceptible to the effects of genetic drift (Moritz et al., 1987; Sunnucks, 2000; Avise, 2004) and thus completely reliable inferences on species’ genetic diversity and structure should ideally be based on multiple types of molecular markers (Canestrelli et al., 2007); moreover, a more abundant sampling for each population and the collection of samples from other parts of the distribution range would allow to deeper examine, with the help of molecular markers, the extent of the geographical isolation, and the possibility of traces of other cryptic species; this could be a further development of this research.

To summarise, the results of this study seem to indicate that there is not sufficient evidence for *O. massai* to be considered as a good species, differentiated from *O. fracticornis* and vicarious to it in Sicily, but that speciation may be taking place; moreover, from our analyses it clearly emerges that the Sicilian population is significantly smaller than the others, which could be the result of insular evolution toward dwarfism (Vernon, 1981).

The Central Apennine population deserves particular attention: it was differentiated in terms of genetics and overall body size; additionally, it appeared similar to the Sicilian population for both characters considered diagnostic for *O. massai* identification, paramere shape and elytral microsculptures. However, no other character (shape, size, allometries, genetics) suggested a particular relationship between Sicilian and Central Apennine populations.

Overall, each population examined presents peculiar aspects of differentiation; in particular, genetic analyses seem to suggest that the populations are evolving as evolutionary independent units.

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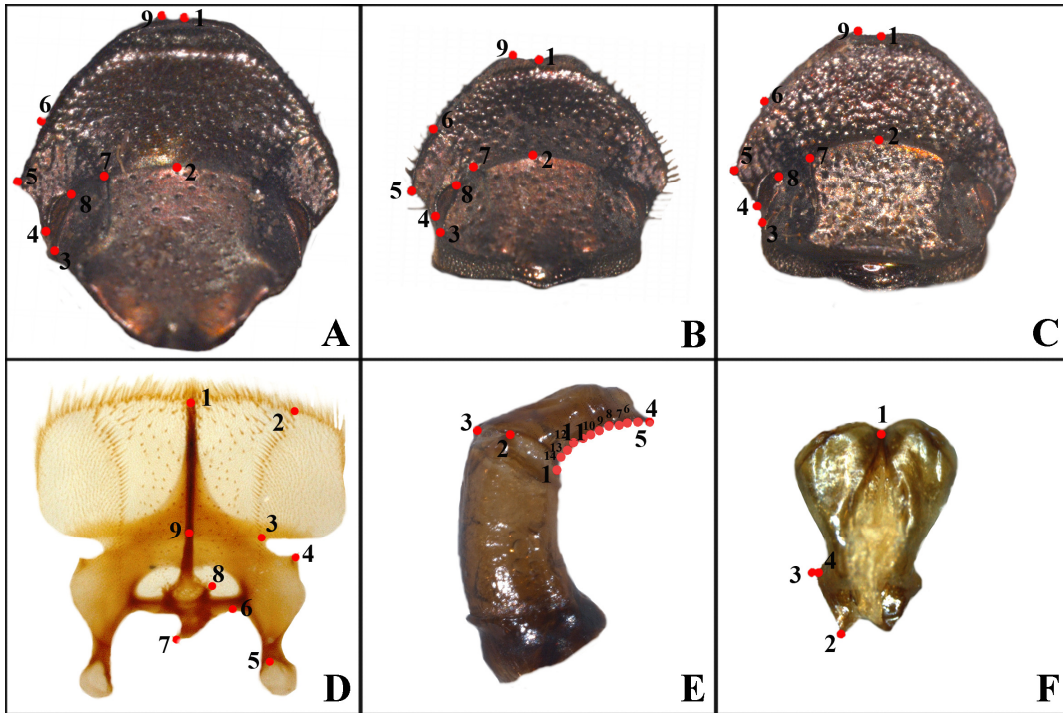


