An unfinished speciation process revealed by geometric morphometrics, horn allometries and biomolecular analyses: The case of the fracticornis-similis-opacicollis species complex of the genus Onthophagus (Coleoptera: Scarabaeidae)

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An unfinished speciation process revealed by geometric morphometrics, horn allometries and biomolecular analyses: the case of the *fracticornis-similis-opacicollis* species complex (Coleoptera: Scarabaeidae).

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Abstract

Species complexes, composed by closely related species of difficult identification for their morphological similarity, provide unique opportunity to study the early phases of morphological and genetic differentiation and the microevolutionary dynamics promoting speciation.

Within the genus *Onthophagus* the presence of many species complexes and sibling species testify to a recent impressive adaptive radiation. We focused on the debated species-complex *Onthophagus* (*Palaeonthophagus*) '*fracticornis-similis-opacicollis*' as a model to study the evolution of morphology, the role of male polyphenism and the microevolutionary dynamics promoting speciation. Even if at present there is a general consensus that *O. fracticornis* is a good species, greater discrepancies arise on the taxonomic status of *O. similis* and *O. opacicollis*: according to some authors these are sibling species with a wide range of sympatry whilst others maintain they are morphotypes of a single, polymorphic species. To investigate the relationships within the complex and to study evolution of morphology and the dynamics of their differentiation, we studied together allopatric and sympatric populations integrating multiple approaches: the geometric morphometric analysis of the shapes of genital and non-genital morphological structures, the comparison of horn static allometries and the biomolecular analysis of a mitochondrial gene. The results provided a complex and often incongruent pattern of morphological and genetic evolution, consistent with a hypothesis of a still unfinished speciation process in sympatric areas and a fully realised speciation by means of ecological segregation in allopatry.

1. Introduction

A species complex is a group of closely related species that, in theory, satisfy the biological definition of species but whose morphology appears very similar and, in some cases, practically indistinguishable. Particularly in these cases, the species status have to be proven, but in many situations even DNA technique and molecular phylogenetic studies, that more and more allow to identify species and to clarify the phylogenetic relationships within this kind of complexes (Hebert et al., 2004; Stelzer et al., 2011), are not able to evidence clear distinctions and boundaries among species; if lineage sorting has not yet been completed, members of a species complex widely share plesiomorphic haplotype while species might not have evolved distinctive genetic patterns yet (Nitta et al., 2011, Conflitti et al., 2012). However, individual species of a complex can be identified by analysing data from multiple approaches (Ciros-Perez, 2001), integrating biomolecular data with sofisticated morphological analyses (i.e. geometric morphometrics and static allometries) and with information on species autoecology and distributional data. The importance of identifying patterns of differentiation between closely related species was already recognized by Darwin (1859) as a basis for understanding evolution by natural selection. Species complex often occur in sympatry (Barraclough & Vogler, 2000), or share portions of the distribution areas sometimes representing hybrid zones; moreover, it has been suggested that sympatric species which are also syntopic may have experienced the same environmental influences, at least throughout their most recent evolutionary history (Dawson et al., 2002).These provide an unique opportunity to deeply investigate evolutionary interactions between species and the microevolutionary dynamics promoting speciation (Hardig et al., 2000).

Onthophagus beetles have experienced an extraordinary evolutionary radiation that has made them one of the most speciose living genera on Earth (more than 2000 species have already been described) (Emlen *et al*., 2005). Many species-complexes and sibling species testify to the recent diversification of this genus (only 23–33 million years ago) (Hanski & Cambefort, 1991; Davis, Scholtz & Philips, 2002; Emlen *et al*., 2005) and likely reflect the under-appreciated role in promoting and driving speciation played by phenotypic plasticity (Pfennig et al., 2010), a widespread character in this genus. *Onthophagus* species-complexes and closely related species (Pizzo et al., 2006a, b, 2009, Macagno et al. 2011) are therefore excellent material for qualitative/quantitative analyses aimed at describing the early phases of differentiation and the evolution of morphology and of male polyphenism and intended at studying microevolutionary dynamics driving speciation.

In this paper, we focused on the species-complex *Onthophagus* (*Palaeonthophagus*) '*fracticornis-similis-opacicollis*' [viz. *Onthophagus similis* (Scriba, 1790); *Onthophagus opacicollis* (Reitter, 1892); *Onthophagus fracticornis* (Preyssler, 1790)]. As for many other *Onthophagus* species, the species of this complex are polyphenic with respect to horn development. Horns are expressed only in males as a result of an explosive proliferation of specific epidermal regions during the prepupal and pupal stages, predominantly triggered by larval feeding conditions: individuals exceeding a critical larval mass moult into large, horned (major) adults which exhibit a fighting and guarding reproductive behaviour, whereas larvae with access to limited feeding resources eclose at smaller sizes and adult males, characterised by a "sneaker" reproductive strategy, express very reduced horns or no horns (minor males) (Moczek & Emlen, 1999, 2000; Moczek, 2009b).

Palaeonthophagus species have Palearctic origins and penetrated Western Europe from the east (Zunino, 1979a, Martin Piera, 1984); *O. fracticornis* occurs in mountainous regions (with a range of 800-1.700 m. a.s.l.) of central and southern Europe, from the southern areas of Scandinavia and Russian regions to northern Mediterranean. The distribution of *O. similis* largely overlaps that of *O. fracticornis*, occupying altitudinal ranges from 500 to 1.600 m. a.s.l. (Lumaret, 1978), whereas *O. opacicollis* is a more thermophilic species occurring in Mediterranean areas at lower altitudes and latitudes (Kirk & Ridsdill-Smith, 1986). The Iberian Peninsula is the most important area of sympatry for the three species, in particular for *O. similis* and *O. opacicollis* (Martín-Piera & López-Colón, 2000).

The systematic rank of the taxa within the complex is still under discussion (Palestrini, 1981; Da Bast, 1983; Martín-Piera 1984, Rahola, 1985; Baraud, 1985, 1987; Lumaret, 1990; Martín-Piera & Boto, 1999; Martín-Piera & López-Colón, 2000; Ljungberg, 2002, Wilson and Angus, 2005, Angus, 2008, Macagno et al., 2011). *O. fracticornis* is considered better differentiated from the other two taxonomic units, which are in turn more closely related. De Bast (1983) studied more than a thousand individuals from Belgian populations of *O. similis* and *O. fracticornis*; even if he was able to distinguish individuals on the basis of standard characters, he noticed about 5/1000 individuals with intermediate characters, not unambigously assignable to a single species. He supposed that a low degree of hybridisation occurred between these species. However, Ljungberg (2002) studying other exemplars from northern Europe (Sweden) and using a combination of different diagnostic morphological characters in females, minor males and major males separately [1], concluded that *O.*

[1] Females of all sizes are best separated on the basis of the shape of the posterior head ridge supplemented by the shape of the cheek; larger males on the basis of horn shape while in smaller males the cheek's shape is the most reliable character

fracticornis and *O. similis* are “far from hopeless to distinguish”. Palestrini (1981) studied the species complex and pointed out that *O. fracticornis* is a well established species. On the basis of the analysis of the genital structures, she also showed that its phyletic affinity with *O. similis* is not as strong as suggested by external morphology or traditional literature (Landin, 1957; Machatschke, 1958; Pierotti, 1959, Zunino, 1979). This result was confirmed both with allozyme analysis (Martín-Piera & Boto, 1999) and COI sequencing (Macagno et al., 2011). At present, investigators generally agree on the taxonomic status of *O. fracticornis* as a good, differentiated species.

Greater discrepancies arise with regards the status of *O. similis* and *O. opacicollis*: according to some authors they are two sibling species with a wide range of sympatry (Rahola, 1985; Lumaret, 1978; Baraud, 1992; Wilson & Angus, 2005; Angus, 2008), whilst for others they are two morphotypes of a single, polymorphic species (Palestrini, 1981; Martín-Piera, 1984; Martín-Piera & Boto, 1999).

Palestrini (1981), by examining a large number of individuals coming from a wide geographic range, asserted that *O. similis* and *O. opacicollis* belong to the same, highly variable species which, from a nomenclatural point of view, must be indicated as *O. similis* (Scriba), this name having priority on *O.opacicollis* Reitt. She evidenced a dimensional gradient showing a size reduction from the south to the north and from the warmer and flatter to the colder and higher areas of the distributional range. Quoting Palestrini (1981) Martín-Piera (1984) argued that this local morphological variation and the incomplete ecological segregation are attributes of a single polymorphic species adapted to a large geographical range of distribution.

Martín-Piera and Lobo (1999) studied allozyme variability of sympatric Iberian populations of *O. similis* and *O. opacicollis* in different environmental conditions, from sea level (where conditions were more suitable for *O. opacicollis*) to middle altitudes (preferred by *O. similis*).

Their results showed the presence of shared allozyme alleles between the two species and suggested the existence of some introgressive exchange of genetic material between *O. similis* and *O. opacicollis*, differing in intensity from site to site.

