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

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13 **Running title:** Shape and size divergence in subgenus *Nebriola*

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

35

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

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41

42 **Introduction**

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

52 Ground beetles (Carabidae) include speciose groups and/or polytypic species whose
53 evolutionary differentiation took place during or after the Quaternary ice ages. The
54 *Nebria gregaria* group on the Queen Charlotte Islands (Alaska) represents a notable
55 example of rapid evolution. Composed of five morphologically similar species, it is
56 considered to be a taxon for which the repeated isolation of populations in glacial
57 refugia has played an important role in the promotion of speciation (Kavanaugh, 1992).
58 However, phylogenetic analyses based on molecular data, obtained from five regions of
59 mitochondrial and genomic DNA, revealed a lack of interspecific differentiation,
60 suggesting that divergences might represent local variation of a single species (Clarke *et al.*,
61 2001). Analogously, the results of genetic divergence within the Palaearctic species
62 *Carabus auronitens* (based on allozyme polymorphism) does not mirror conventional
63 subspecific taxonomy, as there are at least two subspecies (*C. auronitens auronitens* and
64 *C. auronitens festivus*, Turin *et al.*, 2003) that are not separated genetically, and several
65 other subspecies (Deuve, 1994) that are partially contradictory (Drees *et al.*, 2010).

66 In a number of studies, ground beetle morphology seems to be more informative
67 than genetics, and distinct morphological differences may be observed despite little
68 divergence in molecular markers. Sister species and populations of the genus *Pogonus*,
69 for example, could be differentiated using ecological characters, but were not
70 recognized by screening neutral DNA polymorphisms (Dhuyvetter *et al.*, 2007).

71 Analogously, *Carabus arrowianus* exhibits marked morphological diversity among
72 geographical populations in body and genital sizes, and this was found to be supported
73 by significant but not large genetic divergences (Nagata *et al.*, 2009).

74 Several morphometric studies have shown how rapidly changes in body size and
75 shape can evolve. Thus inter- and intraspecific divergences may readily arise. Genitalia,
76 for example, are considered to be among the fastest evolving morphological traits in
77 arthropods (Eberhard, 2010, 2011; Pizzo *et al.*, 2006a, 2006b, 2008). Rapid
78 diversification of male genitalia was described in ground beetles of the genus *Carabus*
79 subgenus *Ohomopterus* (Sasabe *et al.*, 2007, Takami & Sota, 2007), which also
80 exemplifies one of the most outstanding cases of mechanical isolation (Sota & Kubota,
81 1998). Knowledge of the genetic architecture underlying genital evolution is still
82 limited. Recent molecular analyses (genetic linkage maps and analyses of quantitative
83 trait loci) have shown that the interspecific difference in the genital morphologies (three
84 male copulatory structures and two traits derived from female vaginal appendices) were
85 determined by a relatively small number of genes with marked phenotypic effects
86 (Sasabe *et al.*, 2007, 2010).

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

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

96 In ground beetles, geometric morphometrics has been used to discover cryptic
97 species (Faille *et al.*, 2007, Dujardin *et al.*, 2010) and to assess differentiation at a
98 micro-geographical scale within *Carabus auronitens* and *C. nemoralis* (Alibert *et al.*,
99 2001). The technique has also been successfully applied to genetic analyses of the short-
100 range species *Carabus solieri*, the phylogeographic pattern of which (Garnier *et al.*,
101 2004) was then *a posteriori* successfully confirmed by geometric morphometrics
102 analyses based on male genitalia (Garnier *et al.*, 2005, 2006).

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

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

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

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

135

136

137 **Material and methods**

138 The three species considered are morphologically quite similar. *Nebria laticollis* shows
139 almost parallel elytra and has a more curved median lobe of the aedeagus, whereas *N.*
140 *cordicollis* and *N. fontinalis* have the elytra progressively enlarged posteriorly, and
141 moderately curved median lobes. These last two are currently differentiated by the
142 shape of labial palps and setation of the hind legs and last ventrites (Ledoux & Roux
143 2005). They are characterized by similar but distinct ecological habits. *Nebria*
144 *cordicollis* is a petrophilous species dwelling in the alpine belt from 2100–3000 m a.s.l.,
145 where it may be found around snow patches, moraines and other stony habitats (Bisio,
146 1998). *Nebria fontinalis* dwells in cold spring-waters (from +1 to -1°C) and can be
147 found from 1460–2700 m (Focarile & Casale, 1978), while *Nebria laticollis* has similar
148 ecological habits, being found in cold springs and streamlets at 1500–2500 m (Ledoux
149 & Roux, 2005).

