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Interspecific shape divergence in Aphodiini dung beetles: the case of *Amidorus obscurus* and *A. immaturus* (Coleoptera: Scarabaeoidea)

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Abstract The dung beetles *Amidorus obscurus* and *A. immaturus* are nearly indistinguishable, being characterized by a marked constancy in external morphological traits and little sexual dimorphism in adults. We studied two syntopic populations from the Italian Alps by means of geometric morphometric analyses. To identify eventual undetected shape differences between species, we focused upon the head, pronotum and scutellum (three external traits) and the epipharynx. Results indicate that the external traits are rather similar in the two species, whereas the epipharynx is clearly different. Interspecific differences in the aedeagus were also taken into account; they are noteworthy because parameres of *A. immaturus* are different in shape and at least three times longer than those of *A. obscurus*. If it is assumed that the diversification of the two species took place during quaternary ice ages, *A. immaturus* would have evolved marked differences rather quickly, in keeping with the hypothesis of rapid genital evolution. In an ontogenetic trajectory framework, we also considered the morphology of larvae. Interspecific divergence in the shape of the epipharynx is already evident at the preimaginal stage, whereas that of the genital disc is not. Accordingly, we hypothesise that feeding and reproductive traits of the two species diverge morphologically when they become functional. Finally, by considering recent advances in ecological and evolutionary knowledge of dung beetles, the pattern of relative constancy in external morphology exhibited by the tribe Aphodiini, and that of great morphological diversification displayed by Onthophagini, were compared and hypotheses about the origins of these differences discussed.

Keywords: constancy in external morphology; divergent evolution; larval instars; geometric morphometrics; trait functionality.

Introduction

The subfamily Aphodiinae is a very speciose group (about 2000 species) that includes the dung beetle tribe Aphodiini, which, with about a thousand species being described so far (Scholtz et al. 2009), is characterized by a worldwide distribution, with a focus on temperate regions. Aphodiini display a rather uniform foraging behaviour, with both adults and preimaginal instars of most species being coprophagous and dung-dwelling (Dellacasa and Dellacasa 2006).

The remarkable taxonomic diversity of Aphodiini shows a certain constancy in the phenotypes of adults, which are characterized by a small body size (2-20 mm), a rather extended clypeus which covers the mouthparts, a pygidium largely overlapped by the elytra, untrimmed basal margins of elytra, nine-segmented antennae and the posterior tibiae with two apical spurs (Dellacasa and Dellacasa 2006). A previous phylogenetic analysis based on morphology suggested that in this group variation in morphological characters is small (Cabrero-Sañudo 2006). Aphodiini lack evident sexual dimorphism and alternative phenotypes in males, both potentially promoting diversification and speciation, and which are widespread in other dung beetle groups (Moczek 2008, 2010; Pfennig et al. 2010). In the subfamily Scarabaeinae, the tribe Onthophagini (2500 taxa or species, Scholtz et al. 2009, Philips 2011), for instance, displays a tremendous diversity of horn structures with many species developing horns or horn-like structures of some kind, which represent evolutionary modification of the same, original ancestral structure (Moczek 2005, 2008, 2011). In many species of the genus *Onthophagus,* there are large *major* males producing horns and smaller *minor* males which are hornless (a case of male polyphenism; Emlen et al. 2005; Macagno et al. 2009; Tomkins and Moczek 2009, Knell 2011). Phylogenetically close species with similar horns may diverge in static allometries, males of each species developing horns at different body sizes (Emlen et al. 2005; Lukasik et al. 2006; Moczek 2002; Tomkins and Simmons 2002).

In the pattern of uniformity of phenotypes exhibited in Aphodiini, interspecific differences in external morphology may be very subtle and certain species pairs may be virtually indistinguishable. To overcome these difficulties, a geometric morphometric approach can be employed. Geometric morphometrics has proved to be a useful technique for solving a variety of biological problems, and is more powerful than traditional morphometrics, having the ability to identify very subtle differences in shape (Lawing and Polly 2010; Slice 2007).

In northern Europe, dung beetle communities are dominated by Aphodiinae (Errouissi et al. 2004; Jay-Robert et al. 1997, 2008) and the same is true for high-altitude communities of the Alps and Pyrenees (Cabrero-Sañudo and Zardoya 2004; Cabrero Sañudo 2006; Cabrero Sañudo and Lobo 2006).

In the present study, we focused upon two species of the genus *Amidorus* (systematics according to Dellacasa and Dellacasa 2006 used), inhabiting high-altitude alpine pastures of the Ferret Valley (north western Italian Alps): *Amidorus obscurus* (Fabricius, 1792) and *A. immaturus* (Mulsant, 1842). Both species are Palaearctic (Dellacasa and Dellacasa 2006; Palestrini et al. 2008; Tagliaferri 2000, Tarasov 2008), although *A.obscurus* is widespread from Spain to Syria and Transcaucasus, whereas *A. immaturus* is restricted to a smaller area (SE France, NW Italy and Austria), included within the range of *A. obscurus*.

