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Reconstructing ancient Mediterranean crossroads in *Deronectes* diving beetles

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1 **Original article**

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3 **Running header:** Biogeography of Mediterranean *Deronectes* diving beetles

4

5 **Reconstructing ancient Mediterranean crossroads in *Deronectes* diving**
6 **beetles**

7

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25 **ABSTRACT**

26 **Aim** To reconstruct the evolutionary history of a genus of freshwater beetle with a pan-
27 Mediterranean distribution, to test classic hypotheses which proposed a Miocene origin
28 for groups with high biodiversity in the Iberian and Anatolian peninsulas.

29 **Location** Mediterranean basin.

30 **Methods** We sequenced four mitochondrial and one nuclear gene from 51 specimens of
31 30 of the ca. 60 extant species of *Deronectes* (Dytiscidae), all typical of mid-mountain
32 streams from North Africa and Iberia over most of Europe to the Middle East. We used
33 Maximum Likelihood, Bayesian probabilities with an a priori evolutionary rate and a
34 Dispersal-Extinction-Cladogenesis model to reconstruct their biogeographical history.

35 **Results** *Deronectes* has two major lineages which originated in the mid Miocene; one
36 including mostly eastern and another mainly western and central Mediterranean species.
37 From these two areas range expansions, mainly at the end of the Miocene and beginning
38 of the Pliocene, resulted in the many species groups and some of the extant species of the
39 genus. Most of the current diversity and distributions are, however, of Plio-Pleistocene
40 origin, particularly in widespread European species.

41 **Main conclusions** In line with traditional hypotheses, we found an ancient division
42 between eastern and western Mediterranean lineages of *Deronectes*, likely resulting from
43 the isolation of Europe west of the Alps from the Balkans and Anatolia during the early-
44 middle Miocene. The history of the genus was strongly influenced by major geological
45 and climatic events , with successive cycles of fragmentation and subsequent eastward
46 and westward range expansions, resulting in a steady accumulation of species across the
47 basin. Most of these range movements took place through the north side of the
48 Mediterranean, with only local displacements in the south during the Messinian and a
49 recent (Pleistocene) colonization of the Italian peninsula, which remained largely
50 submerged through most of the genus' evolutionary history.

51

52 **Keywords**

53 Biodiversity hotspot, dispersal, diversification, Dytiscidae, Mediterranean, Messinian
54 salinity crisis, phylogeny.

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57

58 **INTRODUCTION**

59 The Mediterranean region, with its complex geological history, is an ideal system to
60 study the effects of palaeogeographical events on evolutionary diversification. The region
61 has had a ‘reticulated’ biogeographical history, in which the constituent landmasses have
62 repeatedly split, collided, and split again in different configurations over time
63 (Rosenbaum *et al.*, 2002; Meulenkamp & Sissingh, 2003; Popov *et al.*, 2004), resulting in
64 repeated episodes of vicariance and dispersal (Oosterbroek & Arntzen, 1992; Sanmartín
65 *et al.*, 2001). Whilst the geological evolution of the basin is relatively well understood,
66 the detailed geographical and temporal origins of most Mediterranean organisms remain
67 unknown, especially in diverse groups such as insects.

68 Traditional hypotheses proposed a Miocene origin for many terrestrial and
69 freshwater Mediterranean lineages, with close relationships between the fauna at the two
70 extreme ends of the basin – the so-called Kiermack disjunction (see e.g. Brehm, 1947 on
71 Iberian and Balkan plants, Bănărescu, 1991 on the Mediterranean freshwater fauna, or
72 Ribera & Blasco-Zumeta, 1998 on insects with disjunct distributions between the steppe
73 areas of NE Spain and those of the eastern Mediterranean and central Asia). Distribution
74 throughout the Mediterranean region became possible for terrestrial and freshwater taxa
75 during the Late Oligocene-Early Miocene, after the formation of a continuous landmass
76 connecting western Europe with the area roughly corresponding to the Balkans and
77 Turkey, separating the Tethys and Paratethys Oceans (Rögl & Steininger, 1983;
78 Oosterbroek & Arntzen, 1992). During the Miocene, the re-establishment of occasional
79 marine connections between the Tethys and Paratethys and successive landmass suture
80 events between the Eastern and Western Mediterranean Basins likely resulted in the
81 diversification of many Mediterranean groups (Oosterbroek & Arntzen, 1992; Montreuil,
82 2007). Alternatively, other biogeographical studies consider North Africa and the
83 Gibraltar Strait, which closed during the Messinian salinity crisis (end Miocene), to be an
84 alternative dispersal route by which lineages could have achieved circum-Mediterranean
85 distributions (e.g. Sanmartin, 2003).

86 Many of these early hypotheses were based on the presence of the same, or very
87 closely related species, on both sides of the Mediterranean in similar ecological
88 conditions (see Ribera & Blasco-Zumeta, 1998 for a review), but without a wider
89 phylogenetic context. Similarly, and given the lack of fossils in most Mediterranean
90 groups, the estimated temporal origin of these relationships were based on circumstantial
91 evidence alone.

92 The widespread use of molecular data to obtain reliable, calibrated phylogenies
93 has resulted in a proliferation of studies on Mediterranean lineages (e.g. Levy *et al.*,
94 2009; Santos-Gally *et al.*, 2012 or Condamine *et al.*, 2013). There are, however, very few
95 data on freshwater invertebrates encompassing the entire Mediterranean area, most works
96 to date focussing on only parts of the basin (e.g. Trizzino *et al.*, 2011 for northern
97 Mediterranean freshwater beetles, or Solà *et al.*, 2013 for eastern Mediterranean
98 freshwater planarians).

99 Whilst there remains a dearth of detailed analyses of lineages with wide
100 Mediterranean distributions, general hypotheses on the origin and composition of the
101 Mediterranean biota as a whole can only be tested by investigating such taxa. In this work
102 we study one of these lineages, the diving beetle genus *Deronectes* Sharp (family
103 Dytiscidae). With ca. 60 described species, *Deronectes* has a predominantly
104 Mediterranean distribution, ranging from North Africa and the Iberian Peninsula over
105 most parts of Europe and the Middle East, with some species reaching central Asia.
106 *Deronectes* are poor dispersers, with species usually restricted to relatively small
107 geographical ranges particularly in mountain regions, making them eminently suitable for
108 biogeographical reconstructions. There are in addition some widespread species with
109 continental-scale distributions (Abellán & Ribera, 2011), demonstrating their potential for
110 range expansion. *Deronectes* usually live amongst gravel, stones or submerged tree roots
111 in small streams with sparse vegetation (Fery & Brancucci, 1997).

112 Previous work based on mitochondrial genes and with incomplete sampling
113 identified two main lineages within the genus, mostly corresponding to species with a
114 western (Iberian Peninsula) or eastern (Anatolia and Middle East) distribution (Ribera *et*
115 *al.*, 2001; Ribera, 2003; Abellán & Ribera, 2011), but the precise relationships of the
116 species, their geographical origin and the temporal framework of their diversification
117 remained obscure. In this work we use a comprehensive molecular phylogeny of the
118 genus to reconstruct its biogeographical history, the geographical origin of major lineages
119 and the events that led to their current distributions through the Mediterranean basin.

120

121 **MATERIAL AND METHODS**

122

123 **Taxon sampling**

124 *Deronectes* contains 58 species and four subspecies (Nilsson & Hájek, 2015), most of
125 them revised by Fery & Brancucci (1997) and Fery & Hosseinie (1998). These authors

126 divided the genus into 10 groups based on morphology, to which Hájek *et al.* (2011)
127 added an 11th for a single species from Turkey (*D. ermani* Hájek *et al.*) (See Appendixes
128 S1a,b in Supporting Information for a checklist of the species and subspecies with
129 distributions). We studied 51 specimens of 30 species of *Deronectes*, with an emphasis
130 on the western clade (20 out of 24 known species) but including representatives of all
131 recognised species groups with the exception of the recently described *D. ermani*. We
132 also included three of the four described subspecies (Appendix S1c). For some species
133 more than one specimen was included to detect possible unrecognised variation. We used
134 37 species of closely related genera of Hydroporini as outgroups, following the
135 phylogenies of Dytiscidae in Ribera *et al.* (2008) and Miller & Bergsten (2014).