Figure 1

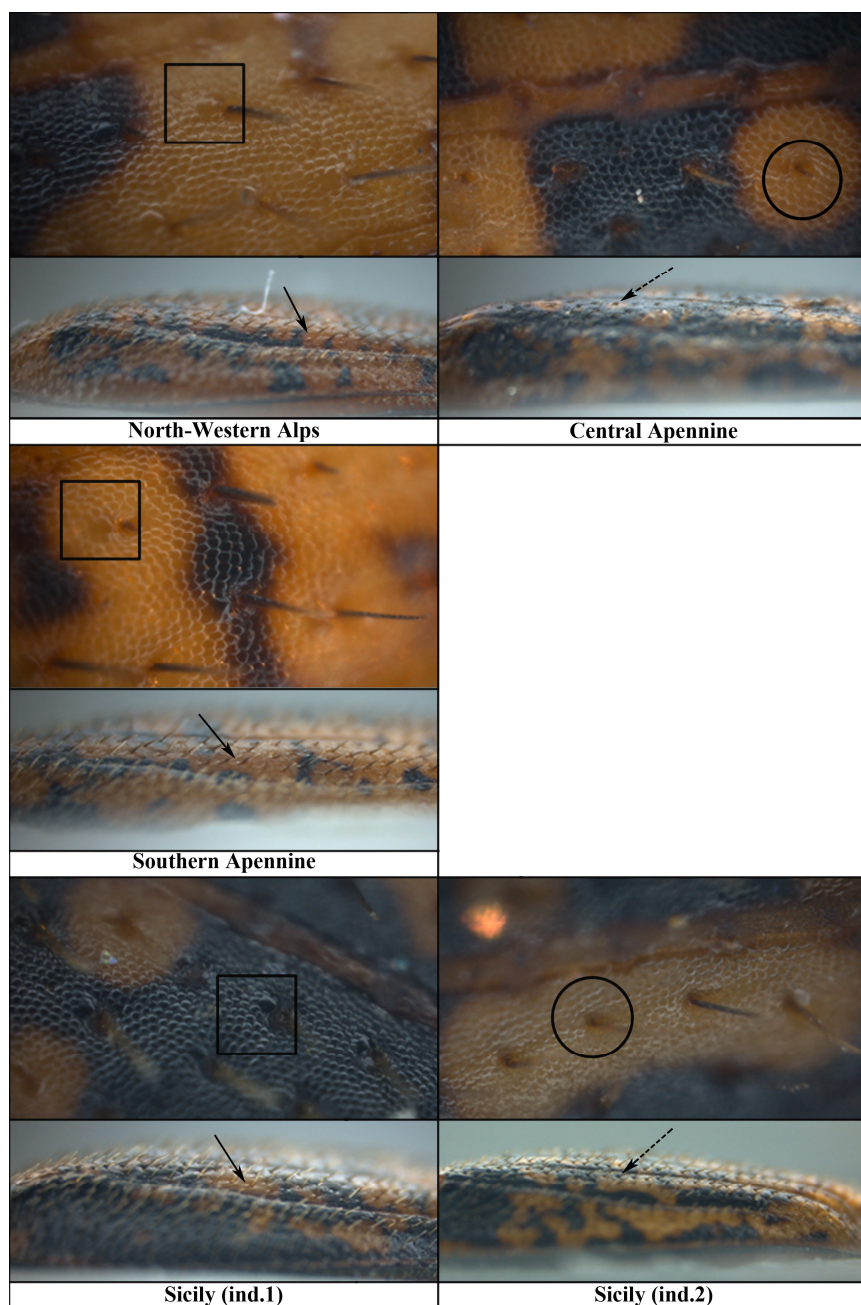


Figure 2

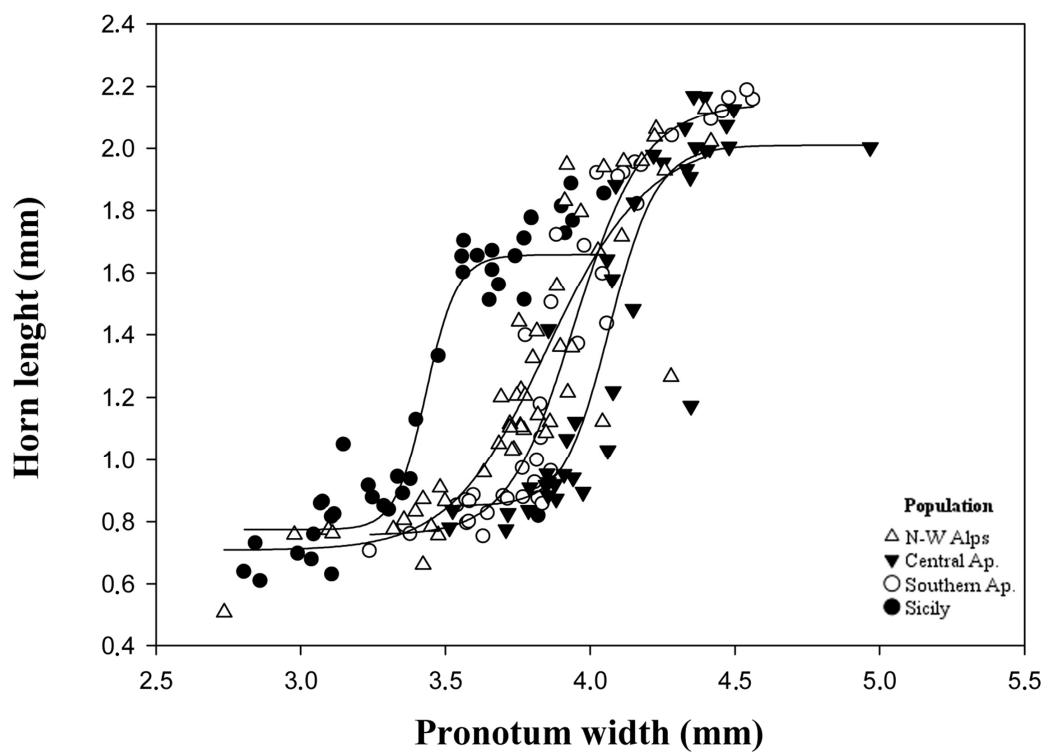