Cytogenetic comparisons evidenced significant karyotipic differences between *O. opacicollis* and *O. similis* when allopatric populations were considered (Wilson and Angus, 2005) and no signs of hybridisation were detected between sympatric Iberian populations (Angus, 2008). However, Angus (2008) studied very few individuals from sympatric Iberian populations.

A more recent study (Macagno et al., 2011) reconstructed the phylogenetic relationships amongst allopatric populations of the *O. fracticornis-similis-opacicollis* complex with a molecular approach (COI sequencing) and assessed the extent of their reciprocal morphological divergence. COI sequencing and the relative phylogenetic reconstruction for the *O. fracticornis-similis-opacicollis* 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, at least in allopatry, were good species. The phylogenetic pattern was characterized by significant differences between *O. fracticornis* and *O. opacicollis–O. similis*, along with less marked differences between *O. opacicollis* and *O. similis*, which appeared as sister species. These results were in keeping with the karyotypic studies carried out by Angus (2008) that suggested that these taxa function as reproductively isolated entities.

To thouroughly investigate the relationships within the complex and to study evolution of morphology and microevolutionary dynamics of its differentiation, we studied toghether allopatric and sympatric populations integrating multiple approaches:

1) The regulation of horn expression appears extremely evolutionary labile, to the extent that static horn allometries of polyphenic males can diverge rapidly amongst closely related

species (Moczek & Nijhout, 2003; Emlen et al., 2007) and even between isolated population of the same species (Moczek & Nijhout, 2003, Pizzo et al., 2011). Horn development has been thought of as a process which might trigger changes in male genital and non-genital morphology, possibly promoting speciation as a by-product (Moczek & Nijhout, 2004; Moczek and Parzer, 2008; Macagno et al, 2011; Pizzo et al,, 2012). It has been demonstrated (Moczek & Nijhout, 2003; Macagno et al., 2011) that horn static allometries have good correspondence with genetic and morphological distances and can therefore be used to demonstrate interspecific relationships in this species complex; we therefore examined and compared horn expression patterns and their divergence among species, and among allopatric and sympatric populations.

2) Geometric morphometric techniques, acknowledged as an approach more powerful than traditional morphometrics in detecting and describing even slight shape variations (Adams et al., 2002), were used to analyse independently interspecific size and shape variation patterns of genital (parameres and sclerites 5 of the endophallus) and non-genital (heads and epipharynges) morphological traits. Genital structures were chosen for the analyses because genitalia evolve quickly and because morphological modifications of copulatory organs are thought to play a major role in reproductive isolation and speciation (Eberhard, 1985, Pizzo et al., 2006, 2008; Macagno et al., 2011).

Geometric morphometric analyses are based on male specimens with the aim of comparing shape and size interspecific differentiation patterns of genital and non-genital structures with the pattern of horn allometries; moreover, species determination was a little simpler for males, in particular for major individuals, where the characteristic shapes of clypeal margins and horns are more evident (Ljungberg, 2002).

3) Interspecific hybridization among closely-related species can be detected by genetic analyses because introgression often results in incongruent phylogenies (Arnold, 1997). Neigel & Avise (1986), when modelling lineage sorting during speciation, found that recently diverged sister species may be paraphyletic with respect to mtDNA lineages for some time after speciation. To detect the existence of potential hybrids in sympatric populations, we reconstructed with multiple methods phylogenetic trees based on mt-DNA (COI) sequences from sympatric and allopatric populations of the species of the complex.

2. Materials and Methods

2.1 Samples

Although the systematics of this species complex (especially the status of *O. similis* and *O. opacicollis*) have not been fully resolved, each taxon is referred to as a 'species' for simplicity throughout the paper.

Male individuals of the *fracticornis- similis- opacicollis* species complex were field-collected in different European localities and then preserved in the entomological collection of the Life Sciences Department at the University of Turin. We had the opportunity to analyse individuals from the same Spanish populations considered in Martin Piera and Boto (1999). Individuals were assigned to one of the three species using standard keys based on external morphological characters (Paulian & Baraud, 1982). For Iberian populations, species determination was very difficult, due to the presence of individuals with ambiguous morphological characters. A lot of Iberian individuals, not unambigously assignable to *O. similis* or *O. opacicollis* following traditional criteria, have been referred to as IUTS

(Individuals with Uncertain Taxonomic Status) throughout the paper (see Table 1). The remaining Iberian individuals were classified as *O. similis* or *O. opacicollis* because they fitted better standard criteria, but their determination should be interpreted more cautiously. Table 1 shows the sampling localities and the number of male specimens of each population used for static allometries and morphometric analyses. Biomolecular analyses have been made using the DNA of 103 individuals belonging to the species complex from a DNA-bank preserved at the Life Sciences Department of the University of Turin (see Table 2). As DNA had already been extracted from heads, only the pronotum and the abdomen of these individuals were preserved: only parameres and sclerites 5 for the 31 male specimens of this sample were available, and they have been included in the geometric morphometric analyses. The shape variability of the pygidial flaps (a structure of the terminal tergite that highly interact with male parameres during copulation) of the remaining 72 female samples used for biomolecular analyses was also analysed with a geometric morphometric approach and compared with biomolecular results and male paramere shape variation.

The populations of Val Ferret, Pisa and Teilhède, whose phylogenetic relationships and morphological divergence patterns were well studied and validated in a recent study (Macagno et al, 2011) were considered as "reference" respectively for *O. fracticornis*, *O.opacicollis* and *O. similis* species throughout all the analyses.

2.2 Morphological preparations

Individuals for morphometric analyses (Table 1) 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 immersing their convex ventral side. Epipharynges were treated following the protocol described in Pizzo et al. (2009), mounted on microscope slides,

and covered with coverslips. Aedeagi were extracted, cleared in boiling KOH 5% for 5 min and first positioned on wet cotton wool taking care to align their edges on the same horizontal plane to take digital photographs of the lateral view of their parameres. Aedeagi were then fixed at their bases on the plasticine support to take digital photographs of the apical view of their parameres.

2D images of each structure were taken using a Leica Z16Apo stereoscopic dissecting scope (Leica Microsystems AG, Wetzlar, Germany) at magnifications of 62.5x (epipharynx, ventral side) , 50x (parameres, apical view), 200x (sclerite 5), 25x (pronotum, dorsal view), 31.3x (head, lateral view), 39.4x (*O. similis* and *O. opacicollis* head, dorsal view), and 25x (*O. fracticornis* head, dorsal view). Pigidial flaps were prepared as in Macagno et al., 2012.

2.3 Landmarks and measurements

In landmark-based morphometric analyses, the morphology of an object is represented by coordinates of sets of landmark points (Bookstein 1991). Landmarks, chosen for their ease of identification, homology between the two species, and ability to capture the general shape of each morphological structure, were digitised using TpsDig 2.15 (Rohlf 2010). To evaluate the confidence of the landmark configuration, a repeatability test was conducted as in Pizzo et al. (2008). Landmarks on the head (N =5), pronotum (N = 4), and epipharynx (N = 8) were digitised as shown in Fig. 1. Landmark configuration on pigidial flaps were digitised as in Macagno et al., 2011b. Landmarks of each specimen were optimally aligned using a Generalised Procrustes Analysis (GPA) to remove the non-shape effects of translation, rotation, and scale (Rohlf 1990, 1999; Rohlf and Slice 1990). As long as variation in shape space is small, the data in tangent space are an almost perfect approximation of the data in shape space; we tested this approximation with TpsSmall 1.20 (Rohlf 2003). We used the

thin-plate spline (TPS) approach (Bookstein 1989, 1991; Rohlf 1999) to generate multivariate descriptions of the shape of each specimen, and to visualise them on deformation grids.

In geometric morphometrics, the value of centroid size (the square root of the sum of squared distances of the set of landmarks from their centroid, measured on calibrated images) can be used as a proxy for the size of a structure (Alibert et al. 2001; Rosenberg 2001; Ubukata 2003; Pizzo et al. 2006a, b; 2008, 2009). Centroid sizes of each structure were calculated using TPSRelw. Allometries of each structure were calculate with SPSS 19.0 software (SPSS Inc., Chicago, IL).

After calibration, linear measurements of pronotum width (as a proxy of body size) and horn length (taken as described in Macagno et al., 2009; see Fig. 2) were taken with the software LAS v. 2.5.0 (Leica Application Suite).

2.4 Horn static allometries

For each population, horn lengths were graphed as a function of body size (Eberhard and Gutierrez 1991; Emlen and Nijhout 2000; Moczek and Nijhout 2003). Earlier studies on the *fracticornis-similis-opacicollis* complex in allopatry (Macagno et al., 2009, 2011) demonstrated that a sigmoidal model was a good fit for male horn length-body size scaling relationship. The visual inspection of the distribution of the male horn length-body size data of all populations analysed in this paper showed an analogous pattern of horn expression; their scaling relationships were determined by fitting to the data the four-parameter non-linear regression

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

(Moczek et al. 2004), where *x* is body size, *y* is horn length, y_0 specifies the minimum horn length, *a* defines the horn length range in the sample, *b* represents 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.