136

137 **DNA extraction and sequencing**

138 Specimens were collected and preserved in absolute ethanol directly in the field. We
139 obtained the DNA non-destructively, either with standard phenol-chloroform extraction
140 or commercial kits (mostly DNeasy Tissue Kit, Qiagen GmbH, Hilden, Germany and
141 Charge Switch gDNA Tissue Mini Kit, Invitrogen, Carlsbad, CA, USA), following
142 manufacturer's instructions. Voucher specimens and DNA extractions are kept in the
143 collections of the Institut de Biología Evolutiva, Barcelona (IBE), Museo Nacional de
144 Ciencias Naturales, Madrid (MNCN) and Natural History Museum, London (NHM).

145 Six gene fragments from five different genes (four mitochondrial and one nuclear)
146 were obtained in four different amplification reactions: 1) 5' end of the Cytochrome c
147 Oxidase Subunit 1 gene (the barcode fragment, Hebert *et al.*, 2003, COI-5'); 2) 3' end of
148 Cytochrome c Oxidase Subunit 1 (COI-3'); 3) 3' end of 16S rDNA plus tRNA transfer of
149 Leucine plus 5' end of NADH dehydrogenase subunit 1 (*nad1*) (16S); 4) an internal
150 fragment of the nuclear gene Histone 3 (H3) (see Appendix S2a for details on primers
151 used and Appendix S2b for PCR conditions).

152

153 **Phylogenetic analyses**

154 Edited sequences were aligned using MAFFT v.6 with the G-INS algorithm and defaults
155 for other parameters (Katoh & Toh, 2008). We used jModeltest 0.1.1 (Posada, 2008) to
156 estimate the evolutionary model that best fitted the data for each gene separately, using
157 AIC (Akaike Information Criterion) scores as selection criteria and default values for
158 other parameters (Katoh & Toh, 2008). To infer the phylogeny of *Deronectes* we used
159 Bayesian probabilities (Bp) and Maximum Likelihood (ML).

160 For Bayesian analyses we used MrBayes 3.2 (Ronquist *et al.*, 2012) implementing
161 the most similar evolutionary models to those selected by jModeltest. For the analyses of
162 combined mitochondrial and nuclear data we used five partitions corresponding to COI-
163 5', COI-3', 16S rRNA+tRNA-Leu, nad1 and H3. We also analysed the mitochondrial
164 (with 4 partitions as above) and nuclear data separately. The program was left running
165 until we obtained a sufficient number of trees after the two independent runs converged,
166 according to the ESS (Effective Sample Size) and PSRF (Potential Scale Reduction
167 Factor) criteria as estimated in Tracer v1.5. (Drummond & Rambaut, 2007) and MrBayes
168 respectively. Convergence and burn-in values were estimated visually after examining a
169 plot of the standard deviation of split frequencies between the two simultaneous runs.

170 For ML analysis we used a fast approximate algorithm as implemented in
171 RAxML 7.1 (Stamatakis *et al.*, 2008) using GTR+G as an evolutionary model and the
172 same partitions as in MrBayes. The optimum topology was that of the best likelihood
173 amongst 100 replicates, and node support was estimated with 1000 bootstrap replicates
174 using the CAT approximation (Stamatakis *et al.*, 2008).

175

176 **Estimation of ages of divergence**

177 We obtained an estimate of divergence dates amongst species with BEAST v1.7
178 (Drummond & Rambaut, 2007). There are no fossils or unambiguous biogeographical
179 events that could be used to calibrate the phylogeny of *Deronectes*, so we used an *a priori*
180 substitution rate for the combined mitochondrial sequence of 0.01 substitutions/site per
181 MY (million years) (standard deviation 0.002), similar to that obtained in related beetle
182 groups for the same combination of mitochondrial protein coding and ribosomal genes
183 (Papadopoulou *et al.*, 2010; Ribera *et al.*, 2010; Andujar *et al.*, 2012). We excluded the
184 nuclear sequence (H3), and to ensure that the topology obtained with mitochondrial
185 sequences was the same as that obtained with the combined matrix we constrained all
186 well supported nodes (with a posterior probability in MrBayes > 0.95 and a bootstrap
187 support in RAxML > 70%) after deleting the outgroups. We used a GTR+I+G
188 evolutionary model, an uncorrelated lognormal relaxed clock and a Yule speciation
189 model. We executed two independent runs with the same settings that were allowed to
190 run until they had converged and the number of trees was sufficient according to ESS
191 values, as measured in Tracer v1.5. The consensus tree of the two runs was compiled
192 with Tree Anotator v1.7 (Drummond & Rambaut, 2007).

193

194 **Diversification**

195 To have an estimation of possible changes in diversification rates through the evolution
196 of the group we used a log-lineage through time approach (LTT) (Barraclough & Nee,
197 2001). Only the western clade could be studied, with an almost complete taxon sampling
198 (20 out of 22 species). We used the R library ‘ape’ (Paradis *et al.*, 2004) to compile the
199 LTT plot using the ultrametric tree obtained in BEAST after deleting duplicated
200 specimens of monophyletic taxa. We used the γ -statistic (Pybus & Harvey, 2000) for
201 measuring the relative timing of diversification, i.e. whether there is a constant
202 diversification rate through the tree, or the interior nodes are closer to the tips, or to the
203 root, than expected under a pure birth process. The γ -values of complete reconstructed
204 phylogenies follow a standard normal distribution. If $\gamma < 0$, the internal nodes can be said
205 to be closer to the root than expected under a pure birth process, and vice versa (Pybus &
206 Harvey, 2000).

207

208 **Ancestral area reconstruction**

209 To estimate ancestral areas of distribution we used a dispersal-extinction-cladogenesis
210 model implemented in the package Lagrange c++ 0.1, a ML inference model in which
211 parameters are estimated for rates of migratory events between areas (range expansions)
212 and local extinctions within areas (range contraction) (Almeida *et al.*, 2012). Lagrange
213 considers branch lengths and allows the definition of a number of areas with an
214 associated probability matrix of dispersal between them (Ree & Smith, 2008). We
215 considered eight geographical areas, based on the current distribution of *Deronectes*
216 species: A) southeastern Iberian Peninsula including Mallorca; B) centre and north of the
217 Iberian Peninsula; C) Italy (including Sicily) and southeastern France; D) Corsica and
218 Sardinia; E) Balkan peninsula; F) Turkey; G) northern and central Europe; and H)
219 Maghreb (See Fig. 1 for the distribution of the main lineages and Appendix S1a and
220 Appendix S1b for the distribution of the species of *Deronectes*). We used the tree
221 obtained in BEAST after pruning duplicated specimens. In all analyses a maximum of
222 four possible ancestral areas was allowed.

223 For the reconstruction of ancestral areas we used three different time slices
224 corresponding to the Pleistocene, Pliocene and Miocene, and a different
225 palaeogeographical scenario for each: present, Piacenzian/Gelasian (1.8-3.4 Ma) and Late
226 Tortonian (7-8 Ma) respectively, adapted from Meulenkamp & Sissingh (2003). For each
227 scenario we identified the geographical barriers between our eight pre-defined areas, and

228 assigned probabilities of dispersal to the land or sea barriers in different combinations,
229 including a null model with all probabilities equal to 1 (Table 1). We used the likelihood
230 of the reconstruction to select the model best fitting the current distribution of the species.
231 A difference equal to or greater than 2 log-likelihood units was considered significant
232 (Ree *et al.*, 2005; Ree & Smith, 2008).

