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On the evolution of shape and size divergence in *Nebria* (*Nebriola*) ground beetles (Coleoptera, Carabidae)

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Ground beetles include many species of rather recent evolutionary origin that are extremely similar in their external morphology. We used geometric morphometrics to quantify the relative degree of morphological divergence in three closely related polytypic alpine species of the genus *Nebria*, subgenus *Nebriola* (*Nebria cordicollis*, *N. laticollis* and *N. fontinalis*). To examine evolutionary patterns, we adopted a hierarchical design, using both shape and size to discriminate between species, subspecies within species, and populations within subspecies. A semilandmark-based approach was used to describe the pronotum, elytron, and median lobe of the aedeagus. The three closely related species diverged significantly in shape and size. There were clear inter-specific differences in the shape of external traits (elytron and pronotum) and of the median lobe of the aedeagus. Furthermore, species also diverged in body size, and in the relative size of traits (evaluated by computation of their static allometries). Shape differences among subspecies and populations were more limited. However, the three subspecies of *N. cordicollis* considered, *N. c. cordicollis*, *N. c. kochi* and *N. c. winkleri*, showed three distinct body sizes and differences in relative size of the elytron. A few size differences were also evident between distinct populations. These results seem to suggest that size may diverge more rapidly than shape in *Nebriola* ground beetles. Both at the species and subspecies level, body size order was not reflected in the relative size order, indicating that body size and relative size of traits do not necessarily evolve in concert.

Key words: Geometric morphometrics, Inter and intraspecific differences, Evolutionary patterns, Ground beetles, Carabidae, subgenus *Nebriola*.

Introduction

Morphological traits are traditionally used to assess taxonomic differences and phylogenetic relationships among arthropods. Morphology may also be useful for the study patterns of divergence among species, subspecies and populations. Within this framework, speciose groups that include polytypic species may be of particular interest. These groups are usually of recent origin, with species and subspecies so similar in their external morphology that molecular analyses are often used to test the validity of taxonomic entities, notably those that have been established on the grounds of traditional qualitative, morphological analyses. Numerous examples are available among the beetles (Coleoptera).

Ground beetles (Carabidae) include speciose groups and/or polytypic species whose evolutionary differentiation took place during or after the Quaternary ice ages. The *Nebria gregaria* group on the Queen Charlotte Islands (Alaska) represents a notable example of rapid evolution. Composed of five morphologically similar species, it is considered to be a taxon for which the repeated isolation of populations in glacial refugia has played an important role in the promotion of speciation (Kavanaugh, 1992). However, phylogenetic analyses based on molecular data, obtained from five regions of mitochondrial and genomic DNA, revealed a lack of interspecific differentiation, suggesting that divergences might represent local variation of a single species (Clarke *et al.*, 2001). Analogously, the results of genetic divergence within the Palaearctic species *Carabus auronitens* (based on allozyme polymorphism) does not mirror conventional subspecific taxonomy, as there are at least two subspecies (*C. auronitens auronitens* and *C. auronitens festivus*, Turin *et al.*, 2003) that are not separated genetically, and several other subspecies (Deuve, 1994) that are partially contradictory (Drees *et al.*, 2010).

In a number of studies, ground beetle morphology seems to be more informative than genetics, and distinct morphological differences may be observed despite little divergence in molecular markers. Sister species and populations of the genus *Pogonus*, for example, could be differentiated using ecological characters, but were not recognized by screening neutral DNA polymorphisms (Dhuyvetter *et al.*, 2007). Analogously, *Carabus arrowianus* exhibits marked morphological diversity among geographical populations in body and genital sizes, and this was found to be supported by significant but not large genetic divergences (Nagata *et al.*, 2009).

Several morphometric studies have shown how rapidly changes in body size and shape can evolve. Thus inter- and intraspecific divergences may readily arise. Genitalia, for example, are considered to be among the fastest evolving morphological traits in arthropods (Eberhard, 2010, 2011; Pizzo *et al.*, 2006*a*, 2006*b*, 2008). Rapid diversification of male genitalia was described in ground beetles of the genus *Carabus* subgenus *Ohomopteru*s (Sasabe *et al.*, 2007, Takami & Sota, 2007), which also exemplifies one of the most outstanding cases of mechanical isolation (Sota & Kubota, 1998). Knowledge of the genetic architecture underlying genital evolution is still limited. Recent molecular analyses (genetic linkage maps and analyses of quantitative trait loci) have shown that the interspecific difference in the genital morphologies (three male copulatory structures and two traits derived from female vaginal appendices) were determined by a relatively small number of genes with marked phenotypic effects (Sasabe *et al.*, 2007, 2010).

Although the relevance of traditional morphological studies to the reconstruction of evolutionary divergence patterns cannot be dismissed, landmark-based geometric morphometrics is now able to score shape and size changes often undetectable by

traditional morphological studies and even classical morphometric approaches (Bookstein, 1991; Rohlf & Marcus, 1993; Marcus *et al.*, 1996; Adams *et al.*, 2004; Slice, 2007; Mitteroecker & Gunz, 2009). Using this technique, morphological traits can be studied to reveal patterns of divergence at different spatio-temporal scales, highlighting both major differences between well-differentiated species and minor differences arising during the first steps of evolutionary divergence.

In ground beetles, geometric morphometrics has been used to discover cryptic species (Faille *et al.*, 2007, Dujardin *et al.*, 2010) and to assess differentiation at a micro-geographical scale within *Carabus auronitens* and *C. nemoralis* (Alibert *et al.*, 2001). The technique has also been successfully applied to genetic analyses of the short-range species *Carabus solieri*, the phylogeographic pattern of which (Garnier *et al.*, 2004) was then *a posteriori* successfully confirmed by geometric morphometrics analyses based on male genitalia (Garnier *et al.*, 2005, 2006).