We used four parameters to characterize the average scaling relationship between horn length and body size in each population. Of those, the threshold body size *c*, or point of inflection of the sigmoid, is understood best with respect to its underlying developmental mechanisms, whereas the other parameters (i.e. slopes or range of horn lengths), are thought to be a little more sensitive to small sample sizes (Moczek & Nijhout, 2003). To examine the degree to which the differences evidenced in sigmoidal regression explained real allometric differences amongst the populations, repeated Welch's t-tests on parameter *c* were carried out (Sokal & Rohlf, 1995; Moczek et al., 2002; Moczek & Nijhout, 2003, Macagno et al., 2011a). All significance levels were adjusted for multiple comparisons using Bonferroni correction.

2.5 Principal Component Analysis

Generalized Procrustes Analysis (GPA), multivariate descriptions and Principal Component Analysis of the shape variables were performed using TpsRelw 1.46 (Rohlf 2008) on landmark configurations drawn on the head, epipharynx, parameres and sclerite 5. Additionally, a PCA was conducted on pygidial flaps of female individuals used for biomolecular analyses. Landmark configuration on pygidial flaps was the same as in Macagno et al., 2012. Visualisation of transformation grids were made using this sampe

software to describe interspecific shape variations. The first two principal component scores were plotted on an axis system using SPSS 19.0 software (SPSS Inc., Chicago, IL); with the same software we also constructed 3D plots including centroid size values of each structure on the third axis. Parallel inspection of principal component plots and transformation grids revealed trends in shape variation of each structure.

2.6 Static allometries of genital traits

Static allometries of genital traits were represented by plotting on an axis system the linear measurement of pronotum width, as a proxy of body size, and the centroid size measurements of each trait, derived from landmark configuration and GPA. Regressions for the reference species (*O fracticornis*-Val Ferret, *O similis*- Teilhède, *O. opacicollis*-Pisa) were calculate with a linear model (Macagno et al, 2011a) and represented as lines in the graph; the position of all other individuals with respect to these references were explored and qualitatively compared on the plots. Static allometries for head and epipharynx were not calculated and considered as a previous study (Macagno et al, 2011a) showed that there is not any significant difference among the allometries of the species of the complex even when allopatric and geographically distant populations were compared.

2.7 Biomolecular analyses

A fragment of the mitochondrial gene cytochrome oxidase subunit 1 (COI) was amplified and sequenced using the primers Pat (5'tccaatgcactaatctgccatatta) and Jerry (5'caacatttattttgatttttttgg) (Simon et al., 1994) from DNA samples of female and male specimens of the species complex conserved in an entomological DNA-bank at the Life

Sciences Department. These specimens were identified as *O. fracticornis*, *O. simlis*, *O. opacicollis* and "Uncertain" (=IUTS) (see Table 2); determination was made with the same standard criteria used in this study. 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, Zoologische Staatssammlung München, Germany) were added to the alignment as the outgroup. We constructed a phylogenetic tree inferred through a Bayesian approach by the Markov chain Monte Carlo method (as implemented in the MrBayes 3.1.12 package [Ronquist and Huelsenbeck, 2003]), using a general time reversible (Tavaré, 1986) model with gamma-distributed rate variation across sites, and a proportion of invariable sites (as in Macagno et al., 2011). A 50% majority rule consensus tree was generated and visualized using TreeView 1.6.6 (http://taxonomy.zoology. gla.ac.uk/rod/treeview.html). Distance-based (Neighbour-joining, NJ) maximum parsimony (MP) and minimum evolution (ME) trees were also generated using MEGA 4.0 software (Kumar et al., 2001). The MP phylogenetic tree was based on the closest neighbour-interchange method. We calculated consensus trees with a 50% majority rule cut-off. Pairwise distances between haplotypes for NJ analysis were obtained under the assumptions of the Jukes–Cantor model.

3. Results

3.1 Horn static allometries

Horn length-body size scaling relations are shown in Fig. 2, and sigmoid regression parameters for each population are reported in Table 3. For the inflection point *c*, values

followed by the same letter do not differed significantly between populations (significance was assessed at $P<0.05$ with multiple Welch's T-tests including Bonferroni correction). This comparison showed that populations of the three species differ significantly for the parameter *c*, thus having "species-specific" *minor-major* switchpoints; on the contrary, the populations composed by IUTS did not show a significant difference in this parameter with respect to *O. similis*.

3.2 Principal Component Analysis of shape variables and static allometries.

Results of Principal Component Analysis are summarised in Table 4. The analyses carried out on each morphological structure showed, as a general trend, a good shape differentiation of *O. fracticornis* from the rest of the sample and a weak , although perceptible, differentiation between *O. similis* and *O. opacicollis*, which tended to overlap. This was particularly true for the parameres (Fig. 3), where *O. fracticornis* formed an independent, strongly differentiated morphogroup, but also the PCA of shape variables from other structures showed a good differentiation of *O. fracticornis* when information on centroid size values was added (sclerite 5: Fig. 4, head: Fig. 5 and epipharynx: Fig.6). Female pygidial flaps analysis was in agreement with these results, showing an evident separation of the *O. fracticornis* individuals along the first principal axis (Fig. 7). The relationships between *O. similis*, *O. opacicollis* and Iberian IUTS evidenced by these analyses resulted in a situation characterised by two partially contradictory patterns: PCA of parameres showed a pattern in agreement with the results of the horn static allometry, where IUTS largely overlapped *O. similis* individuals in the morphospace (Fig. 2 and Fig. 3). The same is true for female pygidial flaps (Fig. 7). On the contrary, PCA of head, epipharynx and sclerite 5 showed that IUTS individuals form a partly recognisable and independent morphogroup which did not present intermediate shapes

between *O. similis* and *O. opacicollis*. The IUTS group seemed a little more similar to *O. opacicollis* than to *O. similis* (Figs. 4, 5 and 6). The contemporary visual inspection of PCA plots, deformation grids and the original photographs allowe to describe some morphological differences characterising IUTS. This is particularly evident for the genital sclerite: The sclerite 5 shape of IUTS appears more thin than that of *O. opacicollis*, which is, in turn, the larger among all and that with the more evident fold on the left side (see Fig. 1). This left side in the IUTS group is shorter and less developed downward than that of *O. similis*. Moreover, IUTS show the less noticeable fold in the left side and the top of the structure is flatter, especially with respect to that of *O. similis*, which is in turn the most curved. Indeed, the sclerite 5 of IUTS seems to exhibit an original and recognizable shape with respect to *O. similis* and *O. opacicollis*. Head shape differences are less marked but it is possible to find some general trend: *O. similis* head show a particular profile, more compressed in the fore-hind direction and with the genea triangular-shaped and the angle between the genea and the eyes and temples more sharp. IUTS, at the opposite, have a more elongated head and the genae-eyes angles obtuse, very less marked. The head of *O. opacicollis* seems to exhibit a intermediate profile between *O. similis* and IUTS, and more rounded genae. However, it is impossible to find any true discontinuity between the shapes of IUTS, *O. similis* and *O. opacicollis*; as a general trend, even the shapes of *O. opacicollis* and *O. similis* morphological structures, independently from the geographical origin of the individuals, tended to be largely overlapping and thus very similar.

Results of static allometries were visualised in Fig. 8 and parameters of allometries from allopatric, reference populations are shown in Table 5. Parameres and sclerites 5 allometries showed a pattern in agreement with paramere PCA, where IUTS tended to overlap *O. similis* reference individuals.

An unexpected and very complex pattern was found for the population of Santiago, classified as *O. similis*, as on the basis of traditional criteria based on head morphology. Accordingly, the PCA of head and epipharynx positioned individuals from Santiago in a region of the morphospace where they overlap Theilhède individuals, even when information on the centroid size was added to the two first principal axes in the plot (Fig. 5 and 6). These results are in conformity with those of horn allometry. On the contrary, parameres and sclerite 5 PCA and, in particular, static allometry of parameres, showed that some individuals are not in the expected morphospace near to *O. similis*, but overlap *O. opacicollis* individuals.

Another unexpected result concerned the populations of Castellane and Isobol, that was mostly separate from Val Ferret population in the static allometry of parameres; this same differentiation was not detectable in the static allometry of the sclerite 5.