233 To account for topological uncertainty we used the best settings as selected above
234 in a Bayes-Lagrange analysis with a selection of 1000 trees from amongst the last 50,000
235 trees of the stationary period (post burn-in) of the BEAST analysis. Using a custom script
236 in R and a spreadsheet we parsed the output and estimated the frequency of each
237 combined area reconstruction for the nodes present in the consensus tree, as well as the
238 individual frequency of each of the eight areas.

239

240 **RESULTS**

241 **Phylogenetic analyses**

242 There were no length differences in protein coding genes, and the length of ribosomal
243 genes ranged between 685-693 bp in the ingroup. The MrBayes runs of combined and
244 nuclear H3 analyses reached a standard deviation of split frequencies below 0.01 at 15
245 and 4 million generations (MG) respectively, and below 0.005 at 18.5 MG in the analysis
246 of the mitochondrial matrix. These were considered the burn-in fractions, after which
247 analyses were left to run until they reached convergence (at 23, 10 and 30 MG
248 respectively).

249 Differences between topologies obtained with Bp and ML were minimal, and
250 affected only the degree of resolution and support of some nodes (Fig. 2; Appendix S3a
251 and S3b). The monophyly of *Deronectes* was strongly supported, as well as its separation
252 into two major clades, one including species predominantly distributed in the eastern
253 Mediterranean (“eastern clade”); and a clade of species predominantly distributed in the
254 western and central Mediterranean (“western clade”) (Figs 1 and 2).

255 The eastern clade was further subdivided into two species groups as follows:

256 1) *D. parvicollis* group, including species from large parts of Asia, Turkey and the
257 Caucasus to southern Siberia and Central Asia. Only one species, *D. parvicollis*
258 (Schaum), extends into Europe (Balkans).

259 2) *D. latus* group, four species from eastern Turkey to the Iberian Peninsula and
260 throughout central and northern Europe, including the British Isles and Scandinavia. This

261 group included the most widespread species of the genus, *D. latus* (Stephens), ranging
262 over most of Europe north of the Pyrenees and the Apennines.

263 Within the western clade we recovered four well-supported species groups plus
264 two isolated species (*D. sahlbergi* Zimmermann and *D. doriae* Sharp), but the
265 relationships amongst them were not well resolved. (Fig. 2). These four clades were:

266 1) *D. opatrinus* group, including mostly species endemic to the Iberian Peninsula,
267 with only one (*D. hispanicus* (Rosenhauer)) reaching northern Morocco and two (*D.*
268 *hispanicus* and *D. opatrinus* (Germar)) southern France.

269 2) *D. aubei* group, with three species and one subspecies distributed from the
270 Cantabrian mountains in northwestern Spain to Sicily, including the Alps and southern
271 Germany.

272 3) *D. moestus* group, including species with a predominantly western
273 Mediterranean distribution, from the Maghreb and the Iberian Peninsula to the Balkans
274 through southern France, Italy and Sicily. The two missing north African species from the
275 western clade (*D. perrinae* Fery & Brancucci and *D. peyerimhoffi* (Régimbart)) most
276 likely belong here, as they are morphologically very similar to *D. moestus* and *D.*
277 *fairmairei* (Leprieur) respectively (Fery & Brancucci, 1997).

278 4) *D. platynotus* group, including two species and two subspecies from the
279 Balkans, Central Europe and northwest Iberia.

280 Most species with more than one sequenced specimen were monophyletic, with
281 some exceptions. There were three paraphyletic complexes of closely related species
282 (Fig. 2): 1) the widespread *D. latus*, with the Iberian endemic *D. angusi* Fery &
283 Brancucci nested within it; 2) the *D. aubei* group, with one clade west of the Rhone river,
284 from the French Massif Central to the Cantabrian Mountains including *D. a. sanfilippoi*
285 Fery & Brancucci and *D. delarouzei* (Jacquelin du Val), and another east of the Rhone,
286 from the Alps to Sicily with *D. a. aubei* (Mulsant) and *D. semirufus* (Germar) (Appendix
287 S1b); and 3) *D. ferrugineus* Fery & Brancucci and *D. wewalkai* Fery & Fresneda, both
288 Iberian endemics. There was also one case (*D. moestus*) with a deep intraspecific
289 divergence, with the specimen from Morocco (MNCN-AI937) sister to *D. brannani*
290 (Schauffus) (a Mallorcan endemic), and both sister to the other specimens of *D. moestus*,
291 from northern Spain to Bulgaria, including specimens of the two recognised subspecies
292 (Fig. 2).

293 The analysis of the nuclear sequence (H3) showed lower resolution and an
294 absence of support at some nodes, with polytomies in some groups (e.g. *D. moestus* or *D.*

295 *latus*) but with a topology compatible with that obtained from the mitochondrial
296 sequence, with a single exception (Appendixes S3c and S3d). Whilst with the
297 mitochondrial sequence the two subspecies of *D. aubei* were recovered as paraphyletic,
298 and respectively sisters to the geographically closest species of the group, the nuclear
299 sequence recovered a monophyletic *D. aubei* as sister to the other two species of the
300 group (*D. delarouzei* and *D. semirufus*).

301

302 **Estimation of ages of divergence and mode of diversification**

303 The origin of extant species of *Deronectes* and the separation of the Eastern and Western
304 clades was estimated to have occurred in the Middle Miocene (ca. 14 Ma, with a 10.0-
305 17.5 95% confidence interval) (Fig. 3). The origin of the well-supported species groups
306 was estimated to have occurred over a relatively short time period at the end of the
307 Miocene and beginning of the Pliocene.

308 Some extant species originated during the Pleistocene, particularly within the *D.*
309 *latus*, *D. aubei* and *D. platynotus* groups, but most species of the Iberian clade (*D.*
310 *opatrinus* group) and the *D. moestus* group were estimated to be of Pliocene or even late
311 Miocene origin. Observed intraspecific variation was also limited to a Pleistocene origin,
312 except in the case of *D. moestus* (Fig. 3).

313 The LTT plot (Fig. 4) reflecting the temporal pattern of diversification of species
314 of the western clade showed a steady increase in lineages over time. The γ -statistic was
315 negative (-0.96) but not significantly different from zero ($P= 0.33$).

316

317 **Ancestral area reconstruction**

318 Amongst models tested, the best likelihood in Lagrange was found for the geography of
319 the Pleistocene, assigning the same penalty value for dispersal through one of the pre-
320 defined land areas or a sea barrier shorter than 100 Km, and a zero probability of
321 dispersal over marine barriers longer than 100 Km or through two or more land areas
322 (Table 1). These settings were used to reconstruct ancestral areas using the 1000 post-
323 burnin trees in BEAST. The use of Pliocene or Miocene palaeogeographical scenarios,
324 either alone or in combination, resulted in significantly worse likelihoods (Table 1).
325 Within the same geographical scenario, results were less sensitive to small changes in the
326 values of the cost of dispersal, with differences of less than two units logL, but always
327 significantly better than the null model of all probabilities equal and equal to one (Table
328 1). In any case, results were very similar for all paleogeographical scenarios, cost

329 matrixes or topologies, with differences only in the relative proportion of some of the
330 reconstructed areas of the deeper nodes, including a large number of species with wide
331 geographical distributions.

332 Most of the nodes present in the consensus tree had a well supported reconstructed
333 ancestral area, with only 5 out of 34 lacking at least one area present in more than 90% of
334 reconstructions, and only 2 (10 and 11 in Fig. 3) where the most likely area was present
335 in less than 80% of the 1000 trees (Table 2). Most of the nodes were also well resolved,
336 with 22 (65%) with only two areas with a frequency higher than 90%, and only three with
337 four areas (the maximum number allowed in the settings) with a frequency higher than
338 90% (Table 2).