The ground beetle subgenus *Nebriola* Daniel, 1903 (genus *Nebria* Latreille, 1802), which encompasses several stenoecious high altitude species prevalently distributed in the Western Alps (Marazzi, 2005), represents an excellent opportunity for testing the potential of geometric morphometrics to unveil inter- and intra-specific evolutionary divergence patterns. Six species are traditionally ascribed to the subgenus *Nebriola*: *Nebria cordicollis* Chaudoir, 1837, *N. fontinalis* Daniel & Daniel, 1890, *N. lariollei* Germiny, 1865, *N. laticollis* Dejean, 1826, *N. morula* Daniel & Daniel, 1891 and *N. pictiventris* Fauvel, 1888. Two other species have been recently described: *Nebria praegensis* Huber & Molenda, 2004, from the German Black Forest (although it was considered as a subspecies of *cordicollis* by Ledoux & Roux, 2005) and *Nebria gosteliae* Huber, Szallies, Baur & Giachino, 2010, from the Italian Pennine Alps.

According to traditional classification criteria, differences between these *Nebriola* taxa involve morphological traits, e.g. the shape of pronotum, elytron, median lobe of the aedeagus, and chaetotaxy (Ledoux & Roux, 2005). Since the habitus is rather constant throughout the subgenus, traditional qualitative morphology is limited in recognizing species and, above all, subspecies. This has caused great uncertainty in establishing the taxonomic rank to be attributed to the taxa described so far (Bari, 1971; Bisio, 1998). Genetic sequences obtained from mitochondrial DNA analyses, although suggestive that several of the species listed above may represent phylogenetically separate entities, failed to give conclusive results about phylogenetic relationships at both subspecies and species levels (Huber *et al.*, 2010).

In this paper we focused on three closely related polytypic species belonging to subgenus *Nebriola*, namely *Nebria cordicollis*, *N. laticollis* and *N. fontinalis.* We 0used geometric morphometrics to quantify the relative degree of morphological divergence and, indirectly, to assess the validity of entities established on the basis of traditional morphological studies. To reveal evolutionary patterns at different spatio-temporal scales, we adopted a hierarchical design, using both shape and size, to discriminate between taxonomic entities at three levels: species, subspecies within species, and populations within subspecies.

Although the paper is focused on divergence patterns of shape and size *per se*, we also test hypotheses explaining the rapid evolution of differences between pairs of originally allopatric sister taxa when they meet (the phenomenon of reinforcement).

Material and methods

The three species considered are morphologically quite similar. *Nebria laticollis* shows almost parallel elytra and has a more curved median lobe of the aedeagus, whereas *N. cordicollis* and *N. fontinalis* have the elytra progressively enlarged posteriorly, and moderately curved median lobes. These last two are currently differentiated by the shape of labial palps and setation of the hind legs and last ventrites (Ledoux & Roux 2005). They are characterized by similar but distinct ecological habits. *Nebria cordicollis* is a petrophilous species dwelling in the alpine belt from 2100–3000 m a.s.l., where it may be found around snow patches, moraines and other stony habitats (Bisio, 1998). *Nebria fontinalis* dwells in cold spring-waters (from +1 to -1°C) and can be found from 1460–2700 m (Focarile & Casale, 1978), while *Nebria laticollis* has similar ecological habits, being found in cold springs and streamlets at 1500–2500 m (Ledoux & Roux, 2005).

The chorology of these species has been thoroughly investigated over the last 30 years. According to Ledoux & Roux (2005), the present taxonomic status of the three species may be summarized as follows: *Nebria laticollis* includes two subspecies from the French and Western Italian Alps; *Nebria fontinalis* includes two subspecies from the Swiss and Italian central Alps; and *Nebria cordicollis* includes nine subspecies from the Swiss and Italian Central Alps and Southwestern Germany (Black Forest). A tenth subspecies, *N. cordicollis winkleri*, described by Bari (1971), is considered by Casale & Vigna Taglianti (1993) and Vigna Taglianti (2005) to be strictly endemic to Monte Camino, Piedmont.

Subspecies-level analyses took into account three subspecies of *N. cordicollis*, namely *N. c. cordicollis, N. c, kochi* and *N. c. winkleri.* Finally, population-level

analyses focused on three populations of *N. c. cordicollis* and *N. c. kochi* separately. The populations were tentatively identified on the basis of the geographic location (*N. c. cordicollis* collected in Anzasca Valley, Ayas Valley and Gressoney Valley, and *N. c. kochi* collected in Orco Valley, Lanzo Valley and Soana Valley). Collection localities are shown in Fig. 1.

Most of the specimens used in the analyses were obtained from northern Italy (Aosta Valley, Piedmont and Lombardy) and Switzerland, or borrowed from museums and private collectors (see acknowledgements). Part of the material was collected in the field during the summers of 2009 and 2010. Details of the number of specimens considered in this hierarchical design at species, subspecies and population level are given in Table 1.

In the laboratory, specimens were cleaned and re-hydrated with distilled water for 4 hours. While the right elytron and the pronotum did not require any further preparatory treatment, the aedeagus was extracted, cleared in boiling KOH 5% for 1 min, and mounted on a piece of translucent acetate using entomological glue. The anatomical structures were then photographed, taking care to present them in the same plane. Photographs of the median lobe of the aedeagus, right elytron and pronotum were taken using a Leica Z16Apo stereoscopic dissecting scope (Leica Microsystems AG, Wetzlar, Germany) and stored using database LAS v 2.5.0 (Leica Application Suite) for measurement. The same datasets were then employed for the geometric morphometrics analyses, employing Rohlf's tps series software.