3.3 Biomolecular data

We amplified and aligned 601 bp sequences resulting in a combined matrix with 134 parsimony informative characters. The closest phylogenetic relationship was between *O. opacicollis* and *O. similis* (p-distance = 0.056) whereas *O. fracticornis* was more differentiated (p-distance = 0.105 from IUTS, p-distance = 0.093 from *O. opacicollis* and 0.109 from *O. similis*). Distances of each species with respect to the outgroup were all in the same range of magnitude (~0.13). Intraclade p-distances were always much lower than interspecific ones ("IUTS clade"= 0.003; *O. fracticornis* = 0.025, *O. opacicollis* = 0.007, *O. similis* = 0.006). The trees generated by the different methods showed two different basic topologies and slight differences in the resolution of terminal clades and robustness of the nodes. In all trees *O. fracticornis-similis-opacicollis* allopatric populations split into three, separated clades, where *O. fracticornis* was the most differentiated species and *O. similis* and

O. opacicollis were more closely related. On the contrary, most individuals from sympatric *O. similis* and *O. opacicollis* populations resulted in the "right" clade together with the other conspecific. IUTS and some *O. similis* (8) and *O. opacicollis* (4) individuals only from Iberian sympatric populations formed a further clade. Very few IUTS fell in *O. opacicollis* (3 from Zufre) or *O. similis* (1 from Avila-El Barraco) clades. In the Bayesian (Fig. 9), neighbor joining (NJ) and minimum evolution (ME) trees, this "IUTS clade" originated from a node before the split between *O. similis* and *O. opacicollis* allopatric populations. In these analyses, *O. fracticornis* and "IUTS clade" nodes were highly supported (near 100%), The split between *O. similis* and *O. opacicollis*, on the contrary, showed low support (boostraps: 58% in Bayesian, 69% in NJ, 60% in ME). MP analysis generated a large number of equally parsimonious trees (136); the tree length was 303. For parsimony informative sites, the consistency index was 0.61, and the retention index 0.96. The trees resulted in a consensus tree where the differentiation of *O. fracticornis* was well supported, but the "*O. opacicollis* clade" originated before the split between the "IUTS clade" and *O. similis*.

4. Discussion

This discussion endeavours to find an explanatory framework that is able to combine and explain the complex and partially inconsistent patterns emerging from the multiple approaches adopted here: biomolecular were able to reveal at a genetic level the existence of an unexpected clade in the complex composed by Iberian IUTS, plus sporadic individuals from sympatric *O. similis* and *O. opacicollis* populations, in addition to the three main, monophyletic clades composed by allopatric *O. similis*, *O. opacicollis* and *O. fracticornis* populations respectively, already evidenced in Macagno et al. (2011a) and confirmed here. This genetic profile fitted with the pattern shown by geometric morphometric analyses of

head and sclerite 5. On the contrary, horn static allometries, generally showing good correspondence with phylogeny, were not able to differentiate *O. similis* from IUTS; this same result emerged from geometric morphometric analyses of parameres and static allometries of the two genital traits. The analysis of female pygidial flaps removed from the same individuals used for biomolecular analysis showed a pattern in agreement with that of parameres but inconsistent with their own molecular phylogeny. However, the MP tree, while recognising the existence of a mt-group composed mainly by Iberian IUTS, exhibited its higher similarity with *O. similis* placing IUTS and *O. similis* on a terminal dicothomy, after the node separating *O. opacicollis* populations.

In detail, the first and most noteworthy result concerned the ability of geometric morphometrics to elucidate mathematically head shape differences among taxa. The plot of the first two principal components returned a pattern in which the three species of the complex occupied recognizable regions of the morphospace, although partially overlapping. IUTS head shapes formed an identifiable morphogroup: head shape was not intermediate between *O. similis* and *O. opacicollis* like as expected, for example, for hybrids but unique and original, nearly as different as it is *O. fracticornis* from the two other species.

This pattern of head shape differences highlights the difficulties found in determining the status of these specimens at the first step of the study, because standard determination keys we used are mainly based on head traits. The good correspondance between our initial determination of a Iberian subsample as IUTS and the existence of a mathematically identifiable IUTS head shape morphogroup and of IUTS genetic clade is almost surprising. Evidently, the taxonomist'eye has a good sensitivity in perceiving subtle morphological variations and placing a discontinuity even when the morphological variation appeared more as a gradient than discrete.

The internal sclerite of genitalia and the epipharynx confirmed the existence of this new, differentiated morphogroup. Werner and Simmons (2008), studying the congeneric species *Onthophagus taurus*, demonstrated that the sclerite 5 (House and Simmons, 2003) directly interact with internal female genitalia: the small spine lies along the bend of the sclerotized spermathecal duct extension, while the longer spine is inserted deeply into the duct opening: the main body and the large spine of sclerite 5 are hollow, and filled with seminal secretion that is injected into the spermathecal duct. Consequently, the extraordinarily optimized function of this trait seems directly involved in fertilization success, likely more so than the mechanical coupling of parameres and pygidial flaps. Any morphological differentiation of sclerite 5 could have effects on differential fitness, reproductive isolation and in driving speciation; this might explain why sclerite 5 perfectly reflects phylogeny, being able to single out IUTS as an independent morphogroup and point out some IUTS overlapping both *O. similis* and *O. opacicollis* morphospaces, as in the phylogenetic tree.

As in a previous study (Macagno et al., 2011a), we found that the pattern of shape variation of the epipharynx was relatively unique, and decoupled from that of parameres and heads and from horn allometries. As in Macagno et al. (2011a), *O. opacicollis* seemed the most differentiated species within the complex, whereas *O. fracticornis* and *O. similis* showed a lower degree of differentiation. IUTS shapes, while in part recognisable, seemed more similar to thoseof *O. opacicollis*. The epipharynx plays an extremely specific role in that it is used in filtering and selecting food particles. Considering the strict association of *Onthophagus* species with particular feeding resources (Halffter and Matthews, 1966), stabilising selective pressures are likely to act on this structure, so that optimal functionality is maintained independent of sex and morph (Palestrini et al. 2000, Pizzo et al., 2009). Previous studies have suggested that epipharynx modifications might rely more on adaptive and selective than on phylogenetic causes (Verdù and Galante, 2004; Macagno et al., 2011a): the three species

have been described as feeding on the same types of dung (Martín-Piera & López-Colón, 2000), but to our knowledge no studies have been carried out 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); moreover, dung pads are not only feeding, but also mating, resources for *Onthophagus* beetles (Halffter & Matthews, 1966). Within this framework, we can hypothesize that the slight differences in epipharynx shape detected among *O. similis*, IUTS and *O. opacicollis* could derive from a mechanism of resource-dependent timing or spatial segregation that could provide a means of divergence between the species. Specifically, the shape modifications of the epipharynx hint at the possibility that adaptations to different trophic resource *use* and a form of ecological segregation might have driven the divergence process among these species.

The phylogenetic pattern hints at the possibility that a new taxon could belong to the species-complex. If is the case, this taxon, recorded only in the Iberian Peninsula, is likely ancestral to the differentiation of *O. similis* and *O. opacicollis*. The anomalous presence of some *O. similis* and *O. opacicollis* within the branches mainly composed by IUTS and, on the contrary, the presence of very few IUTS in the clades occupied mainly by *O. similis* or *O. opacicollis* populations might be consistent with the hypothesis that some introgressive exchange of genetic material between *O. similis* and *O. opacicollis* and between IUTS and Iberian *O. similis* and O. *opacicollis* occurs. This result would be in agreement with that found by Martín-Piera and Boto (1999) who found shared allozymes in the same sympatric Iberian *O. similis* and *O. opacicollis* population analysed in this study. However, we are not able to exclude some sporadic errors in species determination for Iberian individuals at the beginning of the study, due to the difficulty in using standard keys.

Other analyses appeared in contrast to the above picture. Horn static allometries, genital static allometries, parameres and pygidial flap shapes suggested that IUTS are more likely morphologically indistinguishable from *O. similis*. For some aspects the MP tree, even recognising an independent mt-group for IUTS, placed them very closely to *O. similis*.

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). 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. This produces population- and species-specific allometries (Moczek et al., 2002; Moczek & Nijhout, 2003; Moczek, 2009a, b, Macagno et al., 2011a, Pizzo et al., 2011), normally reflecting phylogeny. The three species of the complex showed distinct, species-specific horn static allometries (Fig. 2) where the body size at the point of inflection of the sigmoid curves was smallest in *O. similis* and increased progressively in *O. opacicollis* and *O. fracticornis*. This pattern was in keeping with the phylogeny and congruent with genetic distances for allopatric populations, but failed to detect the differences between *O. similis* and Iberian IUTS evident in other approaches, as if the genetic bases of horn development were remained the same despite the divergence of mitochondrial sequences, and head and sclerite shape. These results might indicate that male polyphenism has not been the driving force promoting differentiation within the complex, and the differentiation of allometries found for allopatric populations might be a secondary by-product of the later geographic isolation.

Genital traits are considered to be evolving very quickly in the genus *Onthophagus*, and often their differentiation pattern clearly marks out the reproductive isolation and the result of a divergence process (Eberhard, 1989, Pizzo et al., 2006, 2008, Macagno et al., 2011a). Both male parameres and female pygidial flaps shared the same pattern exhibited by horn static

allometries of males. Male parameres do interact closely with female pygidial flaps during copulation (Werner and Simmons, 2008). A recent study (Macagno et al., 2011b) suggested that male and female copulatory structures linked mechanically during copulation may diverge in concert with respect to their shapes and can evolve between closely related species and even recently established populations in a remarkably short amount of time. Our results provided further support for this hypothesis on the evolution of genitalia that implicitly or explicitly predict concomitant evolutionary changes of male and female genitalia. However, the incongruent pattern exhibited for male parameres and sclerites, and from shape and size analysis of sclerites, provided additional support to studies giving evidence for mosaic evolution of genitalia (House and Simmons 2005; Song and Wenzel 2008; Werner and Simmons 2008; Pizzo et al., 2012) and suggesting that size and shape of different portions of the same copulatory structures respond to different selective pressures depending on their function during copulation. Size and shape of genital structures and their component parts may be sufficiently developmentally and genetically decoupled to evolve rather independently of each other. Therefore, it seems that local, segment-specific regulation of differential growth and differentiation must underlie genitalic development and evolution, rather than genitalia-specific developmental processes (McPeek et al. 2009, 2011; Macagno et al. 2011b; Pizzo et al., 2012).