In the pattern of uniformity of phenotypes exhibited in Aphodiini, interspecific differences in external morphology may be very subtle and certain species pairs may be virtually indistinguishable. *Amidorus obscurus* and *A. immaturus* provide just such an example of strong morphological uniformity due to low phenotypic plasticity. The two species (and the two sexes) are almost indistinguishable on the basis of the external morphology, and the external traits typically employed in taxa identification (e.g., the clypeal margin of head, the pronotum punctuation, the elytral striae and interstices, the scutellum features) are in fact virtually useless. Instead, males of the two species can be identified on the basis of the shape of the aedeagus, which shows clear differences in the two species (Dellacasa 1983). (It must be underlined, however, that females of both species cannot be identified on the basis of genitalia (Dellacasa and Dellacasa 2006).) As a consequence, the taxonomic history of *A. immaturus* has been troubled (Tagliaferri 2000), being correctly identified only in recent times (Nicolas and Riboulet 1967). This may partly explain why the state of the knowledge of the life history of the two species is insufficient. It is only known that their general ecological habits are alike: both species are montane, oligotopic, with a preference for exposed pastures, and may be collected from spring to autumn in different types of dung (Dellacasa and Dellacasa 2006).

To identify eventual undetected shape differences between species, we focused at first on adults and took into account three external traits (head, pronotum, scutellum) and two internal traits (epipharynx and male genitalia). We examined the overall shape variation of the anatomical parts listed above (with the exception of the male genitalia, see Method for justification) using geometric morphometrics. This method has proven to be a useful technique that is more powerful than traditional morphometrics in identifying very subtle differences in shape (Lawing and Polly 2010; Slice 2007) and is a robust and reliable technique to study morphological variation in invertebrates (Andrade et al. 2009; Becerra and Valdecasas 2004; Crews 2009; Holwell and Herberstein 2010; Holwell et al. 2010). To detect the developmental origin of interspecific differentiation, two larval stages (1^{st} instar and 2^{nd} instar) were also examined by geometric morphometrics, focusing on the shape of the larval epipharynx and on the male genital disc (a precursor of male genitalia).

The pattern of relative constancy in external morphology exhibited by Aphodiini was compared with the pattern of great morphological diversification exhibited by the Onthophagini, which we also studied using geometric morphometrics (Macagno et al. 2009; Pizzo et al. 2006, 2008), with the main aim to identify possible determinants of the evolution of the two divergent morphological patterns.

Material and Methods

Sampling

About two hundred (N= 242) unidentified and unsexed *Amidorus* adults were collected in the Ferret Valley, an alpine valley on the Italian side of the Monte Bianco massif, at the top end of the Aosta Valley, north-western Italy (45°50’60” N; 7°01’00”), in summer 2008. Individuals were reared in isolation in covered plastic containers (9.5 cm diameter, 12.5 cm height) under laboratory conditions for about two months. Then, they were dissected and unequivocally assigned to one species using the aedeagus (males) and the epipharynx (both sexes) as specific diagnostic characters (Roggero, Tocco and Palestrini, unpublished results). Larvae found in the containers of females were therefore identified *a posteriori*, i.e. after the identification of their mothers.

Morphological preparations

Adults and larvae were cleaned in 70% ETOH , stored in vials filled with 99% ETOH and dissected without boiling.

a) Adults

For morphological analyses, we used all *A. immaturus* we collected (N= 24, i.e. 9 males and 15 females) and only a part of the *A. obscurus* sample (N= 66, 32 males and 34 females, randomly chosen). Preparations of male genitalia and epipharynges followed the well known, standardized methods usually employed for Coleoptera (Dellacasa 1983; Dellacasa et al. 2010; Skelley 1993).

The aedeagus of the two species diverges in the shape of the apices of paramers. However, as pointed out by Nicolas and Riboulet (1967), the apices of paramers are membranous and their definite shape can be fully appreciated only when preparations are re-hydrated; this prevented us from using geometric morphometric analysis on this trait.

The epipharynx of adults is a complex, asymmetrical structure on the inner surface of the clypeus with a pubescence that is variously-shaped and developed. It is constituted by a membranous lamina and many sclerotized support structures (Dellacasa 1983; Dellacasa and Dellacasa 2006; Dellacasa et al. 2010), which we used for the geometric morphometric analyses.

Epipharynx, head, scutellum and pronotum images were taken using a stereoscopic dissecting microscope Leica Z16Apo (Leica Microsystems AG, Wetzlar, Germany).

b) Larvae

For the analysis of the epipharynx we used 40 individuals of *A. immaturus* (i.e. 12 male and 7 female 1st instar larvae; 8 male and 13 female 2nd instar larvae) and 38 of *A. obscurus* (i.e. 8 male and 10 female 1st instar larvae; 9 male and 11 female 2nd instar larvae).

In coleopteran larvae, the epipharynx is an external, relatively simple structure (Steinmann and Zombori 1984), in which certain parts (i.e. the tormae) are not entirely developed as in adults. Unlike adults, in the larvae the epipharynx surface is glabrous for the most part.

To identify the preimaginal instars, we used the measures of head width as proposed by Daly (1985), the 1st instar being smaller than the 2nd instar.

To identify the sex of the larvae, we used the genital disc, an unpaired embryonic structure located on the ventral side of the abdomen, which is well-developed and evident in males only (Martinez and Lumaret 2003, 2005; Moczek and Nijhout 2002; Roggero, Tocco and Palestrini, unpublished results.). For the analysis of the genital disc we employed 33 larvae of *A. immaturus* (i.e., 18 individuals of 1st instar and 15 individuals of 2nd instar) and 41 larvae of *A. obscurus* (19 individuals of 1st instar and 22 individuals of 2nd instar).

Landmarks and measurements

In landmark-based morphometric analyses, the morphology of an object is represented by coordinates of sets of landmarks (Bookstein 1991). The landmark points (as defined in Appendix 1) were chosen for their ease of identification, homology in the two species and ability to capture the general shape of each morphological structure, and were digitized using tpsDig 2.16 (Rohlf 2010a).