Measurements

A semilandmark-based approach (Bookstein, 1989, 1991) was used to characterize the shape variation of the pronotum, right elytron and median lobe of the aedeagus. Using tpsDig 2.16 (Rohlf, 2010a), 13 points were placed on the marginal side of the median lobe of the aedeagus, 16 on the right half pronotum and 12 on the right elytron; for each structure, the configuration was chosen following the criteria of homology (Bookstein, 1991). To capture overall shape variation of the curved parts of these anatomical features, the semilandmarks method was applied, coding the points as landmarks and semilandmarks (Figs. 2-4) by tpsUtil 1.47 (Rohlf, 2010b). For the geometric morphometrics analyses, the datasets were chosen taking care that the samples sizes were larger than the number of variables (i.e., the Relative Warps). Using a Generalized Procrustes Analysis (GPA) the effects of translation, rotation, and scale (Rohlf, 1990; Rohlf & Slice, 1990) were removed.

The Maximum Pronotum Width measures (see statistical analyses below) were taken with LAS v 2.5.0 software (Leica Application Suite).

Statistical analyses

Shape

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 each landmark configuration.

Generalized Procrustes Analysis (GPA) was carried on each dataset as implemented in tpsRelw 1.49 (Rohlf, 2010c) for the semilandmarks data, and the aligned configuration, the centroid size values, and Relative Warps scores were retained for further analyses. For each anatomical feature, we drew scatterplots of the shape variation within the sample summarized by the Relative Warps (abbreviated RWs) (PASW Statistics 18, IBM SPSS).

In the semilandmarks-based approach, less than the total Relative Warps scores are sufficient to reach 100% of the overall shape variation (Tocco *et al.*, 2011). Thus, we employed only the RW scores that gave 100% of overall shape variation for the statistical analyses (i.e. 22 out of 28, 15 out of 20, and 18 out of 22 as for pronotum, elytron and median lobe of the aedeagus, respectively), and discarded the others.

Canonical Variate Analysis (CVA) was performed to obtain a classification matrix based on shape variation of traits (Reddy *et al.*, 2005, Sheets *et al.*, 2006, Gómez-Robles *et al.*, 2011) using PASW Statistics 18. In all analyses, the number of Relative Warps was less than that of cases within each group (i.e. specimens, in our case). Percentages of correct classifications were cross-validated through the leave-one-out option, each case being classified by the functions derived from all cases other than the one in question.

Size

Body size

As a proxy for body size, the Maximum Pronotal Width (MaxPW) measure was chosen from those commonly used in morphometric analysis of ground beetles (Ribera *et al.*,

1999; Huber *et al.*, 2010; Laparie *et al.*, 2010) because its consistency, and to avoid the mutual malposition of the different body parts and the subsequent measurement artefacts (Garnier *et al.*, 2005), depending also on how the measure is employed for many other coleopterans (Pizzo *et al.*, 2011, 2012).

Differences among groups (species, subspecies or populations) and between sexes were tested by Nested ANOVA (groups*sex, sexes nested into groups), after data normalization (using pronotum width). Pairwise comparisons among groups were based on estimated marginal means, with Bonferroni adjustment.

Size of each anatomical trait

The centroid size (the square root of the sum of squared distances of a set of landmarks defining the structure from the centroid, or centre of gravity) is approximately uncorrelated with shape for small isotropic landmark variation (Bookstein, 1991; Dryden & Mardia, 1998; Mitteroecker & Gunz, 2009), and is therefore used to estimate size divergence patterns of the structures independent of shape. The centroid size values of the elytron, pronotum and median lobe of the aedeagus were therefore saved as a separate variable; and the ln-transformed values were used as an estimate of traits size.

It is well known (Stern & Emlen, 1999; Gayon, 2000; Frankino *et al.*, 2005; Shingleton *et al.*, 2007, 2008) that the size of each anatomical trait may depend on body size (allometry). To compare the size of different traits it is therefore necessary to consider their size relative to body size (i.e. the *relative size* of the trait). To inspect inter- and intraspecific differences of relative size, we first computed their static allometries. A preliminary inspection of scatterplots of body size (ln-transformed MaxPW) *vs.* ln-transformed centroid size values of pronotum, elytron and median lobe

of the aedeagus did not reveal any significant deviation from linearity. Thus, linear regression models were used to fit these distributions.

Levene's test was used to check the assumptions for parametric tests. To remove the effect of body size, in the ANCOVA the ln-transformed MaxPW was set as a covariate when comparing the ln-transformed centroid sizes within the proposed groups for each anatomical feature. We tested the homogeneity of slopes by adding a fixed factor by covariate interaction (crossed effect) to the full factorial ANCOVA. If the interactions between the covariate and the fixed factor were not significant, slopes were assumed to be equal and a regression line through each group of points, all with the same slope, was fitted. This common slope was a weighted average of the slopes of the different groups. The final test in the ANCOVA was to address the null hypothesis that all Y-intercepts of the regression lines with a common slope were the same. All analyses were conducted by PASW Statistics 18.

Results

Shape

A very good correspondence between shape and tangent space was found for all anatomical traits (slope = 0.999; correlation = 1.000), indicating that the geometrical heterogeneity of the sample was small enough to allow subsequent geometric morphometric analyses.