339 The eastern clade of *Deronectes* was unequivocally reconstructed as having an
340 origin in Turkey (region F), with an expansion to Italy and the Balkan peninsulas (areas C
341 and E) at the origin of the *D. latus* group (node 26 in Fig. 3). There was a subsequent
342 expansion to central and northern Europe and the Iberian Peninsula (areas B and G)
343 during the Pleistocene, at the origin of *D. latus* and *D. angusi* respectively (Fig. 3; Table
344 2).

345 The reconstructed origin of the western clade was more ambiguous. Although
346 centred in the southwestern Mediterranean region, only central and northern Iberia and
347 Corsica and Sardinia (areas B and D) occurred at a frequency higher than 90% in the
348 1000 trees, but the Maghreb (area H) also had a high frequency (89%, Fig. 3; Table 2).
349 Within the western clade, the *D. opatrinus* group (node 4) had a well supported central
350 and north Iberian origin (area B), with an expansion to the southeastern Iberian Peninsula
351 (area A) at the end of the Messinian (nodes 7 and 8), at the origin of the endemic species
352 *D. algibensis* Fery & Fresneda and *D. depressicollis* (Rosenhauer) (Fig. 3). This lineage
353 experienced further expansions during the Pliocene to north Africa and within the Iberian
354 Peninsula.

355 The *D. moestus* group (node 20) was reconstructed as most likely having a
356 Maghrebian or Corso-Sardinian origin (areas H and D), with subsequent expansions to
357 SE Iberia during the Messinian and the rest of the Iberian Peninsula and Mallorca during
358 the Pliocene (Fig. 3; Table 2).

359 The reconstructed origins of the *D. aubei* (node 12) and *D. platynotus* groups
360 (node 18) were more ambiguous. For the first, three areas had a 100% frequency in the
361 set of 1000 trees: central and north Iberia, central and north Europe and the Italian
362 peninsula (areas B, C and G; Table 2). The range expansion of this group apparently took

363 place between the late Miocene and middle Pleistocene. Similarly, two areas were
364 reconstructed with a frequency of more than 90% at the origin of the *D. platynotus* group:
365 central and northern Iberia and the Balkan peninsula (areas B and E) (Fig. 3; Table 2).
366 Two more expansions to the east (Balkan peninsula and Turkey) were unambiguously
367 reconstructed at the origin of *D. doriae* and *D. sahlbergi*, during the Messinian (Fig. 3;
368 Table 2).

369

370 **DISCUSSION**

371 **A Miocene basal split in *Deronectes***

372 According to our results it seems highly likely that the ancestor of extant *Deronectes* was
373 found on the northern shores of the Mediterranean during the early Miocene. Our
374 estimation of the age of the basal split between eastern and western lineages is in good
375 agreement with the increased isolation of Europe west of the Alps from the Balkans and
376 Anatolia during the middle Miocene. During this time, climate change and tectonic
377 movements associated with Carpathian uplift resulted in a succession of sea level
378 fluctuations in the central and eastern Paratethys basins (Ter Borgh *et al.*, 2014). The
379 extension of the Carpathian Foreland in a narrow deep-sea basin towards the west 20-15
380 Ma (Dercourt *et al.*, 1985; Meulenkamp & Sissingh, 2003) could have contributed to the
381 isolation of strictly freshwater species in the two areas. The basal split in *Deronectes*
382 agrees with the estimated age of similar western and eastern lineages within the
383 freshwater beetle genus *Hydrochus* (Hidalgo-Galiana & Ribera, 2011), and with many
384 other comparable splits within Mediterranean lineages, although in most cases no age
385 estimates are available (see e.g. examples in Oosterbroek & Arntzen, 1992).

386 Of the two main lineages of *Deronectes*, the eastern clade was unambiguously
387 reconstructed as having an origin in Anatolia, but the precise origin of the western clade
388 was more uncertain due to the wide geographical ranges of some species within it and the
389 lack of statistical support for the nodes connecting the main groups. During most of the
390 Miocene, the Italian peninsula was mostly submerged or partly merged with the future
391 Balkan and Anatolian peninsulas (Dercourt *et al.*, 1985; Rosenbaum *et al.*, 2002;
392 Meulenkamp & Sissingh, 2003; Popov *et al.*, 2004), something which could explain the
393 absence of ancient lineages in this area, apparently colonised by *Deronectes* only during
394 the Pleistocene.

395

396 **Tortonian disaggregation**

397 We traced the origin of the main species groups within *Deronectes* to the late Tortonian
398 and the transition to the Messinian, in most cases with relatively poor topological
399 resolution, suggesting a rapid succession of isolation events. The diversification of the
400 western clade involved successive splits between the Iberian Peninsula, north Africa and
401 Corsica and Sardinia, resulting in the main species groups recovered in our phylogeny.
402 These species groups were mostly in agreement with those obtained with previous
403 molecular (Ribera *et al.*, 2001; Ribera, 2003; Abellán & Ribera, 2011) and morphological
404 analyses (Fery & Brancucci, 1997; Fery & Hosseinie, 1998). The main differences in our
405 study were the recognition of an Iberian clade, divided into several groups of species not
406 previously thought to be closely related (Fery & Brancucci, 1997), and the composition
407 of the *D. moestus* group (Appendix S1a).

408 The Tortonian was characterised by strong tectonic activity and changes in sea
409 level in the area between southeastern Iberia and the Maghreb (Alvinerie *et al.*, 1992;
410 Martín *et al.*, 2009), favouring vicariance events that led to allopatric speciation in a
411 number of groups (e.g. Jolivet *et al.*, 2006; Hidalgo-Galiana *et al.*, 2011; Faille *et al.*,
412 2014). The development of more continental climates, with changes in precipitation
413 seasonality, have been associated with the appearance of more open vegetation in some
414 areas in southern Europe after the Middle Miocene Optimum, particularly in the
415 southwestern Mediterranean (e.g. Barrón *et al.*, 2010; Casas-Gallego *et al.*, 2015). The
416 causal relationship between climate and vegetation changes is, however, not well
417 established (Bruch *et al.*, 2011).

418 The origin of species of the western group in the Balkans, east of the Paratethys
419 basin, is more uncertain. In the Lagrange analyses they were reconstructed as having a
420 western origin, requiring subsequent range expansion towards the east. However, the
421 relative lack of support allowed an alternative scenario (as seen in the topology of Fig. 2),
422 with the eastern-most species within the western clade (*D. platynotus* group plus the
423 isolated *D. doriae* and *D. sahlbergi*) sister to the remaining western lineages, something
424 which would not require a range expansion from the west, but instead a western
425 migration of the Iberian member of the group (*D. costipennis*) in the Plio-Pleistocene.

426

427 **Messinian crossroads**

428 The onset of the Messinian salinity crisis 5.96-5.33 Ma ago and the establishment of new
429 land corridors seem to have facilitated the expansion of some species of *Deronectes*,
430 although these movements were relatively local and mostly centred in the southwest of

431 the Mediterranean basin. After the closing of the Tortonian sea corridors between
432 mainland Iberia, the Betic-Rifian area and mainland North Africa (Martín *et al.*, 2009)
433 there were expansions of the Iberian lineages towards the southeast (*D. opatrinus* group)
434 and of the *D. moestus* group towards the northwest and the Balearic islands, both likely
435 crisscrossing the Gibraltar area. Both expansions continued during the Pliocene, some
436 species of the Iberian clade towards north Africa, and species of the *D. moestus* group
437 towards southern Europe.

438 In the east, range movements associated with the Messinian likely include the
439 crossing of the Bosphorus strait by *D. doriae*, currently known only from Turkey,
440 Armenia and Iran (Fery & Brancucci, 1997; Nilsson & Hájek, 2015), and possibly *D.*
441 *sahlbergi*, known from Turkey but also from Greece (Nilsson & Hájek, 2015), meaning
442 that Asian populations may have a relatively recent origin.