In adults, landmarks on the head (N = 6), scutellum (N = 5), and epipharynx (N = 9) were digitized as shown in Fig. 1. For the pronotum (N = 16) we used the sliding semi-landmarks method (Bookstein 1997; Perez et al. 2006; Mitteroecker & Gunz 2009), as implemented in tpsRelw v1.49 (Rohlf 2010b) (Fig. 1). In larval instars, landmarks were digitized on the epipharynx (N = 9) and genital disc (N = 7), as shown in Fig. 2.

Statistical analyses

To test whether the variation in shape in each dataset was small enough to have an adequate approximation of the linear tangent space to the non-linear Kendall shape space, we employed tpsSmall v1.20 (Rohlf 2003) on the different landmark configurations.

Generalized Procrustes analysis (GPA) was performed by tpsRelw v1.49 (Rohlf 2010b), retaining the centroid size and relative warp values for further analyses. For each anatomical structure, we drew scatterplots of the shape variation within the sample summarized by the relative warps (SPSS Statistics v18.0, SPSS© Inc.).

Significance of group membership of specimens for each configuration of points was tested by the Multivariate Test of Significance (equivalent to the Hotelling Generalized T^2 test) as implemented by tpsRegr v1.37 (Rohlf 2009), in which the Goodall’s F-test (Goodall 1991) is employed to test whether the mean shapes would differ more than expected by chance (Hallgrimsson et al. 2007). Goodall's F test compares the difference in mean shape between two samples relative to the shape variation found within the samples and is the most powerful approach to test whether the mean shapes differ more than expected by chance. The proportion of Goodall's F statistics from randomly permuted data sets (n = 1000 here) as great or greater than the Goodall's statistic on the original data set is given as the significance probability. Use of the permutation test relaxes some of the restrictive assumptions of Goodall's F test (Goodall 1991; Rohlf 2000). Goodall's F test only considers the total amount of shape variation, and does not consider the directionality of the variation. With small samples (relative to the number of landmark coordinates), this is a useful property.

Discriminant Function Analysis was carried out on the whole data set of relative warp scores to obtain a classification matrix based on shape variation (in keeping with Janžekovic and Kryštufek 2004; Kryštufek and Janžekovic 2005; Pizzo et al. 2006, 2008) using SPSS v18. We used the percentage of correct classifications of cross-validation to further evaluate interspecific differences in shape (in cross-validation, each case is classified by the functions derived from all cases other than the case in question).

Results

Adults

A very good correspondence between shape and tangent space was found for all anatomical traits (slope = 0.99; correlation = 1.0), thus indicating that the geometrical heterogeneity of the sample was small enough to allow subsequent analyses.

External traits

Plots of the first two RW scores (Fig. 3) suggested that external traits (head, pronotum and scutellum) were rather similar in shape. Interspecific differences in the shape of the head, pronotum and scutellum tested through the Multivariate Test of Significance are given in Table 1. Results of permutation tests again suggested a poor interspecific differentiation in all the external anatomical traits (Table 1). In the Discriminant Function Analysis, percentages of correct classification in cross-validation were poor, all being below 70% (head: 64.4%; pronotum: 64.4%; scutellum: 65.6%).

Aedeagus and epipharynx

Interspecific differences in the shape of the aedeagus were visually apparent because of the peculiar shape of the apices of paramers, that are unmistakably different in the two species (Nicolas and Riboulet 1967; Dellacasa 1983). They are short, arched, tapered and pointed in *A. obscurus*, and at least three times longer, mostly rectilinear, evenly broad and rounded distally in *A. immaturus*. The interspecific difference in the shape of epipharynx was also highly significant (Table 1). The plot of the first two RW scores confirmed this pattern because it displayed two distinct, non-overlapping, clusters, clearly demonstrating this trait does vary between species (Fig. 3, bottom, on the right).

The results of the Generalized Goodall F-test and the Permutation Tests (Table 1) were significant, suggesting specimens can be correctly classified on the grounds of the shape of the epipharynx.

Finally, in the Discriminant Analysis most cross-validated grouped cases were correctly classified (96.7%).

Larvae

A very good correspondence between shape and tangent space was obtained for all traits (genital disc slope = 0.99, correlation = 0.99 for both instars; epipharynx slope = 0.99, correlation = 1.00 for both instars), thus indicating that the geometrical heterogeneity of the sample was small enough to allow subsequent analyses.

Genital disc

Plots of the RW values evidenced a marked superimposition of the two species (Fig. 4, top). Interspecific differences in the shape of the genital discs tested through the Multivariate Test of Significance confirmed a marked species similarity (Table 2), suggesting that they cannot be distinguished by the genital disc in either preimaginal stage. The Discriminant Function Analysis correctly classified 64.9% (1st instar) and 75.7 % (2nd instar) of individuals, respectively.

Epipharynx

Plots of the two first RW scores of the 1st instar showed a relative overlap between the two species, whereas that of 2nd instar displayed two distinct clusters, although partly superimposed (Fig. 4, bottom, on the right). Interspecific differences in the shape of epipharynx, however, suggested differences were significant in both larval instars (table 2). Discriminant analysis indicated interspecific differences were more conspicuous in the 2nd instar (97.5%) than in the 1st instar (89.5%).