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

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

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

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

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

182

183 **Measurements**

184 A semilandmark-based approach (Bookstein, 1989, 1991) was used to characterize the
185 shape variation of the pronotum, right elytron and median lobe of the aedeagus. Using
186 tpsDig 2.16 (Rohlf, 2010a), 13 points were placed on the marginal side of the median
187 lobe of the aedeagus, 16 on the right half pronotum and 12 on the right elytron; for each
188 structure, the configuration was chosen following the criteria of homology (Bookstein,
189 1991). To capture overall shape variation of the curved parts of these anatomical
190 features, the semilandmarks method was applied, coding the points as landmarks and
191 semilandmarks (Figs. 2-4) by tpsUtil 1.47 (Rohlf, 2010b). For the geometric
192 morphometrics analyses, the datasets were chosen taking care that the samples sizes
193 were larger than the number of variables (i.e., the Relative Warps). Using a Generalized
194 Procrustes Analysis (GPA) the effects of translation, rotation, and scale (Rohlf, 1990;
195 Rohlf & Slice, 1990) were removed.

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

198

199

Statistical analyses

200

201

Shape

202

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

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

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

217 Canonical Variate Analysis (CVA) was performed to obtain a classification matrix
218 based on shape variation of traits (Reddy *et al.*, 2005, Sheets *et al.*, 2006, Gómez-
219 Robles *et al.*, 2011) using PASW Statistics 18. In all analyses, the number of Relative
220 Warps was less than that of cases within each group (i.e. specimens, in our case).

221 Percentages of correct classifications were cross-validated through the leave-one-out
222 option, each case being classified by the functions derived from all cases other than the
223 one in question.

224

225 *Size*

226

227 *Body size*

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

230 1999; Huber *et al.*, 2010; Laparie *et al.*, 2010) because its consistency, and to avoid the
231 mutual malposition of the different body parts and the subsequent measurement
232 artefacts (Garnier *et al.*, 2005), depending also on how the measure is employed for
233 many other coleopterans (Pizzo *et al.*, 2011, 2012).

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

238

239 Size of each anatomical trait

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

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

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

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

267

268

269 **Results**

270 **Shape**

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

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

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

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

291

292 **Body size**

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

301

302 **Relative size**

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

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

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

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

322

323

324 **Discussion**

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

328

329 **Shape and size of external traits**

330 The shape of the external traits (elytron and pronotum) were clearly different among
331 species, as suggested by the presence of rather distinct clusters of points in the Relative
332 Warp Analyses and by high percentages of correct classification in CVA (higher than
333 95%). The shape of external traits may depend on ecological factors and represent
334 adaptive divergence. It has been shown, for example, that body shape of *Damaster*
335 *blaptoides*, a representative snail-feeding species in Japan, is correlated with the size of
336 land snails consumed by the species, suggesting that food resources as well as
337 geographic isolation may have promoted adaptive divergence of external morphology in
338 snail-feeding carabid beetles (Konuma *et al.*, 2010).

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

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

355

356 **Shape of male genitalia**

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

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

373 2012). As shown by the map, the very similar *N. cordicollis* and *N. laticollis* have a
374 contact area where reinforcement may have operated and, interestingly, these two
375 species show marked differences in median lobe curvature.

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

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

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

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

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

399

400

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414

415

416 **References**

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

609

610 **Figure 1.** Collection localities of specimens used in geometric morphometric analyses.

611 Species are indicated with different symbols. Grey dots = *Nebria cordicollis*; black dots

612 = *Nebria laticollis*; open dots = *Nebria fontinalis*.