Figure 3

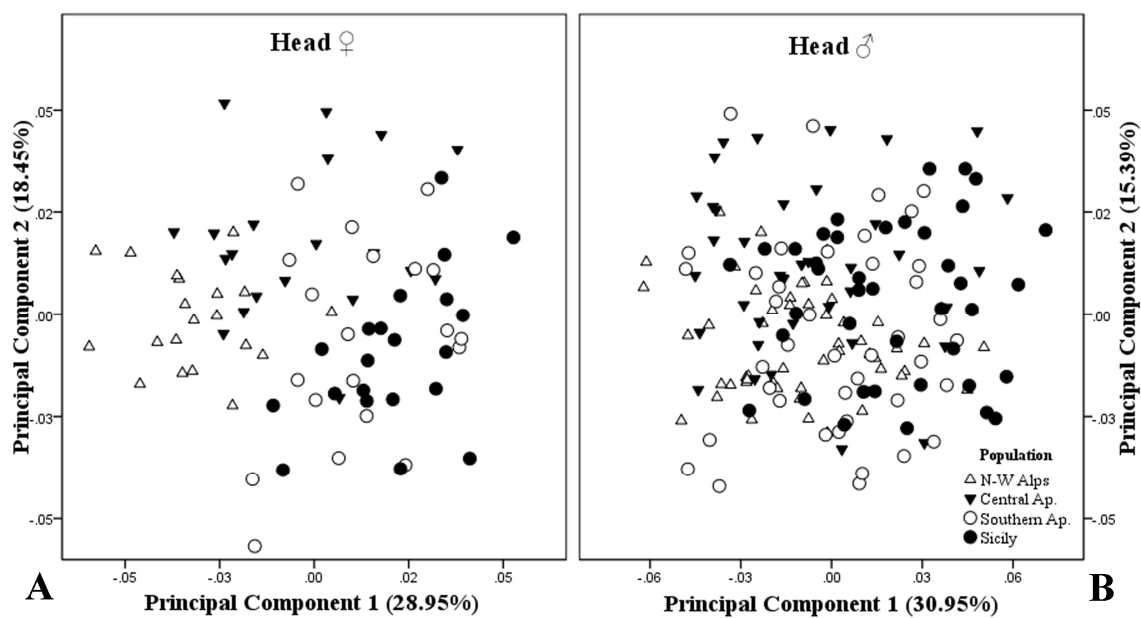


Figure 4

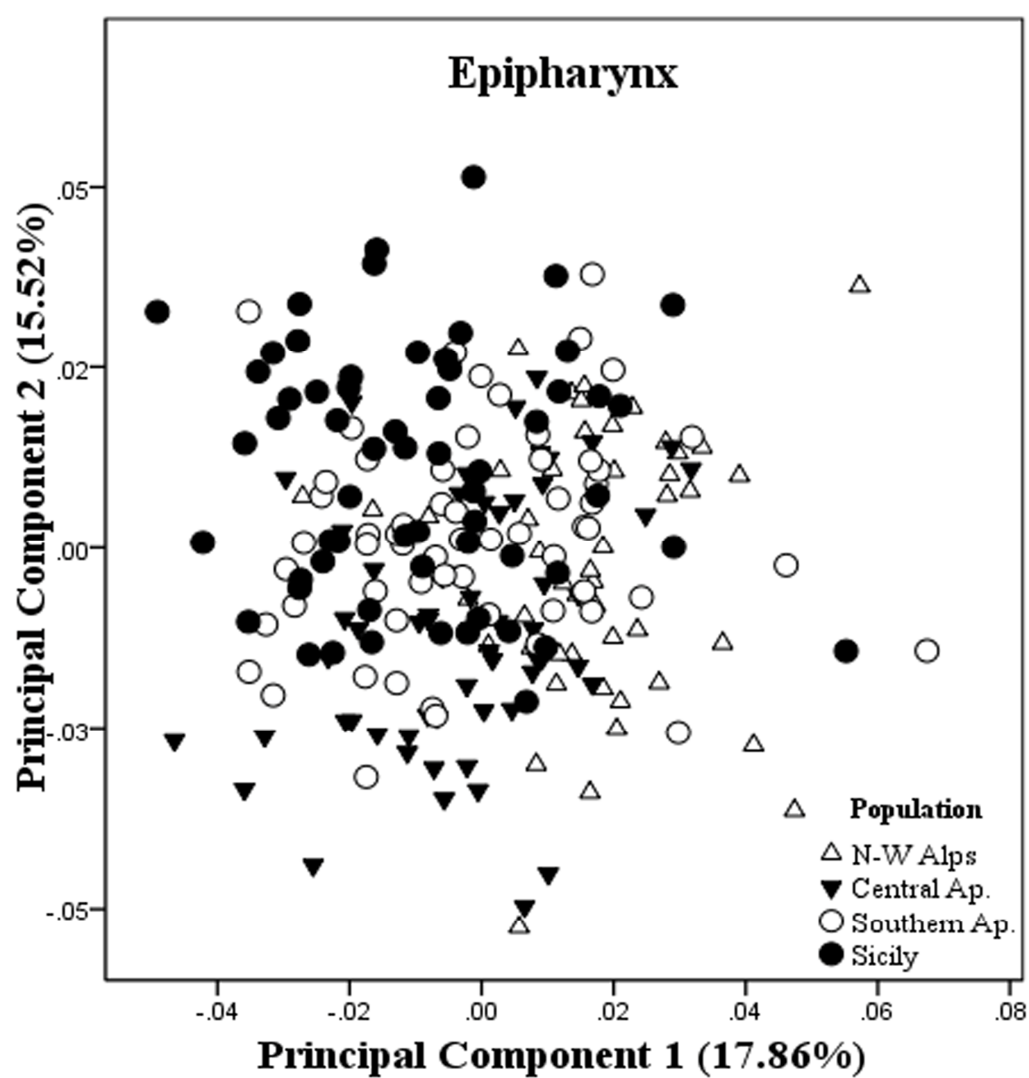