443 We did not find any evidence of large scale range movements during the
444 Messinian in the south or central Mediterranean basin, or along the coast of the Sarmatic
445 Sea (the Paratethys) which could correspond to the "lago mare" dispersal routes proposed
446 by e.g. Bianco (1990). During the Messinian, changes in climate or vegetation on the
447 northern side of the Mediterranean were not very pronounced (Favre *et al.*, 2007), but it
448 is possible that the newly formed land corridors did not have the ecological conditions to
449 allow the dispersal of species restricted to fast flowing freshwater streams (Roveri *et al.*,
450 2014).

451

452 **The establishment of current distributions in the Plio-Pleistocene**

453 The best model for the ancestral reconstruction in Lagrange was that reflecting present
454 geography, suggesting that current distribution patterns within the genus are largely
455 dominated by range movements since the Messinian. This is in contrast with the results
456 obtained with other groups of very poorly dispersing species, such as *Trechus fulvus*
457 group ground beetles (Carabidae), which include many subterranean taxa and have a
458 distribution still dominated by their late Miocene biogeography (Faille *et al.*, 2014). Also
459 supporting the importance of the Plio-Pleistocene in the evolutionary history of
460 *Deronectes* is the high number of species estimated to have originated during this period,
461 reflected by the constancy of the diversification rate estimated from the LTT plot.
462 Exceptions are an island endemic (*D. lareynii* from Corsica), the Moroccan *D. theryi* and
463 the isolated eastern species *D. doriae* and *D. sahlbergi*, all apparently of late Miocene
464 origin. Most of the geographically restricted species in the Iberian clade also have a

465 relatively ancient (Pliocene) origin, most likely driven by vicariance between the main
466 mountain systems (Ribera, 2003). There are other known examples of freshwater
467 Coleoptera with similar biogeographical patterns - for example, in the Hydraenidae
468 (subgenus *Enicocerus*) and Hydrochidae (genus *Hydrochus*) Iberian endemics are mostly
469 of late Miocene origin (Ribera *et al.*, 2010; Hidalgo-Galiana & Ribera, 2011).

470 Such an ancient origin is, however, not a generalised pattern, as in other groups of
471 freshwater Coleoptera most Iberian endemics, many of them restricted to the same
472 mountain systems as endemic *Deronectes*, are apparently of Pleistocene origin. This is
473 the case for most species of the "*Haenydra*" lineage (Ribera *et al.*, 2010; Trizzino *et al.*,
474 2011), some species groups of *Limnebius* (Abellán & Ribera, 2011) (both Hydraenidae),
475 and several Iberian endemic diving beetles from different genera (Dytiscidae, Ribera,
476 2003; Ribera & Vogler, 2004). Similarly, all speciation events within some groups of
477 *Deronectes* are of Pleistocene origin (*D. aubei*, *D. platynotus* and *D. latus* groups), which
478 are also the groups including most non-monophyletic species in our analyses. Most of
479 these can be explained either by incomplete lineage sorting due to their recent divergence
480 (*D. ferrugineus-wewalkai*, *D. latus-angusi*) or the presence of previously unrecognised
481 species-level diversity (*D. moestus* complex), except for the discordance between
482 mitochondrial and nuclear data within the *D. aubei* group. Incomplete lineage sorting is
483 not expected to leave any predictable biogeographical pattern (Funk & Omland, 2003), so
484 is unlikely to be the reason for the grouping of the mitochondrial haplotypes in two
485 clusters, west and east of the Rhone river (the later including the Pleistocene expansion of
486 *D. semirufus* to peninsular Italy and Sicily). This clear geographic pattern is more
487 consistent with introgressive hybridization between closely related taxa sharing the same
488 geographical range, a pattern seen commonly in areas hypothesized to be glacial refugia
489 (e.g. Berthier *et al.*, 2006; Schmidt & Sperling, 2008 or Nicholls *et al.*, 2012). An
490 alternative possibility could be *Wolbachia* infection, known to alter patterns of mtDNA
491 variability (Jiggins, 2003). Our data do not allow further interpretations of this pattern,
492 which may require a more comprehensive taxon sampling in potential refugial areas (e.g.
493 Massif Central or Black Forest) and the sequencing of additional molecular markers.

494 Another western lineage which apparently diversified in the Pleistocene is the *D.*
495 *platynotus* group, which was reconstructed to have expanded westwards from the
496 Balkans, giving rise to the Iberian endemic *D. costipennis*. In Fery & Brancucci (1997)
497 another species (*D. hakkariensis* Wewalka) known from a single specimen from
498 southeastern Turkey was tentatively included in the *D. platynotus* group, although

499 because of its deviating morphology and geographical distribution this relationship was
500 considered doubtful. Unfortunately we could not obtain material of this rare species for
501 our analyses, but if shown to genuinely belong to the *D. platynotus* group, *D.*
502 *hakkariensis* would represent a further expansion to the east, most likely during the
503 Pliocene, constrained by the stem (Messinian) and crown (lower Pleistocene) ages of the
504 group.

505 In the eastern clade, the diversification and expansion of the *D. parvicollis* group,
506 to occupy large areas of the Middle East and central Asia, with one species (*D.*
507 *parvicollis*) expanding westward into the Balkans, most likely took place during the Plio-
508 Pleistocene. The other main lineage within the eastern clade, the *D. latus* group, also
509 expanded during the Pleistocene, but in this case towards the west, first to give rise to the
510 only Italian endemic of the genus (*D. angelinii* Fery & Brancucci) and then to reach
511 Iberia and most of Europe north to Scotland and Scandinavia, as testified by Holocene
512 remains of *D. latus* in Britain and Sweden (Abellán *et al.*, 2011).

513

514 **CONCLUDING REMARKS**

515 Our reconstruction of the evolutionary and biogeographical history of *Deronectes* shows
516 that its diversification has been shaped by geological and climatic changes around the
517 Mediterranean since the Miocene. These have produced successive rounds of
518 fragmentation, subsequent range expansion leading again to further fragmentation - the
519 overall result of which has been a steady accumulation of species. This pattern of range
520 expansions under favourable conditions followed by fragmentation when conditions
521 change has been described for other groups of lotic Coleoptera (Ribera *et al.*, 2010), and
522 may be a more general pattern contributing substantially to the overall richness of the
523 Mediterranean biodiversity hotspot.

524 Within *Deronectes*, most of these eastward and westward range expansions
525 involved overland dispersal through the north side of the Mediterranean basin, with a
526 limited influence of Messinian land corridors and the total absence of these beetles in
527 north Africa from Libya to Egypt. This could be expected given their ecological
528 requirements, but what is more surprising is the irrelevance of the Italian peninsula
529 during most of the evolutionary history of the group. Most of the Italian peninsula
530 remained submerged until the Pliocene (Rosenbaum *et al.*, 2002; Meulenkamp &
531 Sissingh, 2003; Popov *et al.*, 2004), and all species currently found in mainland Italy
532 south of the Alps are of Pleistocene origin. This absence of ancient Italian species is

533 paralleled in freshwater Coleoptera which have Iberian endemics of Miocene or Pliocene
534 origin (*Enicocerus*, *Hydrochus*, Ribera *et al.*, 2010; Hidalgo-Galiana *et al.*, 2011), but not
535 by groups with an abundance of Pleistocene species, which have also Italian endemics
536 ("*Haenydra*" and *Limnebius*; Trizzino *et al.*, 2011; Abellán & Ribera, 2011).
537 Our results clearly show that the timing of key diversification events may differ between
538 taxa even when sharing the same habitat and geographic distribution, differences that
539 have shaped the current distribution of diversity in the Mediterranean hotspot.

