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Original article

Running header: Biogeography of Mediterranean *Deronectes* diving beetles

Reconstructing ancient Mediterranean crossroads in *Deronectes* diving beetles

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ABSTRACT

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

Location Mediterranean basin.

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

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

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

Keywords

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

INTRODUCTION

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

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

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

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

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

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

MATERIAL AND METHODS

Taxon sampling

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

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

DNA extraction and sequencing

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

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

Phylogenetic analyses

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

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

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

Estimation of ages of divergence

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

Diversification

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

Ancestral area reconstruction

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

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

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

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

RESULTS

Phylogenetic analyses

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Estimation of ages of divergence and mode of diversification

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

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

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

Ancestral area reconstruction

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

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

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

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

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

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

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

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

DISCUSSION

A Miocene basal split in *Deronectes*

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

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

Tortonian disaggregation

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

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

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

Messinian crossroads

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

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

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

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

The establishment of current distributions in the Plio-Pleistocene

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

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

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

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

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

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

CONCLUDING REMARKS

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

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

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

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741

SUPPORTING INFORMATION

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

Appendix S1 Additional materials.

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

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

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

Appendix S2 Additional methods.

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

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

Appendix S3 Additional results.

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

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

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

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

BIOSKETCH

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

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

TABLES

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

(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

(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

(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

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

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*	-	-

FIGURE LEGENDS

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

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

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

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

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840 Fig. 2
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850 Fig. 4

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