Discussion

Interspecific divergence between *A. obscurus* and *A. immaturus*

Geometric morphometrics was used to describe interspecific morphological variation and to depict subtle interspecific differences. Plots of relative warp scores, Multivariate Tests of Significance and Discriminant function analyses gave rise, generally, to complementary results: in adults, the shape of external traits (head, pronotum and scutellum) were rather similar in the two species, whereas the epipharynx was clearly different. The aedeagus was not analysed morphometrically because interspecific differences in shape and length of parameres were quite apparent. Therefore, the interspecific shape divergence pattern evidenced in adults was similar to that observed in two *Onthophagus* sister species (*O. taurus* and *O. illyricus*), which were poorly distinguishable on the basis of morphological external traits, but revealed significant differences in the aedeagus and epipharynx (Pizzo et al. 2006, 2009).

The two species examined in this study are phylogenetically very closely related. Piau et al. (1999), on the grounds of the genetic differences observed through mtDNA and PCR-RFLP analyses, suggested the mitotypes of the two species derived from one common mitotype ancestor. Jay-Robert et al. (1997), mostly on the grounds of the characteristic distribution of the two species of *Amidorus*, assumed that certain populations remained isolated within the Alps during quaternary glaciations and produced local speciation in a mountain system.

If so, and with reference, in particular, to the last glaciation (15-20000 years ago), *A. immaturus* would have developed an exaggerated trait, the elongated aedeagus, rather quickly. Within this framework, the evolution of an exaggerated and complex primary sexual trait may have been an adaptation to avoid hybridization during and after the speciation event. All the above is in keeping with the tenet which assumes reproductive organs evolve quickly in insects (rapid genital evolution), and also with the results of several studies regarding the role of genital divergence in speciation (Dapporto 2010a, 2010b; Gilligan and Wenzel 2008; House and Simmons 2003; Mutanen and Pretorius 2007, Mutanen et al. 2007; Parzer and Moczek 2008; Pizzo et al. 2008).

After having detected this pattern in adults, we focused upon interspecific differences in the genital disc and epipharynx of larvae, looking for the ontogenetic stage at which interspecific differentiation arises. It worked out that the male genital disc is neither differentiated in 1st nor in 2nd instars, whereas the epipharynx is significantly differentiated in the 2nd, but not the 1st, instar. It should be taken into account that early larval stages are smaller and therefore less differentiated than later ones, hence probably making interspecific differences in shape morphology less apparent. We believe the low morphological divergence in the epipharinx of the 1st instar (Multivariate Test of Significance gave significant results, Discriminant Function Analysis correctly classified 89.5% of individuals, but the first two relative warp scores were largely overlapping) may be interpreted in this context. It can be hypothesized that evolutionary changes that produced the great interspecific difference in the shape of the aedeagus discussed above took place in the latest preimaginal instar, as in most hemimetabolous insects. The preimaginal instars, in fact, are more prone to changes than adult stages, whose morphology is somehow frozen in the invariance of its exoskeleton (Minelli 2007).

Finally, if we consider in the same context both the imaginal and the preimaginal data, a correlation between interspecific divergence and functionality of the trait seems to arise. The adult male aedeagus is functional (adults are able to reproduce), whereas the larval male genital disc, an aggregate of undifferentiated cells that are the precursor of adult genitalia, is not. Accordingly, the former trait is different in the two species, while the latter is not (although it must be admitted that, at this stage, an eventual diverging trait may not have shown a size or a developmental status accessible to our geometric morphometric analyses). In a similar way, the shape of the epipharynx is functional both in adults and in larvae (that, in fact, spend most of the time feeding on dung) and, accordingly, it is significantly divergent in the two species both in adults and in larvae (in the 2nd instar at least). Our results indicate that interspecific divergence in the shape of the trait may be correlated with its reproductive or feeding functionality, i.e. feeding and reproductive traits of the two species diverge morphologically when, during the ontogenetic development, they become functional.

This study focused on specimens from both species sampled from the same locality. In syntopic conditions, in theory, there could either be hybridisation or increased contrast between species due to selection against hybridisation. However, the morphological differences of genitalia suggest the two species are now reproductively isolated and we are therefore confident the distinct inter-specific divergence found here for some traits could also be recovered in populations where either of the species occurs alone (Piau et al 1999).

Divergent patterns of evolution in Aphodiini and Onthophagini

Aphodiini and Onthophagini display dramatically divergent patterns of diversification in external morphology. Against the relative constancy in external phenotype exhibited by the Aphodiini lineage with lower levels of phenotypic plasticity, Onthophagini present a large interspecific diversity of external morphologies (Parzer and Moczek 2008; Tomkins and Moczek 2009). This diversification pattern still holds at the intra-specific scale. Aphodiini do not exhibit sexual dimorphism, whereas Onthophagini exhibit both sexual dimorphism and male polyphenism (Moczek 2010; Simmons et al. 2007).

Species ecology is known to influence the evolution of certain morphological traits. Stebnicka and Howden (1995), for instance, have identified two typologies of epipharynges in adults (divergent as for pubescence and general shape) that correspond to coprophagous and saprophagous beetles. Aphodiini and Onthophagini display contrasting habitat/climate requirements, the former being typical of habitats in cold/temperate climates whereas the latter is typical of habitats in warm/temperate climates. Onthophagini, in fact, are mainly distributed in the Mediterranean and other temperate or warm areas (Scholtz et al. 2009), whereas Aphodiini dominate northern European dung beetle communities (Jay-Robert et al. 1997) as well as high-altitude communities of the Alps and the Pyrenees (Cabrero-Sañudo and Zardoya 2004; Cabrero Sañudo 2006; Cabrero Sañudo and Lobo 2006). Most of Onthophaginae are considered to be warm-adapted, whereas many Aphodiinae are considered to be cold-adapted (Lobo et al. 2007a, 2007b; Scholtz et al. 2009; Verdù et al. 2007). These differences are reflected in the local distribution of species; in the Alps Scarabaeinae dominate during spring and summer on south-facing slopes, whereas Aphodiinae dominate from spring to autumn on north-facing slopes (Jay-Robert et al. 2008; Zamora et al. 2007).

