Faculty of Science and Engineering

School of Biological and Marine Sciences

2017-09

Pleistocene range shifts, refugia and the origin of widespread species in Western Palaearctic water beetles.

Garcia-Vazquez, D

http://hdl.handle.net/10026.1/9515

10.1016/j.ympev.2017.06.007 Molecular Phylogenetics and Evolution Elsevier BV

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1 Pleistocene range shifts, refugia and the origin of

2 widespread species in Western Palaearctic water

- 3 beetles
- 4

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14	ABSTRACT
15	Quaternary glacial cycles drove major shifts in both the extent and location of
16	the geographical ranges of many organisms. During glacial maxima, large areas of
17	central and northern Europe were inhospitable to temperate species, and these areas are
18	generally assumed to have been recolonized during interglacials by range expansions
19	from Mediterranean refugia. An alternative is that this recolonization was from non-
20	Mediterranean refugia, in central Europe or western Asia, but data on the origin of
21	widespread central and north European species remain fragmentary, especially for
22	insects. We studied three widely distributed lineages of freshwater beetles (the
23	Platambus maculatus complex, the Hydraena gracilis complex, and the genus
24	Oreodytes), all restricted to running waters and including both narrowly distributed
25	southern endemics and widespread European species, some with distributions spanning
26	the Palearctic. Our main goal was to determine the role of the Pleistocene glaciations in
27	shaping the diversification and current distribution of these lineages. We sequenced four
28	mitochondrial and two nuclear genes in populations drawn from across the ranges of
29	these taxa, and used Bayesian probabilities and Maximum Likelihood to reconstruct
30	their phylogenetic relationships, age and geographical origin. Our results suggest that all
31	extant species in these groups are of Pleistocene origin. In the H. gracilis complex, the
32	widespread European H. gracilis has experienced a rapid, recent range expansion from
33	northern Anatolia, to occupy almost the whole of Europe. However, in the other two

34	groups widespread central and northern European taxa appear to originate from central						
35	Asia, rather than the Mediterranean. These widespread species of eastern origin						
36	typically have peripherally isolated forms in the southern Mediterranean peninsulas,						
37	which may be remnants of earlier expansion-diversification cycles or result from						
38	incipient isolation of populations during the most recent Holocene expansion. The						
39	accumulation of narrow endemics of such lineages in the Mediterranean may result						
40	from successive cycles of range expansion, with subsequent speciation (and local						
41	extinction in glaciated areas) through multiple Pleistocene climatic cycles.						
42							
43	Keywords: glacial refugia, Dytiscidae, Hydraenidae, Quaternary glaciations, range						
44	expansion, Mediterranean Peninsulas, Central Asia						
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49 **1. Introduction**

50 The Quaternary was a period of drastic cyclical climatic changes, with multiple 51 glacial-interglacial periods, ultimately driven by variations in the earth's orbit known as 52 Milankovitch cycles. Milankovitch–driven climate oscillations led to large changes in 53 the size and location of the geographic distribution of many species, in some cases 54 resulting in speciation due to the higher probability of isolation of small populations in 55 areas under new selection regimes (Dynesius & Jansson, 2000). These Pleistocene 56 climatic oscillations and the subsequent shifts in ecological conditions, with the 57 repeated fragmentation of populations during glacials and interglacials, have long been 58 hypothesised to have driven the origin of most extant Holarctic species (e.g. Rand, 59 1948; Mayr, 1970).

60 Pleistocene climatic changes were especially drastic in northern latitudes of the 61 Palearctic region, since during the Last Glacial Maximum the European ice sheet 62 covered most areas north of 52°N, with permafrost north of 47°N (Dawson, 1992). 63 Large areas of central and northern Europe therefore became inhospitable to temperate 64 taxa during glacials; in stark contrast to the Mediterranean peninsulas, which retained 65 more temperate climate and vegetation (e.g. Huntley, 1988; Bennett et al., 1991). 66 However, despite the fact that most of central and northern Europe and regions of Asia 67 at similar latitudes were exposed to extremely cold conditions (Dawson, 1992), there 68 were areas on the slopes of mountain ranges and along river valleys where moister 69 conditions prevailed (Soffer, 1990), allowing the local survival of temperate biota in 70 these northern/eastern refugia (e.g. Stewart et al., 2009; Schmitt & Varga, 2012).