540

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547

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- 741

742 **SUPPORTING INFORMATION**

743 Additional Supporting Information may be found in the online version of this article:

744

745 **Appendix S1** Additional materials.

746 **(a)** Checklist of species of *Deronectes*, including geographical distribution, species group
747 according to Fery & Brancucci (1997) and clade in which the species was included
748 according to our results. Nomenclature follows Nilsson & Hájek (2015). In grey, species
749 studied in this work.

750 **(b)** Distribution maps of the species of *Deronectes*.

751 **(c)** List of the specimens included in the phylogeny, with specimen voucher, locality,
752 collector and Genbank accession numbers (in bold, new sequences). In grey,
753 Hydroporinae outgroups, with genus-groups according to Ribera *et al.* (2008).

754

755 **Appendix S2** Additional methods.

756 **(a)** Primers used for the amplification and sequencing. In brackets, length of the
757 amplified fragment.

758 **(b)** Standard PCR conditions for the amplification of the studied fragments.

759

760 **Appendix S3** Additional results.

761 **(a)** Phylogenetic tree obtained with MrBayes with the combined nuclear and
762 mitochondrial sequences and a partition by gene, including all outgroups. Numbers in
763 nodes: Bayesian posterior probabilities.

764 **(b)** Phylogenetic tree obtained with RAxML with the combined nuclear and
765 mitochondrial sequences and a partition by gene, including all outgroups. Numbers in
766 nodes: Bootstrap support values.

767 **(c)** Phylogenetic tree obtained with MrBayes using only the mitochondrial sequence data.
768 Numbers in nodes: Bayesian posterior probabilities.

769 **(d)** Phylogenetic tree obtained with MrBayes using only the nuclear sequence data (H3).
770 Numbers in nodes: Bayesian posterior probabilities.

771

772

773 **BIOSKETCH**

774 David García-Vázquez is a PhD student in the Institute of Evolutionary Biology in
775 Barcelona. This paper is part of his thesis dissertation, focussed on the origin of
776 widespread European species of lotic water beetles. This work is also part of a long-term
777 collaboration between the authors on the evolutionary history of Mediterranean
778 water beetles.

779

780 Editor: Luiz Rocha

781

782 Author contributions: D.G.-V. and I.R. conceived the work; D.T.B., L.F.V. and I.R. led
783 the specimen collection; D.G.-V., R.A. and I.R. obtained the molecular data; D.G.-V. and
784 I.R. analysed the data and led the writing; all authors contributed to the discussion of
785 results and the writing.

786

787

788

789 **TABLES**

790 **Table 1** Dispersal cost schemes used in Lagrange, and likelihood of the different
 791 Lagrange models. (a) Dispersal probabilities across sea or land barriers in the six
 792 combinations used (in bold, combination with the best likelihood score). "land barrier"
 793 refers to one of the pre-defined areas (see Fig. 3). (b) Likelihood of the six dispersal
 794 schemes in (a) for the three tested palaeogeographical scenarios. The matrix with the best
 795 likelihood score for each scenario is shown in bold, with a star for the best overall
 796 scheme. (c) Matrix of dispersal probabilities between our pre-defined geographical areas
 797 for each palaeogeographical scenario according to the costs of scheme #3 in (a) (see Fig.
 798 3 for the maps used for the reconstruction).

800 (a)

barrier	#1	#2	#3	#4	#5	#6
contiguous land areas	1	1	1	1	1	1
land barrier	0.2	0.2	0.4	0.2	0.1	0.2
sea barrier < 100 km	0.4	0.4	0.4	0.2	0.2	0.4
two land barriers	0	0.1	0	0	0	0.2
sea barrier >100 km	0	0.1	0	0	0	0
>2 land or 2 land+sea	0	0.1	0	0	0	0

801

802 (b)

palaeogeographical scenario	#1	#2	#3	#4	#5	#6
Pleistocene (present)	103.2	104.68	101.7*	103.2	103.9	103.7
Pliocene	107.5	108.4	105.1	107.5	108.4	107.8
Miocene (late Tortonian)	107.6	108.5	105.5	107.5	108.5	107.9

803

804 (c)

Pleistocene	A	B	C	D	E	F	G
SE Iberian Peninsula and Mallorca	A	-					
Centre and N Iberian Peninsula	B	1	-				
SE France, Italy and Sicily	C	0.6	1	-			
Corsica and Sardinia	D	0	0	0.6	-		
Balkans	E	0	0.6	1	0	-	
Turkey and Middle East	F	0	0	0.2	0	0.6	-
Northern and central Europe	G	0.6	1	1	0.2	1	0
Maghreb	H	0.6	0.2	0	0	0	0

Pliocene	A	B	C	D	E	F	G
SE Iberian Peninsula and Mallorca	A	-					
Centre and N Iberian Peninsula	B	1	-				
SE France, Italy and Sicily	C	0.6	1	-			
Corsica and Sardinia	D	0.6	0.6	1	-		
Balkans	E	0	0.6	1	0.6	-	
Turkey and Middle East	F	0	0	0.6	0	1	-

Northern and central Europe	G	0.6	1	1	0.6	1	0.6	
Maghreb	H	1	0.6	1	1	0.6	0	0

Miocene (Late Tortonian)	A	B	C	D	E	F	G	
SE Iberian Peninsula and Mallorca	A	-						
Centre and N Iberian Peninsula	B	1	-					
SE France, Italy and Sicily	C	0.6	1	-				
Corsica and Sardinia	D	0	0.6	1	-			
Balkans	E	0	0.6	1	0.6	-		
Turkey and Middle East	F	0	0	0.6	0	1	-	
Northern and central Europe	G	0.6	1	1	0.6	1	0.6	-
Maghreb	H	0.6	0.2	0.6	0.6	0.2	0	0.2

805

806 **Table 2** Ancestral area reconstruction in the Lagrange analyses of the 1000 post-burn-in
807 trees. For each node present in the consensus tree (see Fig. 3) we give the number of trees
808 in which the node appears, the most frequent area or combined areas and the frequency of
809 the individual areas. In bold and with stars, areas with >90% of frequency; in bold, areas
810 between 70-90 % frequency. Area codes: A, south and east of the Iberian Peninsula
811 including Mallorca; B, centre and north of the Iberian Peninsula; C, Italy (including
812 Sicily) and southeastern France; D, Corsica and Sardinia; E, Balkan peninsula; F, Turkey
813 and Middle East; G, northern and central Europe and H, Maghreb.

814

NODE	n° trees	areas	A	B	C	D	E	F	G	H
1	1000	BDFH	5	93*	-	100*	9	100*	-	93*
2	1000	ABDH	56	93*	1	100*	18	41	-	89
3	488	B	-	100*	-	-	11	6	-	-
4	1000	B	48	100*	-	-	-	-	-	-
5	530	B	-	100*	-	-	-	-	-	-
6	1000	B	-	100*	-	-	-	-	-	-
7	272	AB	85	75	-	-	-	-	-	-
8	634	B	57	85	-	-	-	-	-	-
9	805	AB	99*	100*	-	-	-	-	-	-
10	1000	B	55	64	-	-	-	-	-	-
11	636	BEF	-	71	7	-	72	43	-	-
12	1000	BCG	-	100*	100*	-	-	-	100*	-
13	1000	CG	-	-	100*	-	-	-	100*	-
14	999	CG	-	-	100*	-	-	-	81	-
15	970	B	-	100*	-	-	-	-	-	-
16	418	EF	-	34	-	-	89	77	-	-
17	849	E	-	11	-	-	100*	28	-	-
18	1000	BEG	-	100*	-	-	100*	-	72	-
19	1000	EG	-	-	-	-	100*	-	100*	-
20	1000	ABDH	62	53	4	100*	-	-	-	95*
21	1000	ABDH	91*	73	14	87	-	-	-	100*
22	1000	ABCD	98*	80	54	81	1	-	-	21
23	1000	ABCD	100*	100*	100*	100*	-	-	-	-
24	1000	A	100*	-	-	-	-	-	-	1
25	1000	F	-	-	-	-	-	100*	-	-
26	1000	CEFG	-	30	100*	-	100*	100*	70	-
27	998	CEFG	-	34	100*	-	100*	100*	66	-
28	1000	BCEG	-	89	100*	-	100*	-	98*	-
29	907	BCEG	-	100*	100*	-	100*	-	100*	-
30	1000	F	-	-	-	-	-	100*	-	-
31	1000	F	-	-	-	-	-	100*	-	-
32	1000	F	-	-	-	-	-	100*	-	-
33	1000	F	-	-	-	-	-	100*	-	-
34	1000	F	-	-	-	-	-	100*	-	-

815

816

817

818

819 **FIGURE LEGENDS**

820 **Figure 1** Distribution of the main lineages of *Deronectes* according to our phylogenetic
821 results (see Fig. 2).