To summarize, we interpret the set of results with a hypothetical scenario in which *O. fracticornis* separated first during the colonisation of western Europe and differentiated following its preference for mountainous regions, evolving in separated, stenoecious populations acting as evolutionary, independent islands: this is clearly shown both in morphological and genetic analyses performed in Pizzo et al., 2011 and in this paper, in the strong populational structure of the molecular tree (Fig. 9) and in the static allometry of parameres (Fig. 8). The ancestral form giving rise to *O. similis* and *O. opacicollis* moved

westwards colonising the Iberian Peninsula, where it is still recognisable from a genetic and morphological point of view in the IUTS. Along with this colonisation, ecological segregation and possibly a differential trophic resource use could have provided a means for the progressive divergence of *O. similis* and O. *opacicollis*, the first toward hilly and transitional climates and the second toward more thermophilic Mediterranean areas at lower altitudes and latitudes (Kirk e Ridsdill-Smith, 1986); in the areas where the species do not co-occur, this process is realised and the taxa appear to be good, fully differentiated species. In the Iberian Peninsula, the divergence process and the ecological segregation may not be fully concluded and a certain level of introgressive exchange of genetic material persist among the descendant of the ancestral form - the IUTS - and *O. similis* and *O. opacicollis*, resulting in a reticulate genetic network. This view partially fits with that of Martìn-Piera and Boto (1999) who argued that local morphological variation, incomplete ecological segregation and the presence of shared allozymatic alleles found in Iberian populations were still attributes of a polymorphic, highly adaptable species rather than characters of two, completely separated yet closely related species.

In conclusion, the results provided a complex and often incongruent pattern of morphological and genetic evolution, that in our view is consistent with a hypothesis of a still unfinished speciation process in sympatric areas and a fully realised speciation by means of ecological segregation in allopatry.

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Figure captions

Fig. 1. Landmark configurations on each morphological structure analysed: head (first row, nine landmarks), epipharynx (second row, nine landmarks), sclerites 5 (third row, five landmarks) and parameres (fourth row, five landmarks).

Fig. 2. Horn static allometries of each population, showing the sigmoidal allometric relationship between pronotum width and horn length. The red lines show how the pronotum and head measurements were taken from each specimen. The population of Isobol (see Table 1) was not considered in this plot due to the low number of individuals.

Fig. 3. Scatterplot of principal components 1 and 2 resulting from a PCA of parameres shape variables and parameres centroid size. For each principal component, percentage of variance explained is indicated in brackets. Deformation grids showing shape modifications described by the first two principal components of *O. similis*, *O. opacicollis* and IUTS (a), and *O. fracticornis* (b) are reported above the 2D graph. Specimens of the species *O. fracticornis* are not shown in the 3D scatterplot.

Fig. 4. Scatterplot (2D and 3D) of principal components 1 and 2 resulting from a PCA of sclerite 5 shape variables and sclerite 5 centroid size. For each principal component, percentage of variance explained is indicated in brackets.

Fig. 5. Scatterplot (2D and 3D) of principal components 1 and 2 resulting from a PCA of head shape variables and head centroid size. For each principal component, percentage of variance explained is indicated in brackets.

Fig. 6. Scatterplot (2D and 3D) of principal components 1 and 2 resulting from a PCA of epipharynx shape variables and epipharynx centroid size. For each principal component, percentage of variance explained is indicated in brackets.

Fig. 7. Scatterplot (2D and 3D) of principal components 1 and 2 resulting from PCA of pygiadial flaps shape variables and pygidial flaps centroid size. For each principal component, percentage of variance explained is indicated in brackets.

Fig. 8. Paramere and sclerite 5 log centroid size versus log pronotum width scatterplot. Static allometries for "reference" populations from Val Ferret (*O. fracticornis*), Pisa (*O. opacicollis*) and Teilhéde (*O similis*) are shown.

Fig. 9. The 50% majority rule consensus tree from the Bayesian Monte Carlo Markov chain analysis of mtDNA cytochrome oxidase subunit 1 (COI). *Of, Onthophagus fracticornis*; *Oo, Onthophagus opacicollis*; *Os, Onthophagus similis*, IUTS Individual with uncertain taxonomic status. Specimens belonging to *Onthophagus vacca* and *Onthophagus nuchicornis* European populations were used as an outgroup (indicated as *Palaeonthophagus spp*. in the trees, courtesy of Dirk Ahrens).

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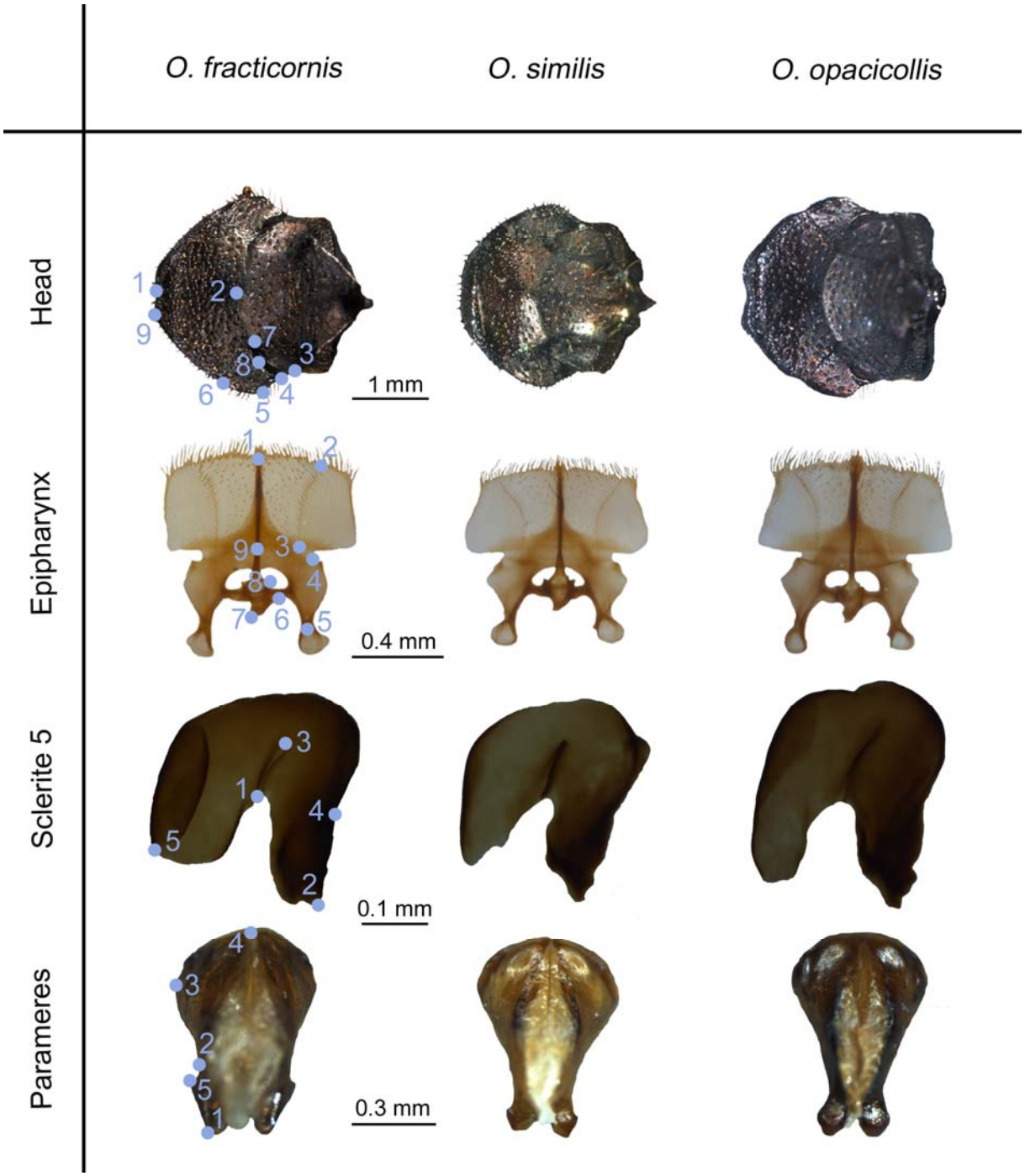
O. fracticornis
O. similis
O. opacicollis
Head
Epipharynx
Sclerite 5
Parameres
1 mm
0.4 mm
0.1 mm
0.3 mm