71 Two main scenarios could account for the origin of the current central and 72 northern European fauna. Firstly, there is the traditional model of postglacial range 73 expansion from Mediterranean refugia (e.g. Hewitt, 2000), in which central and 74 northern Europe were colonized by northward range expansions mainly from the 75 Iberian, Italian, Balkan and Anatolian peninsulas at the end of the last glaciation. 76 According to this model, populations of most European species were confined to 77 refugial areas in southern Mediterranean peninsulas during glacial maxima, from which 78 they would have re-colonized the continent during interglacials (although Hewitt (2000) 79 also recognised the important role of the Carpathians as providing potential refugia). 80 Whilst such a scenario is well established for some taxa, it is not ubiquitous. A second 81 possibility is that the colonization of central and northern Europe at the end of the Last 82 Glacial was from non-Mediterranean source areas in eastern Europe and Asia (Bilton et

al., 1998). According to this view, the isolation of the Mediterranean peninsulas during
glacial cycles led to speciation, preventing gene flow with the new colonisers of central
and northern Europe during subsequent interglacials. For taxa conforming to this model,
southern peninsulas are centres of endemism rather than being a source of colonists
(Bilton et al., 1998; Schmitt & Varga, 2012).

88 Such biogeographical isolation of Mediterranean peninsular populations has 89 been suggested previously for small mammals (Bilton et al., 1998) and some insects (e.g. Cooper et al., 1995). Amongst aquatic Coleoptera, the absence of fossil remains of 90 91 southern species in the abundant central and northern European Quaternary subfossil 92 record (Abellan et al., 2011) supports a view of Mediterranean peninsulas as areas of 93 endemism, rather than significant sources of postglacial colonists. Data from extant 94 species also suggest that current southern endemics have not contributed to the diversity 95 of northern areas (e.g. Hydrochus (Hydrochidae), Hidalgo-Galiana & Ribera, 2011; or 96 *Enicocerus* (Hydraenidae), Ribera et al., 2010). Some central and northern European 97 species may have had their origin in Mediterranean peninsulas, but in such cases it 98 appears that the taxa concerned were those whose refugia were located in the 99 northernmost areas of the peninsulas, on the margins of deglaciated areas (e.g. Ribera et 100 al., 2010 for *Enicocerus*, and García-Vázquez & Ribera, 2016 for *Deronectes*), 101 successful expansion possibly being aided by physiological adaptations in such species 102 (Calosi et al., 2010; Cioffi et al., 2016).

103 Despite increased understanding of the evolution of the European insect fauna in 104 recent decades, data on the origin of widespread central and northern European species, 105 which should have necessarily experienced recent expansions of their geographical 106 ranges, remain severely limited. Here we study a suite of such species, using molecular 107 phylogeographic data to clarify their temporal and geographic origin and to better 108 understand the role of the Pleistocene glacial cycles in driving their diversification. We 109 examined species groups from three genera of freshwater beetles, in two different 110 families, whose representatives colonised water independently: 1) the Hydraena gracilis 111 complex ("Haenydra" lineage, family Hydraenidae); 2) the Platambus maculatus 112 complex (family Dytiscidae) and (3) Oreodytes sanmarkii (C.R. Sahlberg) and O. 113 davisii (Curtis) (family Dytiscidae). All taxa concerned are typical of running waters, 114 and include both widespread European and narrowly distributed southern endemic 115 species (Trizzino et al., 2013; Nilsson & Hájek, 2017a,b). They do, however, differ in

functional traits and evolutionary histories (see below), facts which contribute to thegenerality of our conclusions.

Using a combination of mitochondrial and nuclear data we reconstruct the phylogenetic relationships, age and geographical origin of the western Palaearctic species of these three widely distributed lineages, to better understand the effects of Quaternary glacial cycles on their diversification and current distributions.