Divergence between species was evident. Plots of the first two RW scores (explaining about 60% of the variance) suggested that all the considered traits were different in shape, displaying for each trait three distinct, partly overlapping clusters of

points (Fig. 5). Considering those RW values that explained more that the 5% of variation, for the pronotum five RWs accounted for about 86.53% of explained variance, for the elytron four RWs gave 83.46%, and for the medial lobe of aedeagus the five RWs gave 87.8%. In the CVA, percentages of correct classification in cross validation were high (more than 95% in most cases), especially for the median lobe of the aedeagus, which gave rise to 100% of correct predicted classifications for two groups out of three (Table 2).

Divergence between subspecies and populations was much less evident. Plots of the first two RW scores did not show distinct clusters (not shown here). According to the CVA results, high percentages of correct classification (about 90%) were found for the pronotum of the subspecies *N. cordicollis winkleri* and the Lanzo Valley population of the subspecies *N. cordicollis kochi* only, while the other values had lower significance (Table 2).

Body size

Mean body sizes and nested ANOVA results are reported in Table 3. It should be noted that, whatever the level considered, no significant differences between sexes were detected. At the species level, *N. fontinalis* was significantly smaller than each of the other two species. The subspecies of *N. cordicollis* showed three distinct body sizes, *N. c. winkleri* being significantly larger than *N. c. kochi* which in turn was significantly larger than *N. c. cordicollis*. Differences between populations were not significant, with the noticeable exception of the population of *N. c. cordicollis* from Anzasca Valley, which was significantly smaller than that from Gressoney Valley.

Relative size

Levene's test ensured that the adjustments of allometric functions to a linear scaling in the form $y = y_0 + ax$ were statistically correct in the case of the median lobe of the aedeagus of species, elytra of species and subspecies, and pronotum of species, subspecies and populations.

The interaction of ln MaxPW * group was never significant, with the exception of the median lobe of the aedeagus of one species (*N. fontinalis*) and the pronotum of one population of *N. cordicollis cordicollis*. In these two cases, the species and the population with different slope of the regression line was removed and Levene's tests and ANCOVAs re-run on the remaining two groups.

Subsequent pair-wise comparisons of intercepts (sequential Bonferroni corrections applied) highlighted widespread significant size divergences between all species. In particular: the relative size of the pronotum and elytron of *N. cordicollis* was significantly larger than those of *N. fontinalis*, and both of these were significantly larger than those of *N. laticollis*; and the relative size of the median lobe of the aedeagus of *N. cordicollis* was significantly larger than that of *N. laticollis* (Table 4).

Divergences were also significant in subspecies for the elytron (*N. cordicollis kochi* having a larger elytron than *N. c. winkleri* and *N. c. cordicollis*) (Table 4). Some significant pairwise differences in the relative size of the pronotum were also ascertained for populations of *N. c. kochi*.

Discussion

Our geometric morphometric analyses made clear that the three closely related polytypic species considered, i.e. *Nebria cordicollis*, *N. fontinalis* and *N. laticollis*, significantly diverged in shape and size.

Shape and size of external traits

The shape of the external traits (elytron and pronotum) were clearly different among species, as suggested by the presence of rather distinct clusters of points in the Relative Warp Analyses and by high percentages of correct classification in CVA (higher than 95%). The shape of external traits may depend on ecological factors and represent adaptive divergence. It has been shown, for example, that body shape of *Damaster blaptoides*, a representative snail-feeding species in Japan, is correlated with the size of land snails consumed by the species, suggesting that food resources as well as geographic isolation may have promoted adaptive divergence of external morphology in snail-feeding carabid beetles (Konuma *et al.*, 2010).

Species also diverged in body size, *N. fontinalis* being significantly smaller than each of the other two species, and in relative size of external traits, the sizes of the pronotum and elytron of *N. cordicollis* being significantly larger than those of *N. fontinalis*, and both of these species showing significantly larger traits than *N. laticollis* (pair-wise comparisons of intercepts). Variations in body size may also be driven by ecological determinants. The body sizes of most *Ohomopterus* species are positively correlated with annual mean temperature, representing the converse to Bergmann's rule as in many other poikilotherms (Sota *et al.*, 2000). Ecological requirements of the three *Nebriola* species were partly distinct (*N. cordicollis* was a petrophilous species found in moraines and other stony habitat whereas *N. fontinalis* and *N. laticollis* were typical of

cold streamlets). Their geographic distributions were also partly different (*N. laticollis* is found in the French and Western Italian Alps, whereas *N. fontinalis* and *N. cordicollis* are found in the Swiss and Italian Central Alps). We therefore suggest that in these stenoecious high altitude *Nebriola* species, both size and shape of external traits may have diverged, at least in part, as a result of geographical isolation and different natural selection regimes.

Shape of male genitalia

The shape of the median lobe of the aedeagus was apparently different in the three species, with poorly- or non-overlapping clusters of points in the Relative Warp Analyses, and percentages of 100% for two species (i.e . *N. cordicollis* and *N. laticollis*) out of three in CVA. These results are consistent with the important role of genitalia in inter-specific divergence processes. Rapid interspecific genital divergence has generally been attributed to shape, rather than size, variation, and a number of studies have emphasized the evolutionary independence of genital shape and size (Arnqvist & Thornhill, 1998; Macdonald & Goldstein, 1999; Sota *et al.*, 2007). In dung beetles, a pilot study on genital evolution in *O. taurus* provided the first evidence that male genitalia (paramere morphology) has diverged across recently established exotic populations (Pizzo *et al.*, 2008). In contrast to body size, the evolution of genital morphology is considered to be independent from adaptation to the external environment and mainly driven by sexual selection (Eberhard, 2010, 2011).