613

614

615 **Figures 2-4.** Landmark configurations of pronotum, median lobe of aedeagus, and right

616 elytron. **2**, pronotum: points 1, 5, 12–14 and 16 are landmarks, points 2–4, 6–11 and 15

617 are semilandmarks; **3**, elytron: points 1–3 and 10–12 are landmarks, points 4–9 are

618 semilandmarks; **4**, median lobe of aedeagus: points 1–3 and 10–13 are landmarks,

619 points 4–9 are semilandmarks.

620

621

622 **Figure 5.** Shape divergence among species. Scatterplots of first two Relative Warps

623 scores obtained from Relative Warp Analysis of *Nebria cordicollis* (grey dots), *Nebria*

624 *fontinalis* (open dots) and *Nebria laticollis* (black dots).

625

626 **Table legends**

627

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

629

630 **Table 2.** Shape divergence. CVA of species, subspecies and populations considered.

631 Percentages of predicted group membership from cross validation are given.

632 Percentages higher than 90% are in bold type.

633

634 **Table 3.** Mean body sizes expressed as means \pm SD of Maximum Pronotum Width in

635 mm (proxy for the body size) of the three species, subspecies and populations

636 considered. Differences between sexes were tested by Nested ANOVA (groups*sex).

637 Pairwise comparisons among groups were based on estimated marginal means, with

638 Bonferroni adjustment. No significant differences between sexes and no significant

639 interactions between sexes and groups were detected. *** <0.001 ** <0.01.

640

641 **Table 4.** Relative trait size divergence between species, subspecies and populations

642 shown as the difference between intercepts of static allometries of the median lobe of

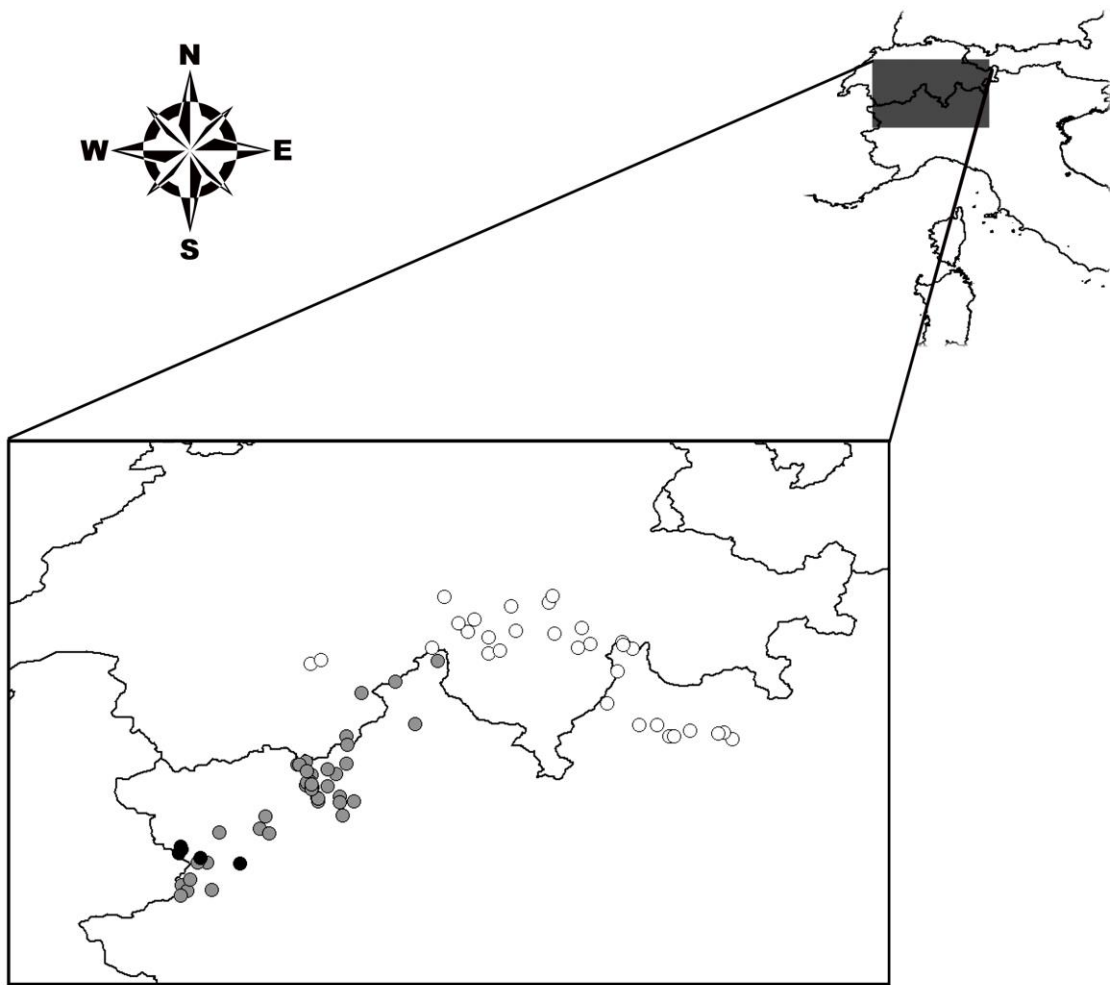
643 the aedeagus, elytron, and pronotum (full-factorial ANCOVAs with sequential