Fig 1

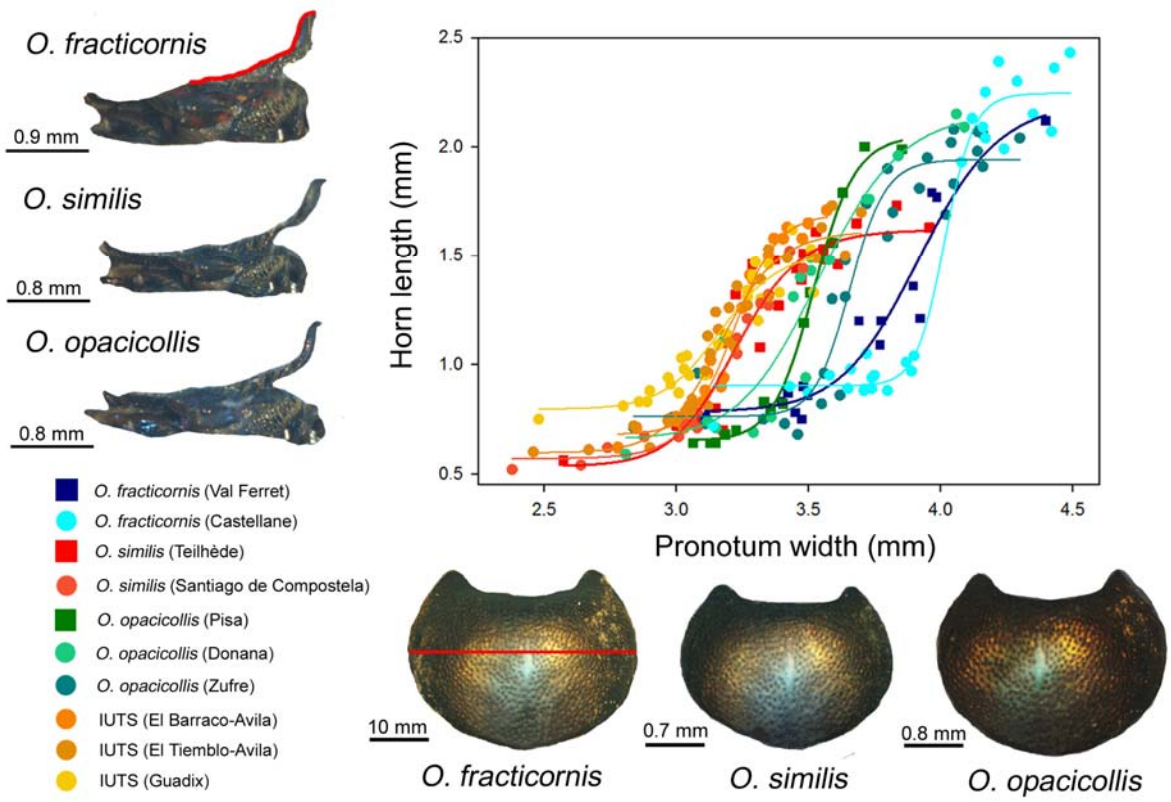
O. fracticornis
0.9 mm
O. similis
0.8 mm
O. opacicollis
0.8 mm
O. fracticornis (Val Ferret)
O. fracticornis (Castellane)
O. similis (Teilhède)
O. similis (Santiago de Compostela)
O. opacicollis (Pisa)
O. opacicollis (Donana)
O. opacicollis (Zufre)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)
IUTS (Guadix)
Horn length (mm)
Pronotum width (mm)
10 mm
0.7 mm
0.8 mm
O. fracticornis
O. similis
O. opacicollis

Fig 2

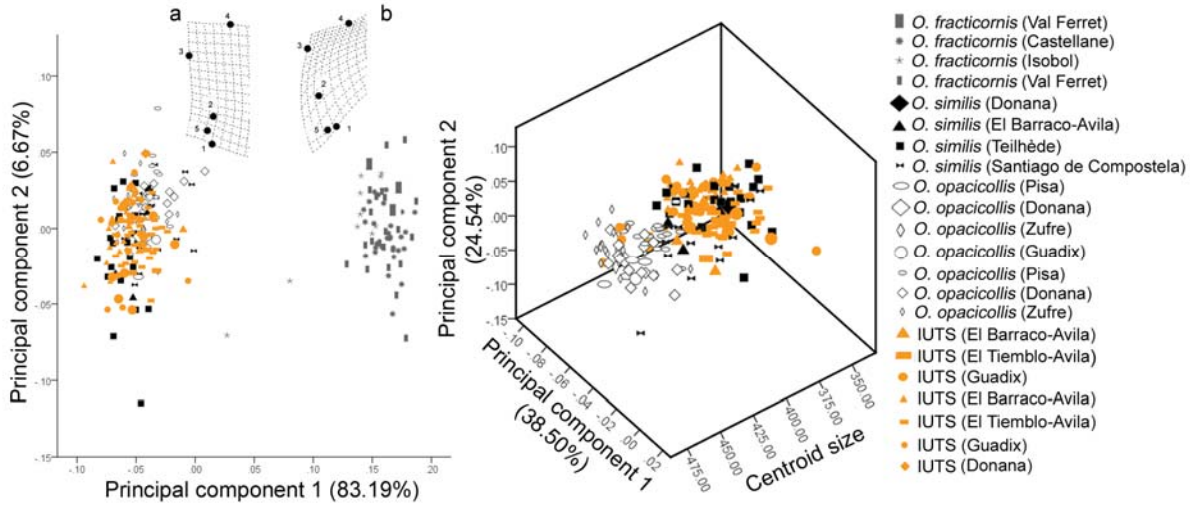
a
b
Principal component 2 (6.67%)
Principal component 1 (83.19%)
Principal component 2 (24.54%)
Principal component 1 (38.50%)
Centroid size
O. fracticornis (Val Ferret)
O. fracticornis (Castellane)
O. fracticornis (Isobol)
O. fracticornis (Val Ferret)
O. similis (Donana)
O. similis (El Barraco-Avila)
O. similis (Teilhède)
O. similis (Santiago de Compostela)
O. opacicollis (Pisa)
O. opacicollis (Donana)
O. opacicollis (Zufre)
O. opacicollis (Guadix)
O. opacicollis (Pisa)
O. opacicollis (Donana)
O. opacicollis (Zufre)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)
IUTS (Guadix)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)
IUTS (Guadix)
IUTS (Donana)

Fig 3

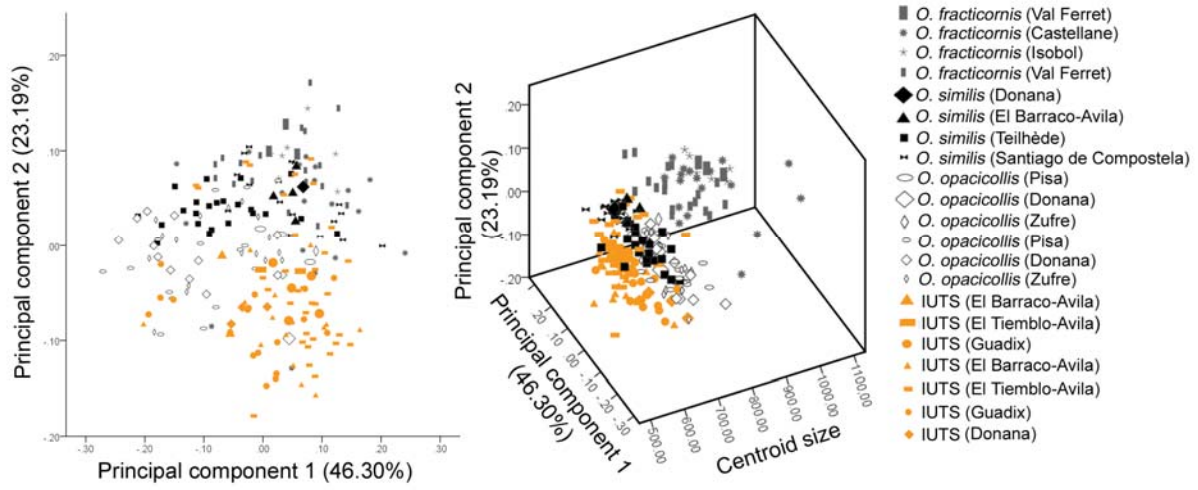
Principal component 2 (23.19%)
Principal component 1 (46.30%)
Principal component 2 (23.19%)
Principal component 1 (46.30%)
Centroid size
O. fracticornis (Val Ferret)
O. fracticornis (Castellane)
O. fracticornis (Isobol)
O. fracticornis (Val Ferret)
O. similis (Donana)
O. similis (El Barraco-Avila)
O. similis (Teilhède)
O. similis (Santiago de Compostela)
O. opacicollis (Pisa)
O. opacicollis (Donana)
O. opacicollis (Zufre)
O. opacicollis (Pisa)
O. opacicollis (Donana)
O. opacicollis (Zufre)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)
IUTS (Guadix)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)
IUTS (Guadix)
IUTS (Donana)