822

823 **Figure 2** Phylogeny of *Deronectes*, as obtained with MrBayes with the combined nuclear
824 and mitochondrial sequence and a partition by gene. Numbers on nodes, Bayesian
825 posterior probabilities/Bootstrap support values in RAxML. Habitus photograph, *D.*
826 *fosteri* Aguilera & Ribera (from Millán *et al.*, 2015).

827

828 **Figure 3** Ultrametric time calibrated tree obtained with Beast. Coloured branches show
829 ancestral distributions as estimated from the analysis of 1000 post-burn-in trees. Above
830 nodes (in brackets) the most frequent area or combined areas reconstructed as the
831 ancestral area of the node (see Table 2). Numbers inside nodes refer to Table 2.

832

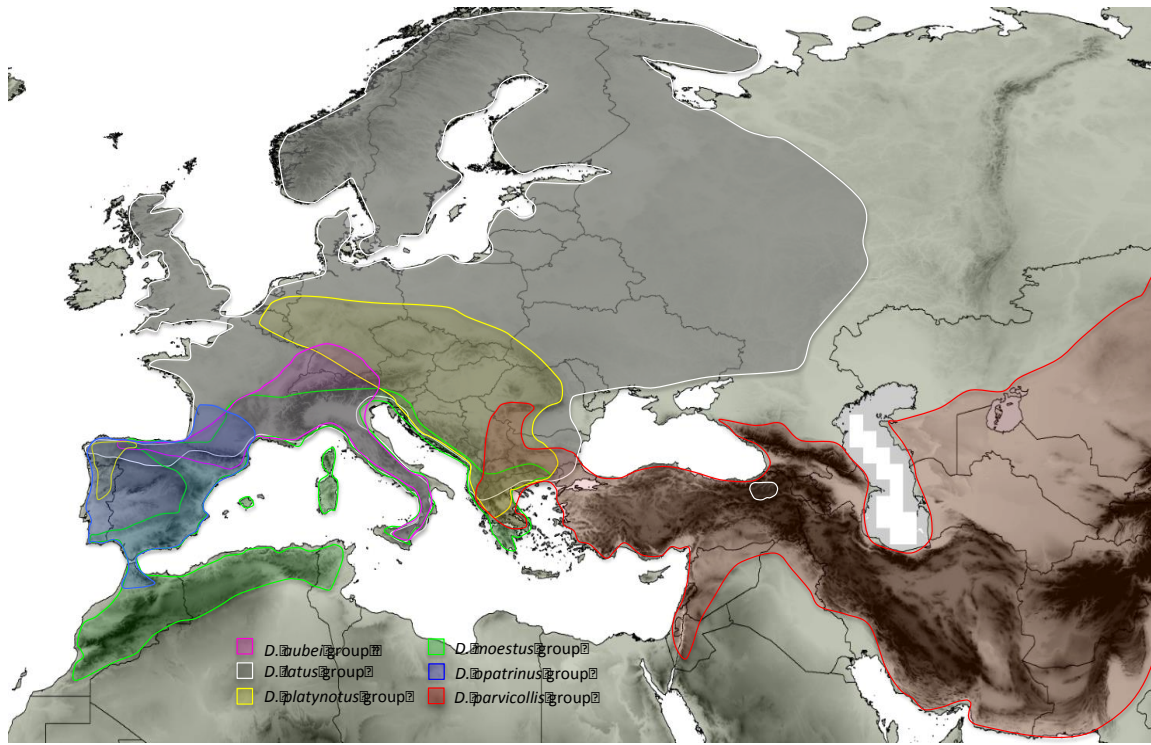
833 **Figure 4** Lineage Through Time plot (LTT) of the western clade obtained from the
834 ultrametric tree in Fig. 3.

835

836

837 Fig. 1

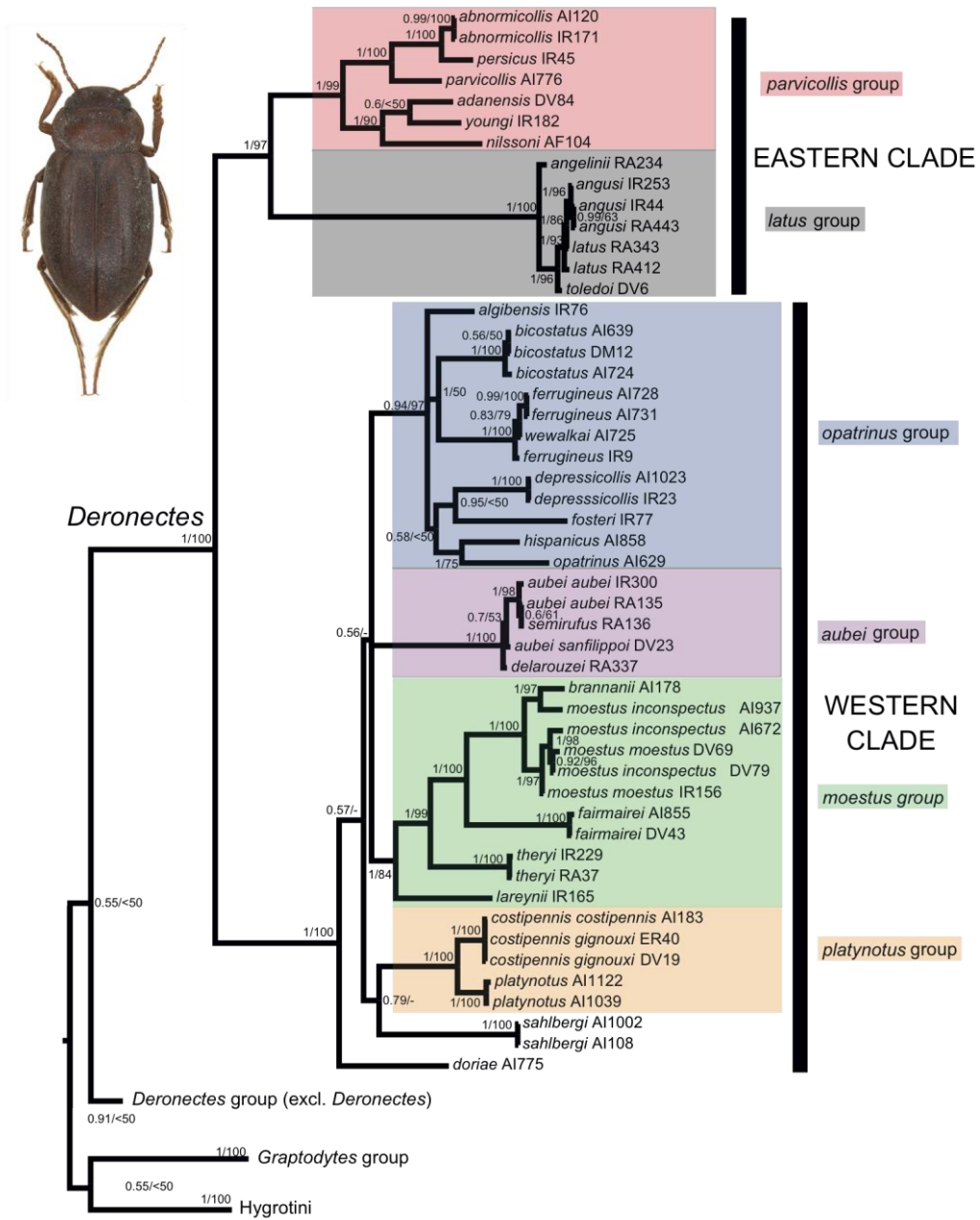
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839