644 Bonferroni correction applied). In each comparison the first group has the largest trait

645 size. Only comparisons which passed both Levene's tests and the interaction of ln Max

646 PW*group tests ($P > 0.05$) are shown. *** <0.001 ** <0.01.

647



648

649 **Figure 1.** Collection localities of specimens used in geometric morphometric analyses.650 Species are indicated with different symbols. Grey dots = *Nebria cordicollis*; black dots651 = *Nebria laticollis*; open dots = *Nebria fontinalis*.

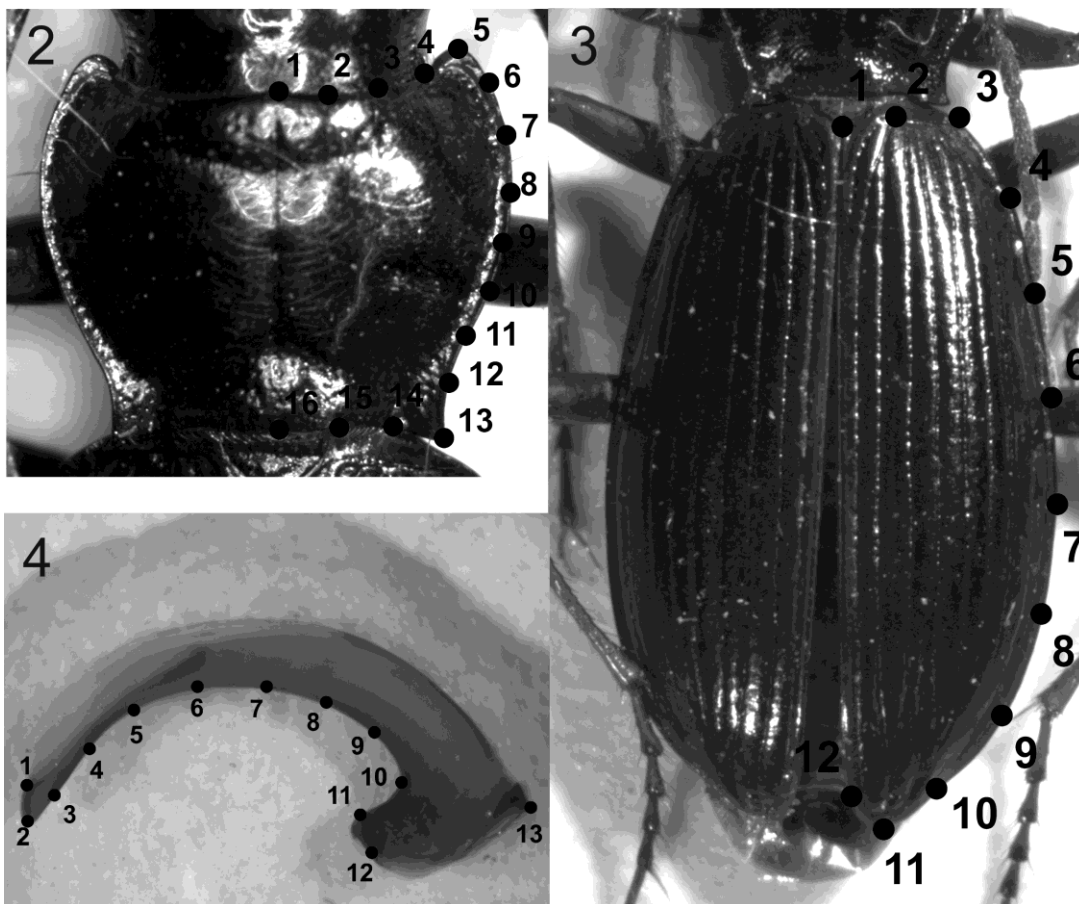
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659 **Figures 2-4.** Landmark configurations of the pronotum, median lobe of the aedeagus
 660 and right elytron. **2**, pronotum: the points 1, 5, 12-14 and 16 are landmarks, the points 2-
 661 4, 6-11 and 15 are semilandmarks; **3**, elytron: the points 1-3 and 10-12 are landmarks,
 662 the points 4-9 are semilandmarks; **4**, median lobe of the aedeagus: the points 1-3 and
 663 10-13 are landmarks, the points 4-9 are semilandmarks.