Our results on male genitalia may support the hypothesis of “reinforcement” – the rapid evolution of differences between pairs of allopatric sister taxa when they finally meet across a more or less wide area (Usami *et al.*, 2006; Sota & Tanabe, 2010; Masly,

2012). As shown by the map, the very similar *N. cordicollis* and *N. laticollis* have a contact area where reinforcement may have operated and, interestingly, these two species show marked differences in median lobe curvature.

Considering all the above, in keeping with Nagata *et al.* (2009), we hypothesize that in the subgenus *Nebriola*, Pleistocene fluctuation may have promoted the divergence of species by geographical isolation and natural selection, and that reinforcement phenomena may have occurred in a few instances.

Shape and size results, taken together, indicate that the three taxa are valid species. This suggests that, in spite of the great constancy of the morphological *habitus* within the subgenus *Nebriola*, traditional qualitative morphology was effective in recognizing the different taxa at the species level.

Our hierarchical design also considered differences among subspecies and populations. In both cases, eventual divergences in shape, if any, were poorly detectable. This result is in keeping with the great uncertainty in subspecies designation expressed by several authors (Bisio, 1998; Ledoux & Roux, 2005) and suggests great caution in considering *Nebriola* subspecies as valid taxonomic units. However, the subspecies of *N. cordicollis* showed three distinct body sizes and also exhibited significant differences in relative size of the elytron. A few size differences were also evident between distinct populations. All the above seem to suggest that, with respect to external traits, size might have been the first morphological characteristic to diverge in evolution of these beetles.

Finally, it should be noted that *N. fontinalis*, the smallest species, did not show the smallest external traits. Likewise, the subspecies *N. cordicollis winkleri*, which has the largest body size, did not have the largest elytron. These results suggest that inter-

specific divergence of body size and relative size of traits do not necessarily evolve in concert, in keeping with previous data from dung beetles (Macagno *et al.*, 2011).

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[Figure captions; set from these separated captions; also for Tables]

Figure 1. Collection localities of specimens used in geometric morphometric analyses. Species are indicated with different symbols. Grey dots = *Nebria cordicollis*; black dots = *Nebria laticollis*; open dots = *Nebria fontinalis*.

Figures 2-4. Landmark configurations of pronotum, median lobe of aedeagus, and right elytron. **2,** pronotum: points 1, 5, 12–14 and 16 are landmarks, points 2–4, 6–11 and 15 are semilandmarks; **3,** elytron: points 1–3 and 10–12 are landmarks, points 4–9 are semilandmarks; **4,** median lobe of aedeagus: points 1–3 and 10–13 are landmarks, points 4–9 are semilandmarks.

Figure 5. Shape divergence among species. Scatterplots of first two Relative Warps scores obtained from Relative Warp Analysis of *Nebria cordicollis* (grey dots), *Nebria fontinalis* (open dots) and *Nebria laticollis* (black dots).