Figure 5

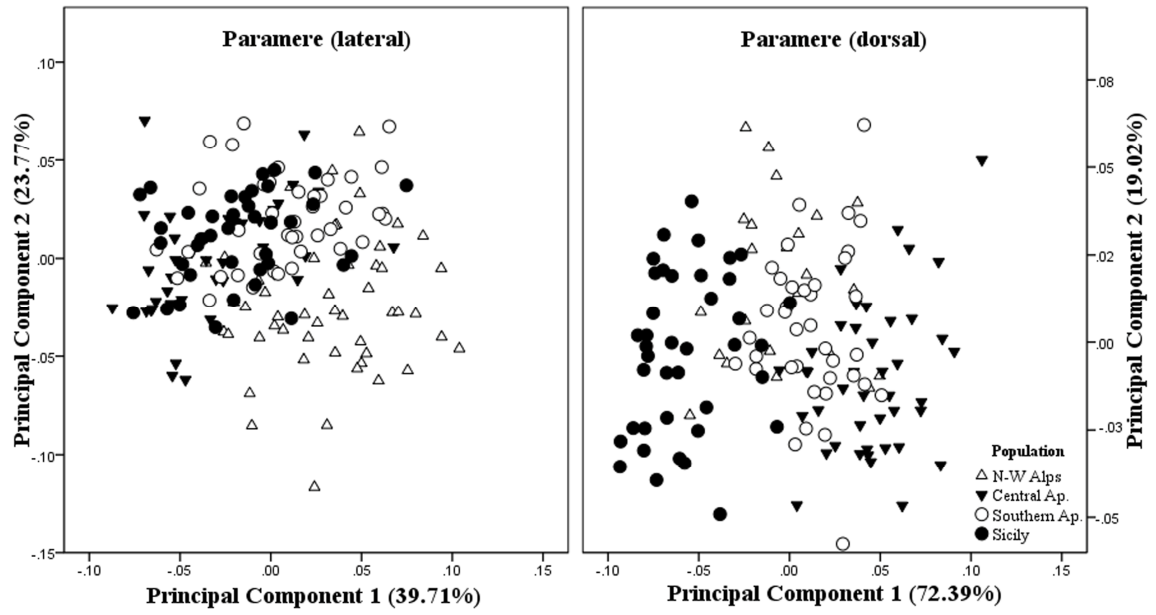


Figure 6