840 Fig. 2

841

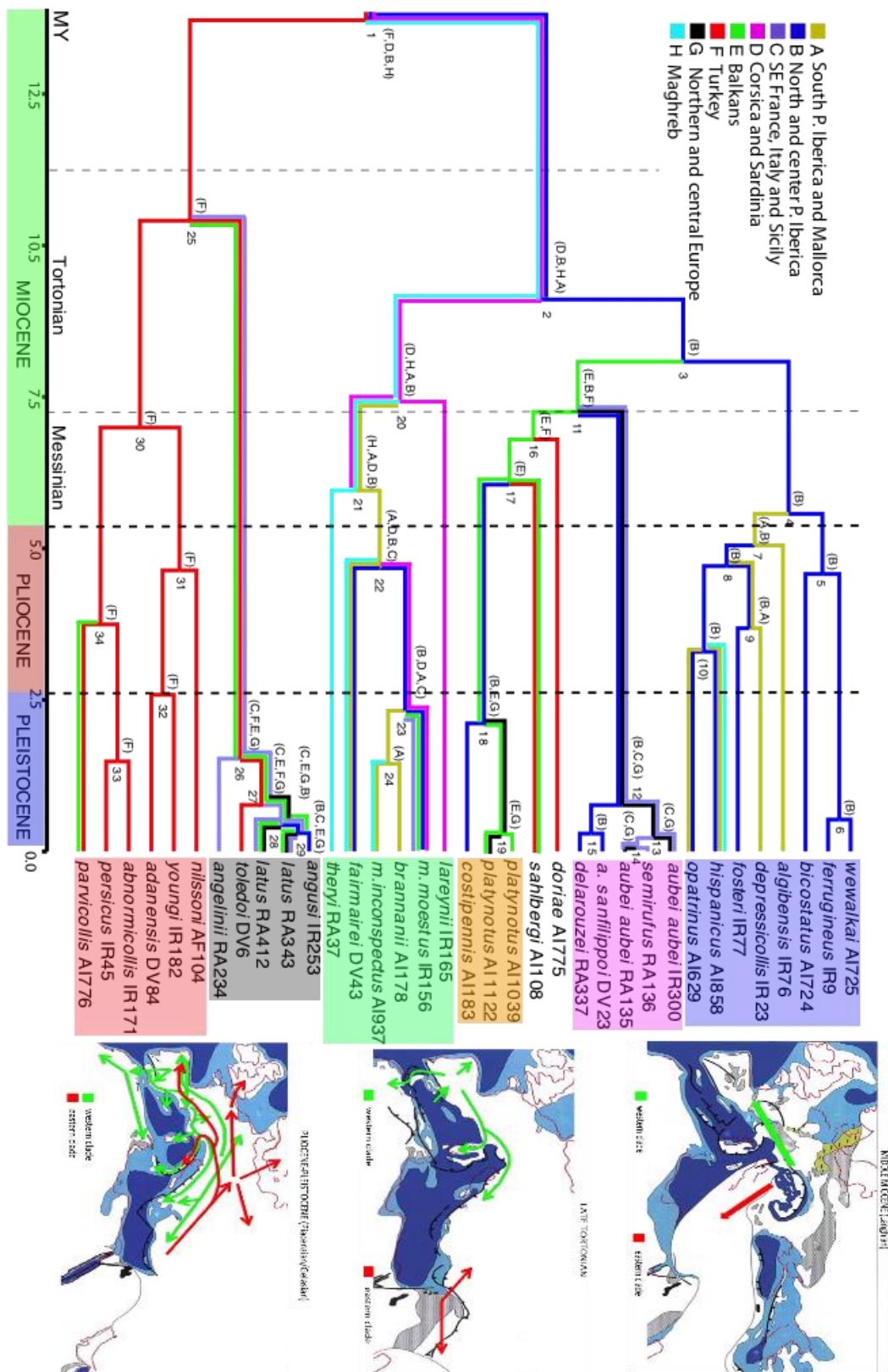


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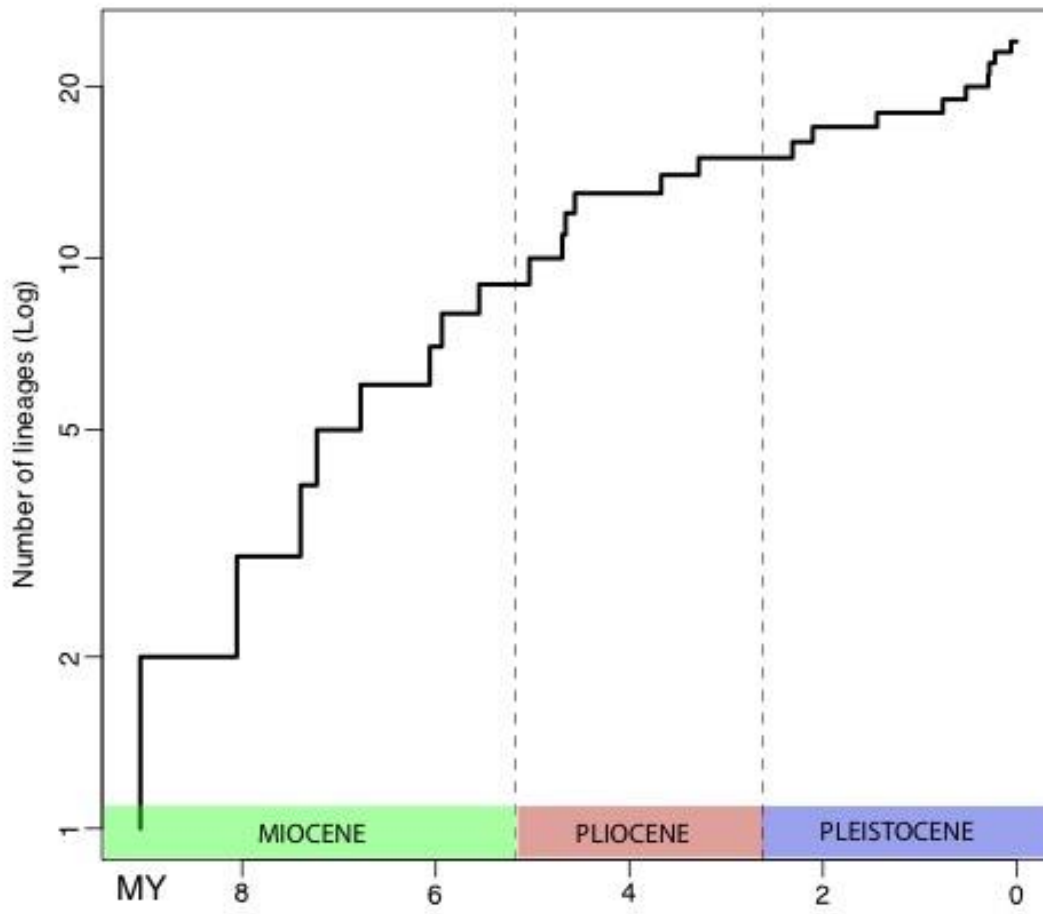
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845 Fig. 3



846
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849

850 Fig. 4



851