We suggest habitat/climate factors have acted to set divergent breeding and thermoregulatory strategies in the two groups. Within these different strategies, sexual selection (breeding strategies) and natural selection (thermoregulatory strategies) may have contributed to drive the divergent morphological evolution of the two groups producing medium-size horned species (Onthophagini) on the one hand and small-sized unhorned species (Aphodiini) on the other.

i) Breeding ecology

Except for a few cases, Aphodiini adults live and breed in the dung mass where they do not construct nests (dwellers) (Dellacasa and Dellacasa 2006; Hanski and Cambefort 1991). Conversely, Onthophagini dig burrows under the dung mass that are provisioned with dung to form a ball (the brood ball, destined for larval food) where an egg is laid (tunnelers). In Onthophagini, horns of large males are used to defend the nest and prevent other males from copulating with females, which may spend long periods underground caring for the brood (Hunt and Simmons 2002, Knell 2011).The different expression of sexually dimorphic ornamentation is the major form of interspecific variation in Onthophagini. Aphodiini males do not need to defend the nest and, therefore, they do not need to develop horns. It has been suggested that nesting behavior has been mediated more by environmental conditions than phylogeny (Scholtz et al. 2009). We hypothesized therefore that these two divergent breeding strategies may have arisen as an answer to the different ecological and climatic conditions experienced. The non-nesting strategy, in particular, seems to be the best one for species living in cold habitats by enabling individuals to breed several times within the short breeding period characteristic of the habitat (Scholtz et al. 2009).

ii)Thermoregulatory ecology. Species of both Aphodiini and Onthophagini are usually smaller than the body size above which the regulation of body temperature through internal processes is thought to be physiologically possible (Bartholomew and Heinrich 1978; Prange 1996; Roxburgh et al. 1996, Chow and Klok 2011) and therefore have to rely on other mechanisms to respond to changes in external temperature. One mechanism to respond to such changes is to actively seek out locations with appropriate temperatures, or to move between warm and cold temperature locations so as to achieve a preferred average temperature. Shepherd et al. (2008) found that horn possession in two Onthophagini species dramatically alters this thermal preference behavior. They also hypothesized that the formation of long horns diverts resources away from structures crucial for active heat transfer, such as thoracic muscles, and compromises the later ability to effectively thermoregulate in the face of temperature fluctuations. To our knowledge, no thermoregulatory data are available for Aphodiini, but it is reasonable to think that an efficient thermo-regulatory behavior is needed to secure survival in cold climates. This might also explain why Aphodiini do not develop horns. Moreover, Shepherd et al. (2008) also demonstrated that body size significantly affects thermoregulation and suggested that larger individuals, by virtue of their size, can operate at higher temperatures, whereas smaller individuals prefer to operate at lower temperatures. This may also serve to explain why, as a rule, Aphodiini are smaller than Onthophagini.

Summing up, we suggest habitat/climate factors may have underpinned divergent reproductive and thermoregulatory strategies in the two groups and that strategies of the Onthophagini enabled the development of horns (and other forms of sexual dimorphism), whereas those of Aphodiini were not.

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Appendix 1.

Landmark setting. Nomenclature of the different parts of traits where landmarks were set follow Dellacasa and Dellacasa 2006, and Steinmann and Zombori 1984.

ADULT	
Epipharynx	
1	Along midline, on tylus
2	Apex of left chaetoparia
3	At proplegmatium
4	At base of laeotorma
5	On first apex of crepis
6	On longer apex of crepis
7	At midline, on nesium
8	At joining point of proplegmatium and left chaetoparia
9	Apex of left apotorma
Pronotum	
1	Along midline, on fore margin
2-3	Semilandmarks
4	At base of lateral angle of fore margin
5	At side angle of fore margin
6-10	Semilandmarks
11	On hind side angle of pronotum
12-15	Semilandmarks
16	Along midline, on hind margin
Head	
1	Midline, on fore margin
2	Lateral apex of fore margin
3	Lateral carina, on margin
4	Anterior base of left gena
5	Apex of gena
6	Posterior base of gena
Scutellum	
1	Left apex
2	Right apex
3	Medial point of right side
4	Hind apex
5	Medial point of left side
LARVA	
Epipharynx	
1	Midline, on fore margin
2	On left clythra
3	On side margin of left lobe
4	At superior margin of laeotorma
5	At inner base of laeotorma
6	At base of anterior epitorma
7	At base of apotorma
8	At apex of apotorma
9	At apex of anterior epitorma
Genital Disc	
1	Right base of disc
2	Right base of disc, anterior angle
3	Right apex of disc
4	Midline , anterior margin
5	Left apex of disc
6	Left base of disc, anterior angle
7	Left base of disc

Table 1. Results of Multivariate Test of Significance regarding external traits (head, pronotum and scutellum) and the epipharynx in adults of *A. immaturus* and *A. obscurus*. The proportion of Goodall's F statistics from randomised data sets as great or greater than the Goodall's statistic from the original data set is given as the significance probability (1000 random permutations were used).