664

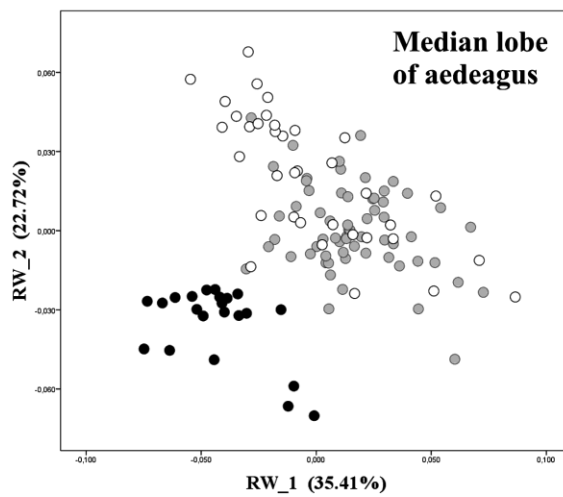
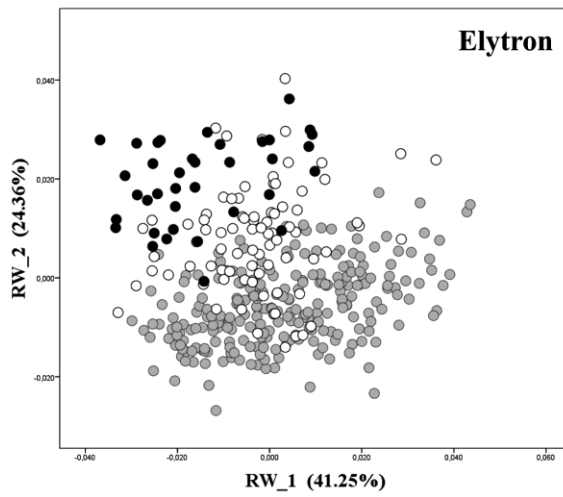
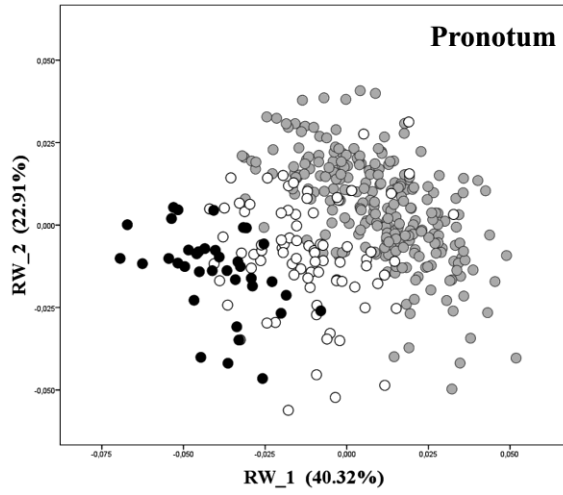


Figure 5. Shape divergence among species. Scatterplots of the first two Relative Warps scores obtained from the Relative Warp Analysis of *Nebria cordicollis* (grey dots), *Nebria fontinalis* (open dots) and *Nebria laticollis* (black dots).