122

123 **2. Material and Methods**

124 2.1. Taxonomic background and taxon sampling

125 a) *Hydraena gracilis* complex

126 The genus Hydraena, currently with ca. 900 species distributed worldwide 127 (Trizzino et al., 2013) is the largest genus within the family Hydraenidae and probably 128 the most diverse amongst the aquatic Coleoptera (Jäch & Balke, 2008). Within 129 Hydraena, the "Haenydra" lineage includes ca. 90 species with a north Mediterranean 130 distribution (Trizzino et al., 2013). They are usually found in clean, fast flowing waters, 131 often in mountain streams, from the Iberian Peninsula to Iran and the Urals, but are 132 absent from North Africa (Ribera et al., 2011; Trizzino et al., 2011; Trizzino et al., 133 2013; Jäch, 2015). Many species of this lineage have very restricted distributions, often 134 limited to a single valley or mountain system, but there are also a few species with very 135 wide geographical ranges.

In this work we focus on the most widespread species of "*Haenydra*", *Hydraena gracilis* Germar and its closest relatives in the *H. gracilis* complex *sensu* Jäch (1995),
which includes seven recognised species and one subspecies (Trizzino et al., 2013). *Hydraena gracilis* is widely distributed across almost the whole of Europe, ranging

Hydraena gracilis is widely distributed across almost the whole of Europe, ranging
 from southern France eastwards to Ukraine and northwards to Finland, including the

140 from southern France eastwards to Ukraine and northwards to Finland, including the

141 British Isles (Fig. 1). Previous molecular studies, albeit on a limited number of

specimens (Ribera et al., 2011), suggested that despite its widespread distribution,

143 genetic differences across its geographic range were minimal. Jäch (1995), however,

144 found morphological differences between specimens from the Balkans and the rest of

145 Europe, supporting the recognition of the subspecies *H. gracilis balcanica*

146 d'Orchymont. Hydraena gracilis is absent from the Iberian and Anatolian peninsulas,

- 147 where it is replaced by different species of the complex (Fig. 1). *Hydraena*
- 148 gracilidelphis Trizzino, Valladares, Garrido & Audisio is the westernmost species of
- this group, endemic to the Iberian Peninsula (mainly in the north but with some records

150 in the southwest) and the French Pyrenees (Trizzino et al., 2012). The Anatolian

- 151 Peninsula and adjacent areas are occupied by three species: *H. anatolica* Janssens
- 152 distributed in northern and eastern Anatolia and parts of the Caucasus and northwestern
- 153 Iran; H. graciloides Jäch in northern Turkey; and H. crepidoptera Jäch known only
- 154 from two northern Turkish provinces (Kastamonu and Sinop). The other two species of
- 155 the complex, *H. nike* Jäch and *H. elisabethae* Jäch, are endemic to two Aegean islands;
- 156 Samothraky and Thassos respectively (Trizzino et al., 2013).

We studied a total of 48 specimens from five of the seven species of the *H*. *gracilis* complex (we could not obtain fresh specimens of the two Aegean Island endemics) from 37 different localities, covering the full geographical range of the studied species (Fig. 1; Table S1). As outgroups we used three closely related species of the wider *H. gracilis* lineage within "*Haenydra*" (Trizzino et al., 2011; Table S1).

162

163 b) *Platambus maculatus* complex

The genus *Platambus* contains 66 recognised species (Nilsson & Hájek, 2017a)
and has a wide distribution, being present in the Palearctic, Nearctic, Neotropical and
Oriental regions, and is currently divided into eight species-groups (Nilsson & Hájek,
2017a). Amongst these the *P. maculatus* group - as defined by Nilsson (2001) - is the
largest, with 24 species distributed across Asia and Europe. In a molecular phylogeny of
Agabinae Ribera et al. (2004) recovered a paraphyletic *Platambus*, with the *P. maculatus* group separated from other Asian and American species.

171 Here we focus on the most widespread species of the group; P. maculatus 172 (Linnaeus) and its closest relative, P. lunulatus (Fischer von Waldheim), which we refer 173 to as the Platambus maculatus complex. Platambus maculatus has a wide Palearctic 174 distribution, from western Iberia to northern Iran and Mongolia (including Italy, the 175 Balkans and Anatolia), Scandinavia and the British Isles (Nilsson & Hájek, 2017b; Fig. 176 2). The species has a very variable elytral pattern, which led to the description of many 177 forms all of which are currently considered synonyms of *P. maculatus* (Nilsson & 178 Hájek, 2017a,b). Most conspicuous amongst these is P. maculatus "graellsi" 179 (Gemminger & Harold) from the northwest and centre of the Iberian Peninsula (Millán 180 et al., 2014). The other species of the complex, P. lunulatus, is distributed from the 181 Anatolian Peninsula and parts of the Caucasus and Middle East to Egypt (Karaman et 182 al., 2008; Nilsson & Hájek, 2017b) (Fig. 2).