	Generalized Goodall F-test			Permutation
	F	df	P	tests*
Head	2.2128	8, 680	0.0248	5.30%
Pronotum	1.8078	28, 2464	0.0059	12.90%
Scutellum	1.1568	8, 704	0.3230	31.60%
Epipharynx	33.8985	20, 1760	0.0000	0.10%

*Percent of Goodall F values ≥observed

Table 2. Results of Multivariate Tests of Significance regarding the genital disc and the epipharynx in larvae of *A. immaturus* and *A. obscurus*. The proportion of Goodall's F statistics from randomised data sets as great or greater than the Goodall's statistic from the original data set is given as the significance probability (1000 random permutations were used).

		Generalized Goodall F-test			Permutation
	Instar	F	df	P	tests
Genital disc	1	2.0632	10, 350	0.0268	11.0%
	2	1.1339	10, 350	0.3357	33.70%
Epipharynx	1	3.1417	14, 504	0.0001	0.10%
	2	5.6075	14, 532	0.0000	0.10%

*Percent of Goodall F values ≥observed

Figures

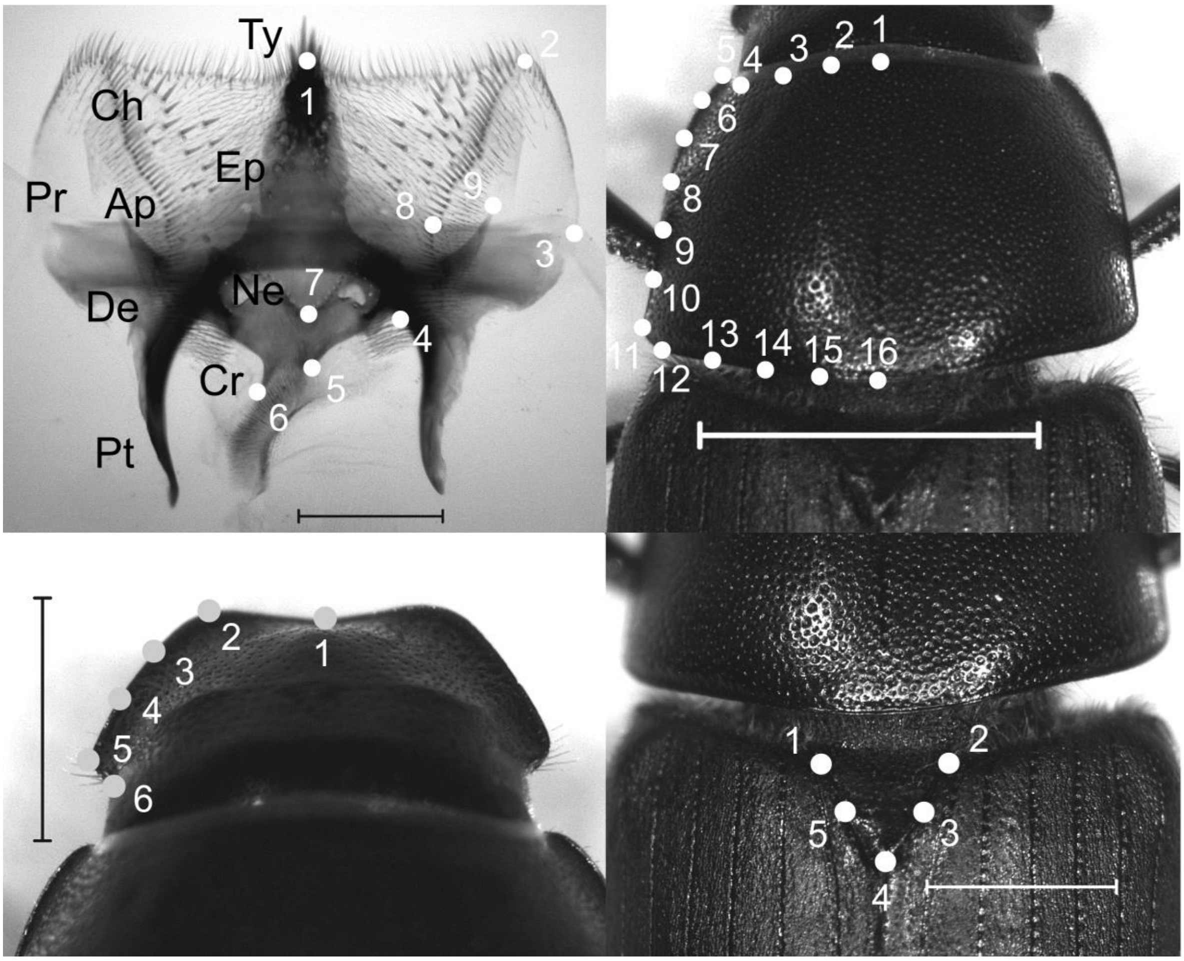

Fig. 1. *Amidorus obscurus*, for each anatomical structure the number (N_L) and position of landmarks are shown. **A**: Epipharynx, scale bar = 2 mm. On the left: the acronyms of the parts of the structure. On the right: the position of the chosen landmarks ($N_L = 9$); on left the acronyms of the different parts are shown: tylus (Ty), chaetopariae (Ch),epitorma (Ep), proplegmatium (Pr), apotorma (Ap), dexiotorma (De), nesium (Ne), crepis (Cr), and pternotorma (Pt). **B**: Pronotum ($N_L = 16$), scale bar = 2 mm. **C**: Head ($N_L = 6$), scale bar = 1 mm. **D**: Scutellum ($N_L = 5$), scale bar = 1 mm. In the pronotum configuration (B), the points 2, 3, 6, 7, 8, 9, 10, 12, 13, 14 and 15 were treated as semi-landmarks, the others as landmarks.