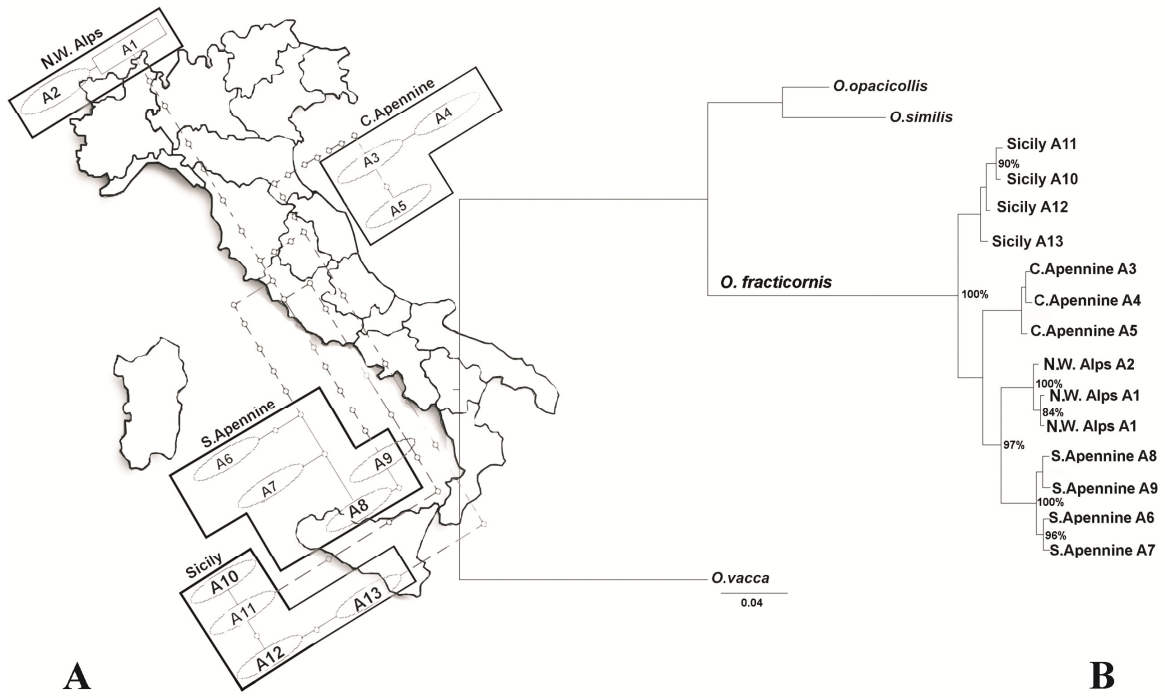


Figure 7

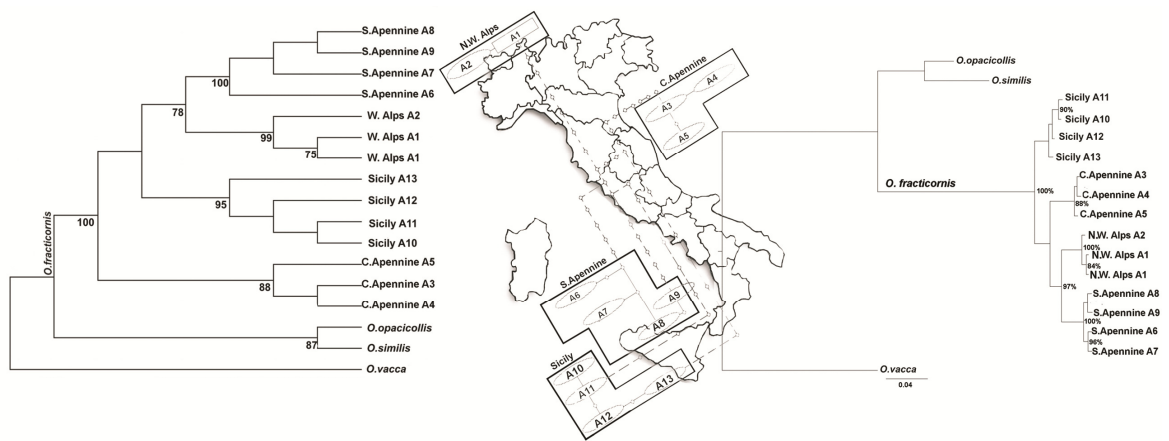


Figure 8