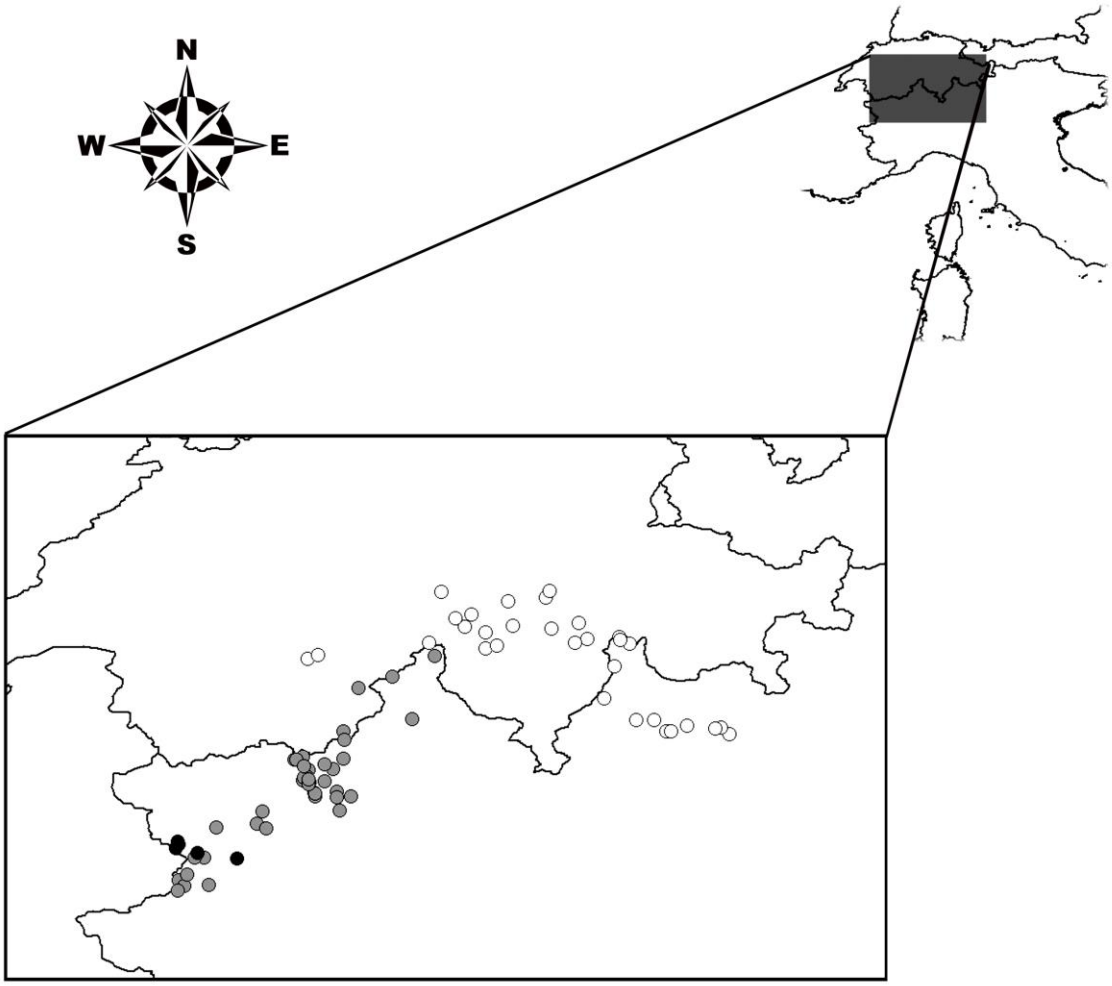
Table legends

Table 1. Number of specimens used in geometric morphometric analyses.

Table 2. Shape divergence. CVA of species, subspecies and populations considered. Percentages of predicted group membership from cross validation are given. Percentages higher than 90% are in bold type.

Table 3. Mean body sizes expressed as means ± SD of Maximum Pronotum Width in mm (proxy for the body size) of the three species, subspecies and populations considered. Differences between sexes were tested by Nested ANOVA (groups*sex). Pairwise comparisons among groups were based on estimated marginal means, with Bonferroni adjustment. No significant differences between sexes and no significant interactions between sexes and groups were detected. *** <0.001 ** <0.01.

Table 4. Relative trait size divergence between species, subspecies and populations shown as the difference between intercepts of static allometries of the median lobe of the aedeagus, elytron, and pronotum (full-factorial ANCOVAs with sequential Bonferroni correction applied). In each comparison the first group has the largest trait size. Only comparisons which passed both Levene's tests and the interaction of ln Max PW*group tests (P> 0.05) are shown. *** <0.001 ** <0.01.



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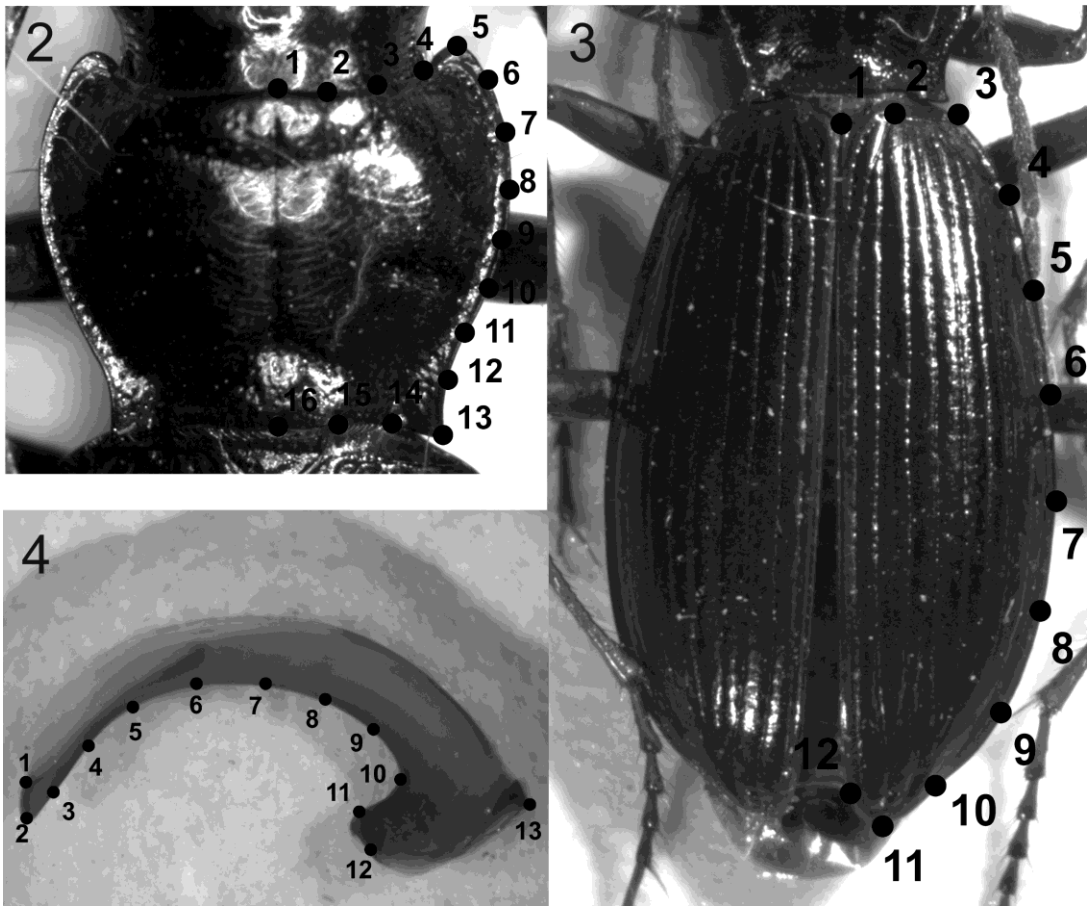
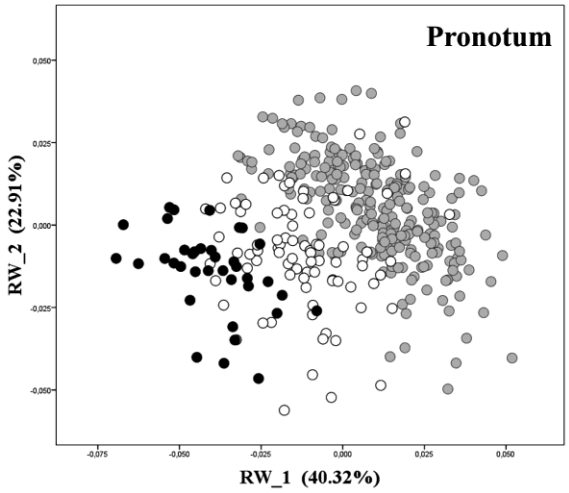
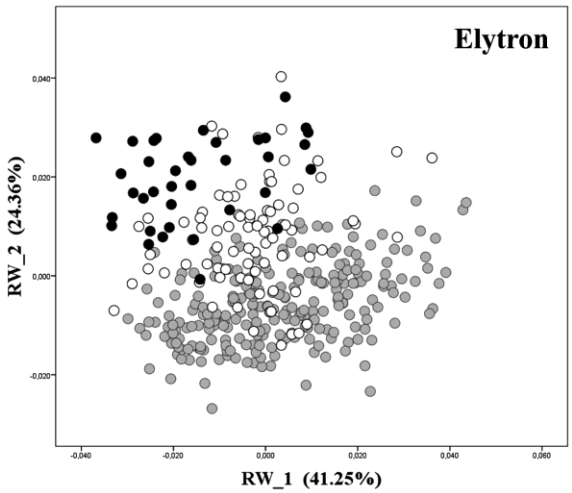