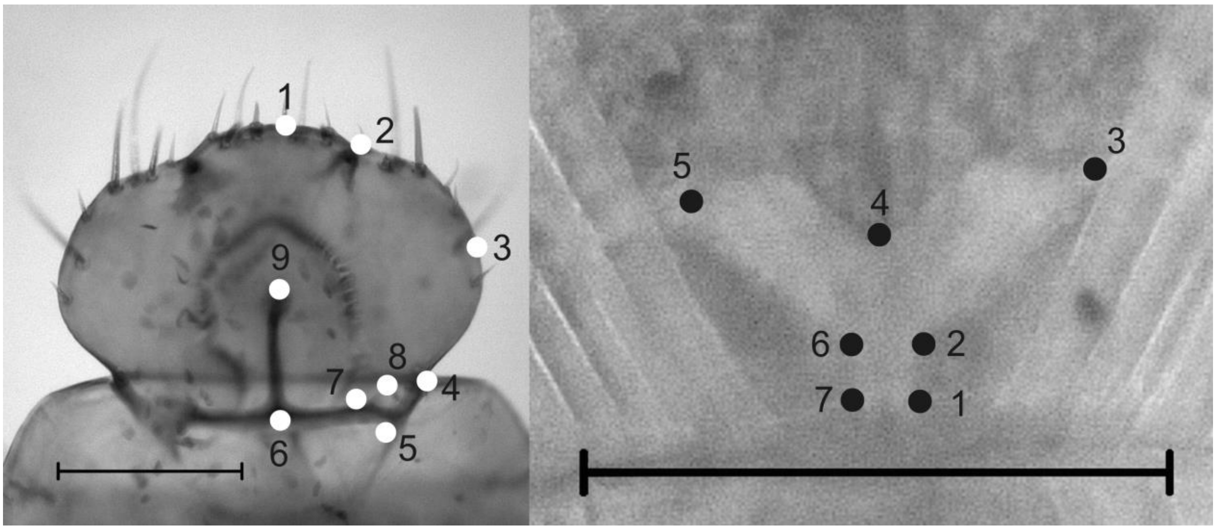

Fig. 2. Epipharynx of the larval instars with 9 landmarks (**A**), and male genital disc with 7 landmarks (**B**). Scale bars = 0.2 mm.

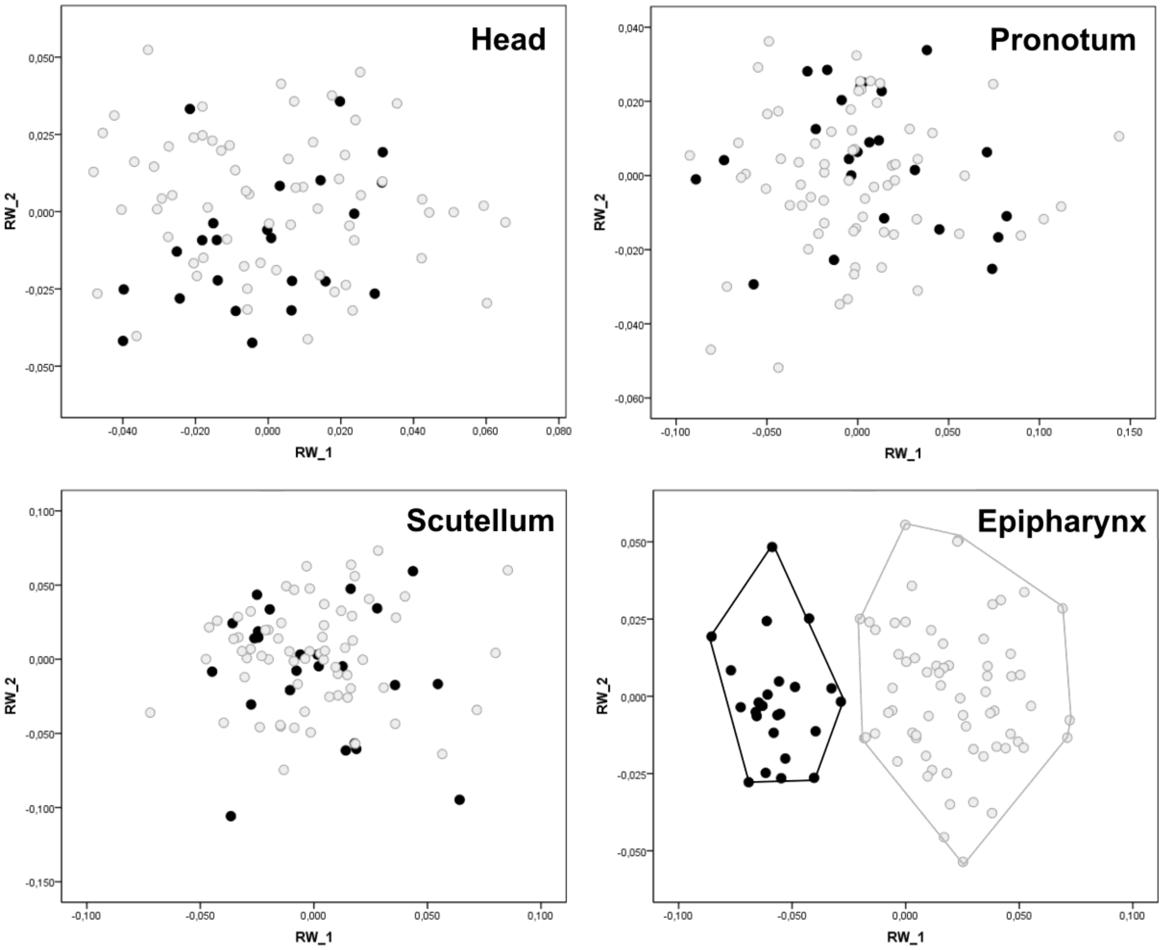

Fig. 3. Scatterplots of the first two relative warp scores obtained from the Relative Warps Analysis of the head (**A**, percentages of the explained variance: RW_1 = 35.39%, RW_2 = 25.63%,), pronotum (**B**,