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

Level		Pronotum	Elytron	Median lobe of aedeagus
Species	<i>N. cordicollis</i>	238	238	63
	<i>N. fontinalis</i>	82	82	36
	<i>N. laticollis</i>	37	37	22
	Tot	357	357	121
Subspecies	<i>N. cordicollis cordicollis</i>	93	93	22
	<i>N. cordicollis kochi</i>	110	110	21
	<i>N. cordicollis winkleri</i>	35	35	20
	Tot	238	238	63
Populations	<i>N. cordicollis cordicollis</i>			
	Anzasca Valley	38	38	18
	Ayas Valley	32	32	21
	Gressoney Valley	34	34	23
	Tot	104	104	62
	<i>N. cordicollis kochi</i>			
	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			
		<i>N. cordicollis</i>	<i>N. fontinalis</i>	<i>N. laticollis</i>	Total
Pronotum	<i>N. cordicollis</i>	98.8	1.2	0.0	100
	<i>N. fontinalis</i>	3.7	96.3	0.0	100
	<i>N. laticollis</i>	0.0	5.4	94.6	100
Elytron	<i>N. cordicollis</i>	98.0	2.0	0.0	100
	<i>N. fontinalis</i>	4.9	90.2	4.9	100
	<i>N. laticollis</i>	0.0	2.7	97.3	100
Median lobe of aedeagus	<i>N. cordicollis</i>	100.0	0.0	0.0	100
	<i>N. fontinalis</i>	2.8	97.2	0.0	100
	<i>N. laticollis</i>	0.0	0.0	100.0	100
Subspecies		Predicted Group Membership			
		<i>N. cordicollis kochi</i>	<i>N. cordicollis winkleri</i>	<i>N. cordicollis cordicollis</i>	Total
Pronotum	<i>N. cordicollis kochi</i>	78.2	3.6	18.2	100
	<i>N. cordicollis winkleri</i>	8.6	91.4	0.0	100
	<i>N. cordicollis cordicollis</i>	17.2	0.0	82.8	100
Elytron	<i>N. cordicollis kochi</i>	53.6	18.2	28.2	100
	<i>N. cordicollis winkleri</i>	14.3	74.3	11.4	100
	<i>N. cordicollis cordicollis</i>	20.4	15.1	64.5	100
Median lobe of aedeagus	<i>N. cordicollis kochi</i>	57.1	28.6	14.3	100
	<i>N. cordicollis winkleri</i>	25.0	65.0	10.0	100
	<i>N. cordicollis cordicollis</i>	4.5	9.1	86.4	100
Populations <i>N. cordicollis cordicollis</i>		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 <i>N. cordicollis kochi</i>		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 \pm 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						
	<i>N. cordicollis</i>	<i>N. fontinalis</i>	<i>N. laticollis</i>	Differences among groups F		Significant pairwise comparisons (P<0.05)
Species	2.306 \pm 0.131	2.138 \pm 0.079	2.269 \pm 0.114	67.027	***	(1) vs (2) (2) vs (3)
	<i>N. cordicollis kochi</i>	<i>N. cordicollis winkleri</i>	<i>N. cordicollis cordicollis</i>	Differences among groups F		Significant pairwise comparisons (P<0.05)
Subspecies	2.301 \pm 0.110	2.485 \pm 0.086	2.247 \pm 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 <i>N. cordicollis cordicollis</i>	2.220 \pm 0.116	2.235 \pm 0.116	2.284 \pm 0.114	5.299	**	(1) vs (3)
	Orco Valley	Lanzo Valley	Soana Valley	Differences among groups F		Significant pairwise comparisons (P<0.05)
Populations <i>N. cordicollis kochi</i>	2.294 \pm 0.128	2.309 \pm 0.081	2.346 \pm 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 $\ln \text{Max PW}^* \text{group}$ tests ($P > 0.05$) are shown. *** < 0.001 ** < 0.01 .

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