Figures 2-4. Landmark configurations of the pronotum, median lobe of the aedeagus and right elytron. **2,** pronotum: the points 1, 5, 12-14 and 16 are landmarks, the points 2-4, 6-11 and 15 are semilandmarks; **3,** elytron: the points 1-3 and 10-12 are landmarks, the points 4-9 are semilandmarks; **4,** median lobe of the aedeagus: the points 1-3 and 10-13 are landmarks, the points 4-9 are semilandmarks.


Pronotum
RW_2 (22.91%)
RW_1 (40.32%)


Elytron
RW_2 (24.36%)
RW_1 (41.25%)

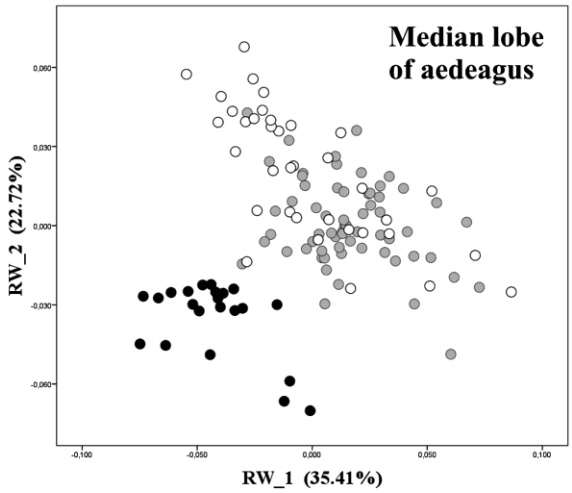

Median lobe
of aedeagus
RW_2 (22.72%)
RW_1 (35.41%)

Figure 5. Shape divergence among species. Scatterplots of the first two Relative Warps scores obtained from the Relative Warp Analysis of *Nebria cordicollis* (grey dots), *Nebria fontinalis* (open dots) and *Nebria laticollis* (black dots).

Table 1. Number of specimens used in geometric morphometric analyses.

Level		Pronotum	Elytron	Median lobe of aedeagus
Species	*N. cordicollis*	238	238	63
	N. fontinallis	82	82	36
	N. laticollis	37	37	22
	Tot	357	357	121
Subspecies	*N. cordicollis cordicollis*	93	93	22
	N. cordicollis kochi	110	110	21
	N. cordicollis winkleri	35	35	20
	Tot	238	238	63
Populations	*N. cordicollis cordicollis*			
	Anzasca Valley	38	38	18
	Ayas Valley	32	32	21
	Gressoney Valley	34	34	23
	Tot	104	104	62
	N. cordicollis kochi			
	Orco Valley	63	63	40
	Lanzo Valley	44	44	36
	Soana Valley	33	33	18
	Tot	140	140	94

Table 2. Shape divergence. CVA of species, subspecies and populations considered. Percentages of predicted group membership from cross validation are given. Percentages higher than 90% are in bold type.

Species		Predicted Group Membership			
		N. cordicollis	*N. fontinalis*	*N. laticollis*	Total
Pronotum	*N. cordicollis*	**98.8**	1.2	0.0	100
	N. fontinalis	3.7	**96.3**	0.0	100
	N. laticollis	0.0	5.4	**94.6**	100
Elytron	*N. cordicollis*	**98.0**	2.0	0.0	100
	N. fontinalis	4.9	**90.2**	4.9	100
	N. laticollis	0.0	2.7	**97.3**	100
Median lobe of aedeagus	*N. cordicollis*	**100.0**	0.0	0.0	100
	N. fontinalis	2.8	**97.2**	0.0	100
	N. laticollis	0.0	0.0	**100.0**	100