RW_1 = 69.64%, RW_2 = 12.32%), scutellum (**C**, RW_1 = 30.58%, RW_2 = 19.91%) and epipharynx (**D**, RW_1 = 37.27%, RW_2 = 10.49%) of adults of *A. immaturus* (black symbols) and *A. obscurus* (grey symbols). The number of relative warps obtained was 8, 28, 8 and 20 for the head, pronotum, scutellum and epipharynx, respectively.

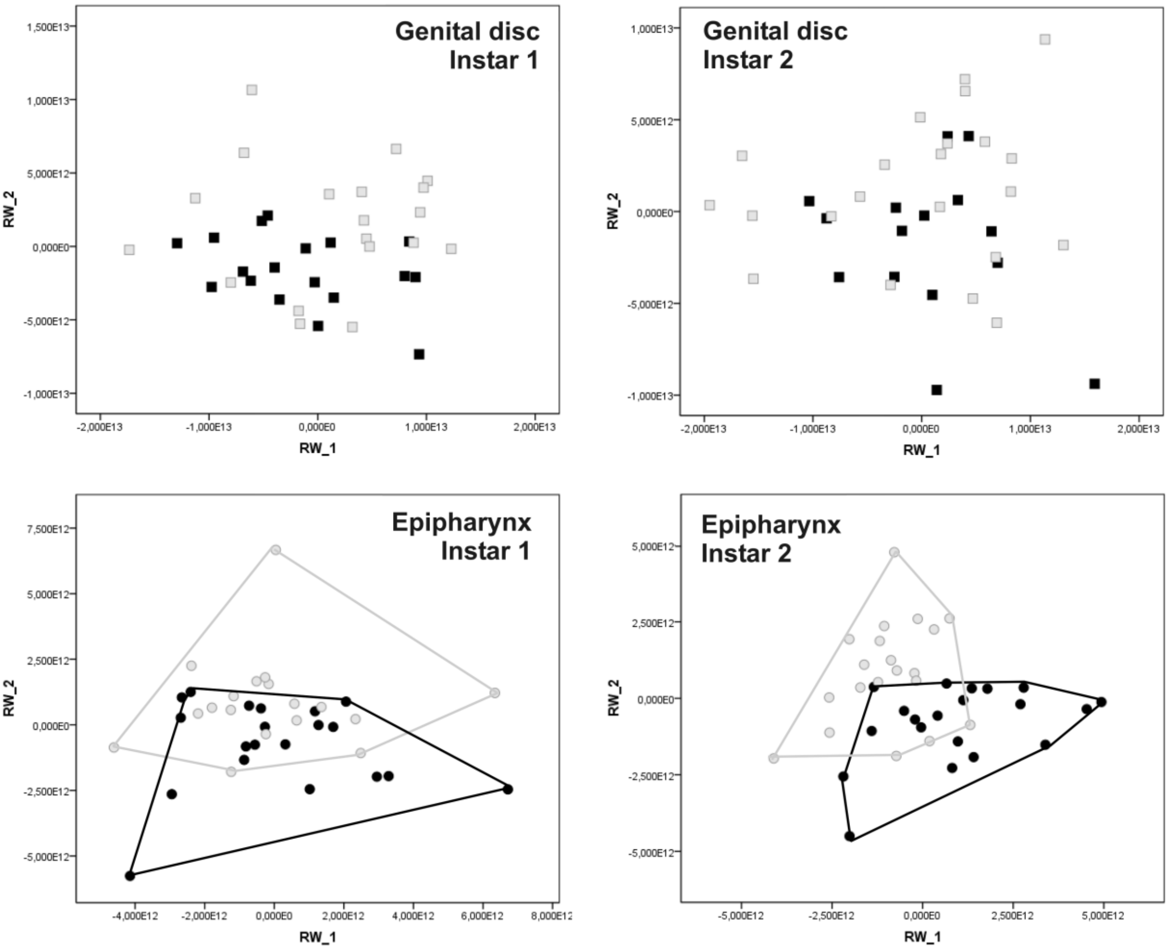

Fig. 4. Scatterplots of the first two relative warp scores obtained from the Relative Warps Analysis of male genital discs and epipharynges of larvae of *A. immaturus* (black symbols) and *A. obscurus* (grey symbols). Genital disc: 1st instar (**A**, percentages of the explained variance: RW_1 = 60.93%, RW_2 = 15.19%) and 2nd instar (**B**, RW_1 = 64.08%, RW_2 = 16.37%). Epipharynx:1st instar (**C**, RW_1 = 28.40%, RW_ 2 = 17.72%) and 2nd instar (**D**, RW_1 = 25.60% and by RW_2 = 20.41%). The number of relative warps obtained was 10 for genital discs and 14 for epipharynges of both instars.