Fig 4

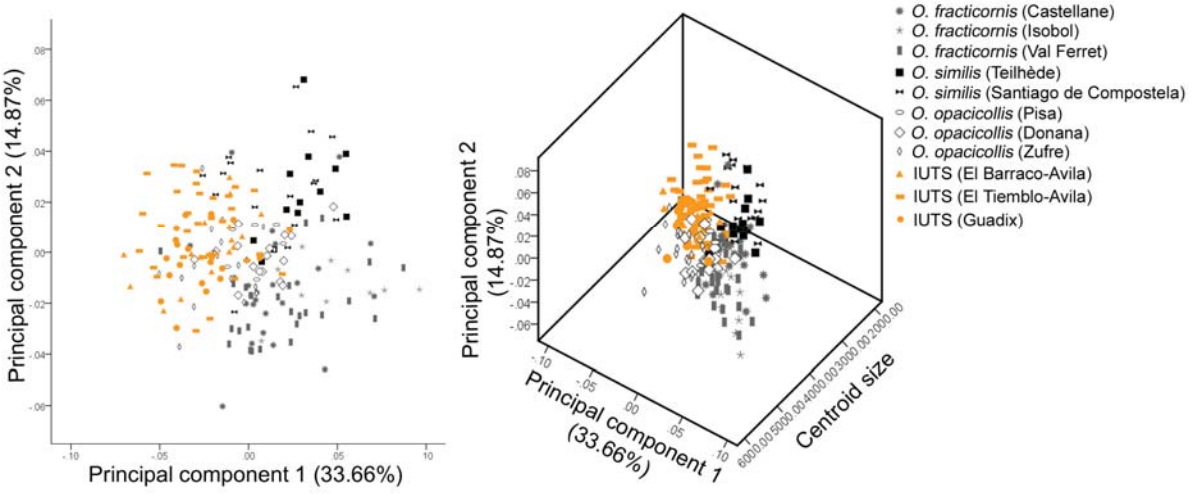
Principal component 2 (14.87%)
Principal component 1 (33.66%)
Principal component 2 (14.87%)
Principal component 1 (33.66%)
Centroid size
O. fracticornis (Castellane)
O. fracticornis (Isobol)
O. fracticornis (Val Ferret)
O. similis (Teilhède)
O. similis (Santiago de Compostela)
O. opacicollis (Pisa)
O. opacicollis (Donana)
O. opacicollis (Zufre)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)
IUTS (Guadix)

Fig 5

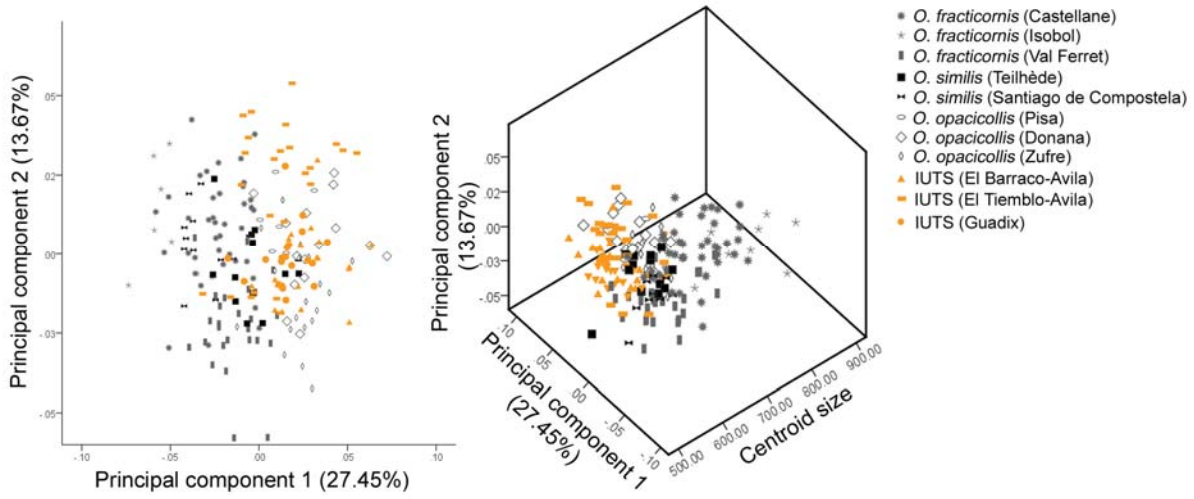
Principal component 2 (13.67%)
Principal component 1 (27.45%)
Principal component 2 (13.67%)
Principal component 1 (27.45%)
Centroid size
O. fracticornis (Castellane)
O. fracticornis (Isobol)
O. fracticornis (Val Ferret)
O. similis (Teilhède)
O. similis (Santiago de Compostela)
O. opacicollis (Pisa)
O. opacicollis (Donana)
O. opacicollis (Zufre)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)
IUTS (Guadix)

Fig 6

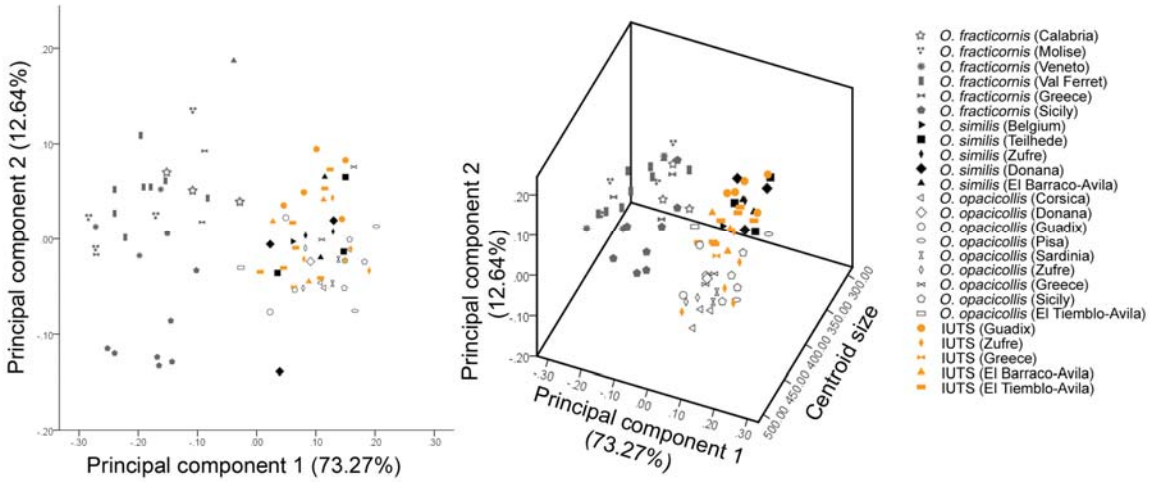
Principal component 2 (12.64%)
Principal component 1 (73.27%)
Principal component 2 (12.64%)
Principal component 1 (73.27%)
Centroid size
O. fracticornis (Calabria)
O. fracticornis (Molise)
O. fracticornis (Veneto)
O. fracticornis (Val Ferret)
O. fracticornis (Greece)
O. fracticornis (Sicily)
O. similis (Belgium)
O. similis (Teilhede)
O. similis (Zufre)
O. similis (Donana)
O. similis (El Barraco-Avila)
O. opacicollis (Corsica)
O. opacicollis (Donana)
O. opacicollis (Guadix)
O. opacicollis (Pisa)
O. opacicollis (Sardinia)
O. opacicollis (Zufre)
O. opacicollis (Greece)
O. opacicollis (Sicily)
O. opacicollis (El Tiemblo-Avila)
IUTS (Guadix)
IUTS (Zufre)
IUTS (Greece)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)