Subspecies		Predicted Group Membership			
		N. cordicollis kochi	*N. cordicollis winkleri*	*N. cordicollis cordicollis*	Total
Pronotum	*N. cordicollis kochi*	78.2	3.6	18.2	100
	N. cordicollis winkleri	8.6	**91.4**	0.0	100
	N. cordicollis cordicollis	17.2	0.0	82.8	100
Elytron	*N. cordicollis kochi*	53.6	18.2	28.2	100
	N. cordicollis winkleri	14.3	74.3	11.4	100
	N. cordicollis cordicollis	20.4	15.1	64.5	100
Median lobe of aedeagus	*N. cordicollis kochi*	57.1	28.6	14.3	100
	N. cordicollis winkleri	25.0	65.0	10.0	100
	N. cordicollis cordicollis	4.5	9.1	86.4	100

Populations *N. cordicollis cordicollis*		Predicted Group Membership			
		Anzasca Valley	Ayas Valley	Gressoney Valley	Total
Pronotum	Anzasca Valley	63.2	31.6	5.2	100
	Ayas Valley	25.0	68.8	6.2	100
	Gressoney Valley	5.9	5.9	88.2	100
Elytron	Anzasca Valley	73.6	13.2	13.2	100
	Ayas Valley	6.3	81.2	12.5	100
	Gressoney Valley	11.8	11.8	76.4	100
Median lobe of aedeagus	Anzasca Valley	61.1	27.8	11.1	100
	Ayas Valley	38.1	61.9	0.0	100
	Gressoney Valley	21.7	8.7	69.6	100

Populations *N. cordicollis kochi*		Predicted Group Membership			
		Orco Valley	Lanzo Valley	Soana Valley	Total
Pronotum	Orco Valley	82.5	9.5	8.0	100
	Lanzo Valley	6.8	**90.9**	2.3	100
	Soana Valley	9.1	9.1	81.8	100
Elytron	Orco Valley	73.0	15.9	11.1	100
	Lanzo Valley	20.5	79.5	0.0	100
	Soana Valley	15.2	6.1	78.7	100
Median lobe of aedeagus	Orco Valley	65.0	17.5	17.5	100
	Lanzo Valley	25.0	55.6	19.4	100
	Soana Valley	16.7	16.7	66.6	100

Table 3. Mean body sizes expressed as means ± SD of the Maximum Pronotum Width in mm, (proxy for the body size) of the three species, subspecies and populations considered. Differences between sexes were tested by Nested ANOVA (groups*sex). Pairwise comparisons among groups were based on estimated marginal means, with Bonferroni adjustment. No significant differences between sexes and no significant interactions between sexes and groups were detected. *** <0.001 ** <0.01.

Level						
	N. cordicollis	***N. fontinalis***	***N. laticollis***	**Differences among groups F**		**Significant pairwise comparisons (P<0.05)**
Species	2.306 ± 0.131	2.138 ± 0.079	2.269 ± 0.114	67.027	***	(1) vs (2) (2) vs (3)
	N. cordicollis kochi	***N. cordicollis winkleri***	***N. cordicollis cordicollis***	**Differences among groups F**		**Significant pairwise comparisons (P<0.05)**
Subspecies	2.301 ± 0.110	2.485 ± 0.086	2.247 ± 0.109	50.445	***	(1) vs (2) (1) vs (3) (2) vs (3)
	Anzasca Valley	**Ayas Valley**	**Gressoney Valley**	**Differences among groups F**		**Significant pairwise comparisons (P<0.05)**
Populations ***N. cordicollis cordicollis***	2.220 ± 0.116	2.235 ± 0.116	2.284 ± 0.114	5.299	**	(1) vs (3)
	Orco Valley	**Lanzo Valley**	**Soana Valley**	**Differences among groups F**		**Significant pairwise comparisons (P<0.05)**
Populations ***N. cordicollis kochi***	2.294 ± 0.128	2.309 ± 0.081	2.346 ± 0.139	1.959	n.s.	-

Table 4. Relative trait size divergence between species, subspecies and populations shown as the difference between intercepts of static allometries of the median lobe of the aedeagus, elytron and pronotum (full-factorial ANCOVAs with sequential Bonferroni correction applied). In each comparison the first group has the largest trait size. Only comparisons which passed both Levene's tests and the interaction of ln Max PW*group tests (P> 0.05) are shown.*** <0.001 ** <0.01.

Trait	Level	Comparison	Mean Difference	Sig.
Pronotum	Species	*N. cordicollis* vs *N. fontinalis*	0.020	**
		N. cordicollis vs *N. laticollis*	0.083	***
		N. fontinalis vs *N. laticollis*	0.063	***
	Subspecies	*N. cordicollis kochi* vs *N. cordicollis cordicollis*	0.004	ns
		N. cordicollis winkleri vs *N. cordicollis kochi*	0.005	ns
		N. cordicollis winkleri vs *N. cordicollis cordicollis*	0.009	ns
	Populations *N. cordicollis kochi*	Orco Valley vs Lanzo Valley	0.040	***
		Soana Valley vs Orco Valley	0.006	ns
		Soana Valley vs Lanzo Valley	0.046	***
Elytron	Species	*N. cordicollis* vs *N. fontinalis*	0.066	***
		N. cordicollis vs *N. laticollis*	0.039	***
		N. fontinalis vs *N. laticollis*	0.073	***
	Subspecies	*N. cordicollis kochi* vs *N. cordicollis winkleri*	0.055	**
		N. cordicollis kochi vs *N. cordicollis cordicollis*	0.021	**
		N. cordicollis winkleri vs N. cordicollis cordicollis	0.017	ns
Median lobe of aedeagus	Species	*N. cordicollis* vs *N. laticollis*	0.052	***