Fig 7

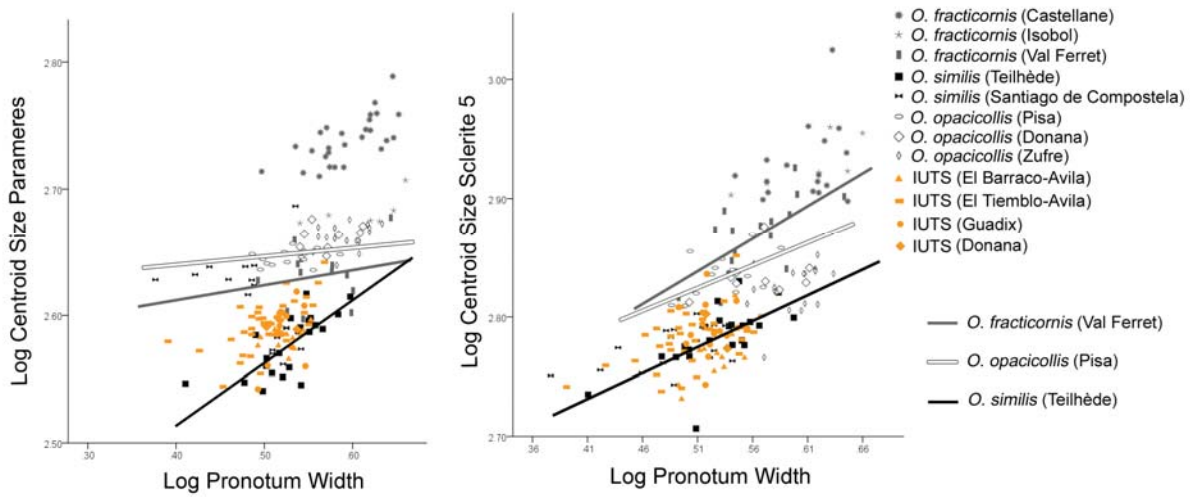
Log Centroid Size Parameres
Log Pronotum Width
Log Centroid Size Sclerite 5
Log Pronotum Width
O. fracticornis (Castellane)
O. fracticornis (Isobol)
O. fracticornis (Val Ferret)
O. similis (Teilhède)
O. similis (Santiago de Compostela)
O. opacicollis (Pisa)
O. opacicollis (Donana)
O. opacicollis (Zufre)
IUTS (El Barraco-Avila)
IUTS (El Tiemblo-Avila)
IUTS (Guadix)
IUTS (Donana)
O. fracticornis (Val Ferret)
O. opacicollis (Pisa)
O. similis (Teilhède)

Fig 8

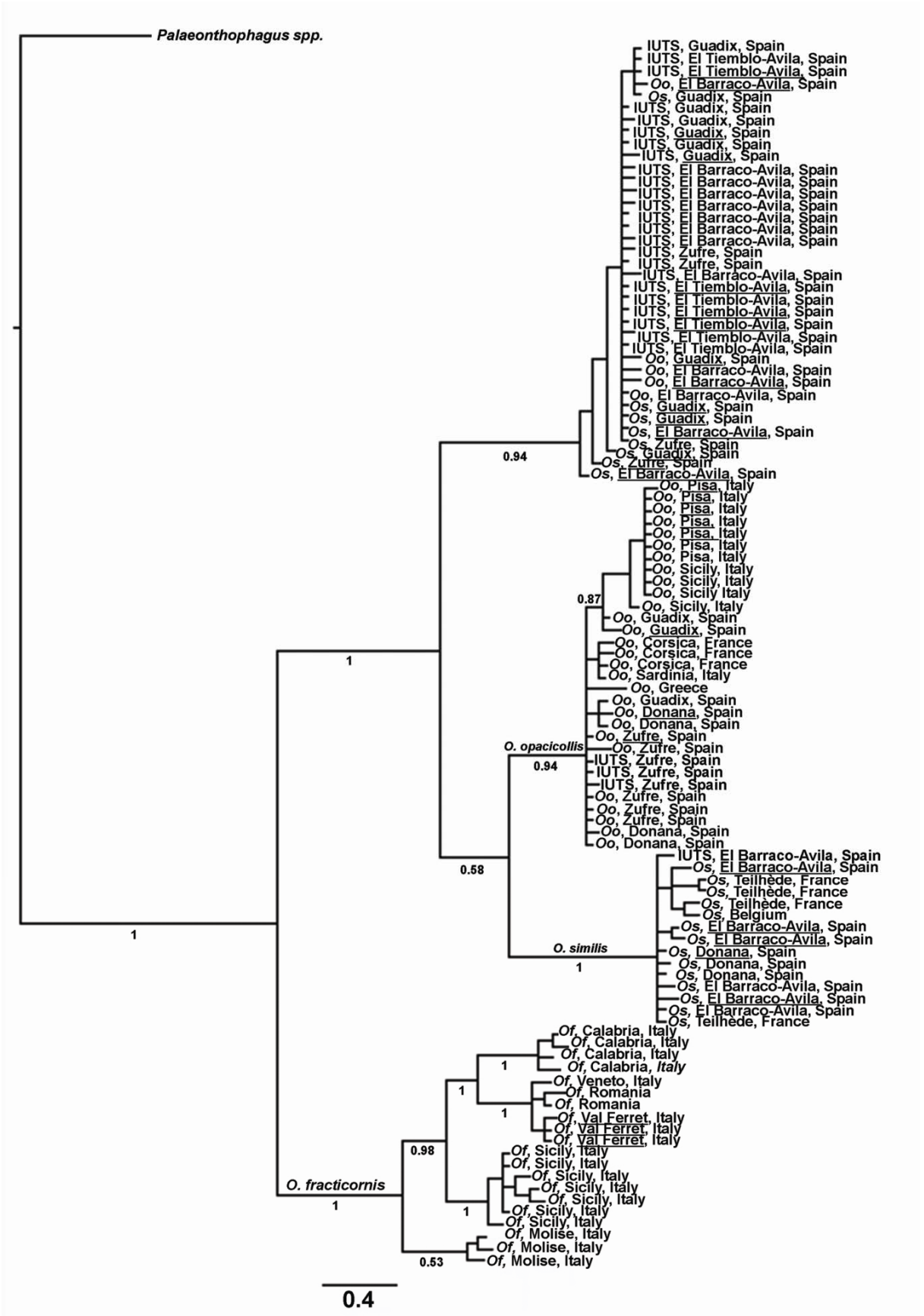
Palaeonthophagus spp.
IUTS, Guadix, Spain
IUTS, El Tiemblo-Avila, Spain
IUTS, El Tiemblo-Avila, Spain
Oo, El Barraco-Avila, Spain
Os, Guadix, Spain
IUTS, Guadix, Spain
IUTS, Guadix, Spain
IUTS, Guadix, Spain
IUTS, Guadix, Spain
IUTS, Guadix, Spain
IUTS, El Barraco-Avila, Spain
IUTS, El Barraco-Avila, Spain
IUTS, El Barraco-Avila, Spain
IUTS, El Barraco-Avila, Spain
IUTS, El Barraco-Avila, Spain
IUTS, El Barraco-Avila, Spain
IUTS, El Barraco-Avila, Spain
IUTS, Zufre, Spain
IUTS, Zufre, Spain
IUTS, El Barraco-Avila, Spain
IUTS, El Tiemblo-Avila, Spain
IUTS, El Tiemblo-Avila, Spain
IUTS, El Tiemblo-Avila, Spain
IUTS, El Tiemblo-Avila, Spain
IUTS, El Tiemblo-Avila, Spain
IUTS, El Tiemblo-Avila, Spain
Oo, Guadix, Spain
Oo, El Barraco-Avila, Spain
Oo, El Barraco-Avila, Spain
Oo, El Barraco-Avila, Spain
Os, Guadix, Spain
Os, Guadix, Spain
Os, El Barraco-Avila, Spain
Os, Zufre, Spain
Os, Guadix, Spain
Os, Zufre, Spain
Os, El Barraco-Avila, Spain
0.94
Oo, Pisa, Italy
Oo, Pisa, Italy
Oo, Pisa, Italy
Oo, Pisa, Italy
Oo, Pisa, Italy
Oo, Pisa, Italy
Oo, Pisa, Italy
Oo, Sicily, Italy
Oo, Sicily, Italy
Oo, Sicily Italy
Oo, Sicily, Italy
0.87
Oo, Guadix, Spain
Oo, Guadix, Spain
Oo, Corsica, France
Oo, Corsica, France
Oo, Corsica, France
Oo, Sardinia, Italy
Oo, Greece
Oo, Guadix, Spain
Oo, Donana, Spain
Oo, Donana, Spain
Oo, Zufre, Spain
O. opacicollis
Oo, Zufre, Spain
IUTS, Zufre, Spain
0.94
IUTS, Zufre, Spain
IUTS, Zufre, Spain
Oo, Zufre, Spain
Oo, Zufre, Spain
Oo, Zufre, Spain
Oo, Donana, Spain
Oo, Donana, Spain
1
1
0.58
IUTS, El Barraco-Avila, Spain
Os, El Barraco-Avila, Spain
Os, Teilhède, France
Os, Teilhède, France
Os, Teilhède, France
Os, Belgium
Os, El Barraco-Avila, Spain
Os, El Barraco-Avila, Spain
O. similis
Os, Donana, Spain
1
Os, Donana, Spain
Os, Donana, Spain
Os, El Barraco-Avila, Spain
Os, El Barraco-Avila, Spain
Os, El Barraco-Avila, Spain
Os, Teilhède, France
Of, Calabria, Italy
Of, Calabria, Italy
Of, Calabria, Italy
1
Of, Calabria, Italy
1
Of, Veneto, Italy
Of, Romania
1
Of, Romania
Of, Val Ferret, Italy
Of, Val Ferret, Italy
Of, Val Ferret, Italy
0.98
Of, Sicily, Italy
Of, Sicily, Italy
Of, Sicily, Italy
Of, Sicily, Italy
Of, Sicily, Italy
1
Of, Sicily, Italy
Of, Sicily, Italy
Of, Molise, Italy
Of, Molise, Italy
0.53
Of, Molise, Italy
O. fracticornis
1
0.4

Fig 9