We sequenced 106 specimens from 67 different localities of the two recognised
species of the *P. maculatus* complex, including the "*graellsi*" form (Fig. 2; Table S1).
As outgroups we used seven specimens from different species of the *Platambus*

186 maculatus group, as defined in Nilsson & Hájek (2017b).

187

188 c) European species of the genus *Oreodytes*

189 The Holarctic genus Oreodytes Seidlitz contains 30 recognised species, four of 190 them split into two subspecies (Fery, 2015; Nilsson & Hájek, 2017a). Oreodytes species 191 live in cold streams or lakes margins, generally at high altitude or latitude (Balfour-192 Browne, 1940; Zack, 1992; Nilsson & Holmen, 1995). The genus is distributed in the 193 Palearctic and Nearctic regions, with six species occurring in Europe. In a previous 194 study, Ribera (2003) recovered a paraphyletic *Oreodytes*, although with low bootstrap 195 support. Oreodytes was divided into two lineages corresponding to the main distinction 196 in body size and shape, i.e. larger and more elongate species (including *O. davisii*) 197 versus smaller and rounder ones (including O. sanmarkii).

198 Of the six European species of Oreodytes, the most widespread is O. sanmarkii, 199 distributed over large parts of the Palearctic from the Iberian Peninsula to the Russian 200 Far East, and reaching the Nearctic in northern Canada (Larson et al., 2000; Nilson & 201 Hájek, 2017a,b) (Fig. 3). In southern Europe, the species is known from the Iberian 202 Peninsula, northern provinces of Italy and the Balkans south to Bulgaria and Macedonia 203 (Fery, 2015; Nilson & Hájek, 2017b). The species shows a high level of variability in 204 colouration over its large distributional range (e.g. Larson, 1990) but only one 205 subspecies, O. sanmarkii alienus (Sharp), endemic to the Iberian Peninsula, is currently 206 recognised (Balke, 1989; Nilson & Hájek, 2017a,b). Also with a wide Palaearctic 207 distribution is O. davisii, known from the British Isles to Ukraine and the Caucasus, 208 including Scandinavia, the Mediterranean peninsulas and Turkey (Nilsson & Hájek, 209 2017b) (Fig. 3). In the case of this species an Iberian form has also been recognised as a 210 subspecies on morphological grounds, O. davisii rhianae Carr (Carr, 2001; Nilsson & 211 Hájek, 2017a,b). The other European species of Oreodytes are O. septentrionalis 212 (Gyllenhal), distributed from the Iberian Peninsula to eastern Siberia and Mongolia 213 (Nilsson & Hájek, 2017b); O. alpinus (Paykull), with a northern Palearctic distribution, 214 being present from lochs in northern Scotland (Foster, 1992) and Scandinavia to 215 Kamchatka in the Russian far East (Nilsson & Kholin, 1994); O. meridionalis Binaghi

- 216 & Sanfilippo, endemic to the southern Apennines (Rocchi 2007; Nilsson & Hájek,
- 217 2017b); and the recently described *O. angelinii* Fery from Greece (Fery, 2015).

We studied 58 specimens from 35 different localities of all the European species of *Oreodytes* with the exception of the Italian endemic *O. meridionalis* and the newly described *O. angelinii*, with a focus on *O. davisii* and *O. sanmarkii* and the two Iberian subspecies (*O. d. rhianae* and *O. s. alienus* respectively). We also included in the analysis 16 specimens of different Asian and American species of the genus as outgroups (Fig. 3; Table S1).

224

225 2.2. DNA extraction and sequencing

226 Specimens were collected and preserved in absolute ethanol directly in the field. 227 We extracted the DNA non-destructively with commercial kits (mostly "DNeasy Tissue 228 Kit", Qiagen GmbH, Hilden, Germany and "Charge Switch gDNA Tissue Mini Kit", 229 Invitrogen, Carlsbad, CA, USA) following the manufacturers' instructions. Specimens 230 and DNA extractions are deposited in the collections of the Institut de Biología 231 Evolutiva, Barcelona (IBE), Museo Nacional de Ciencias Naturales, Madrid (MNCN) 232 and Natural History Museum, London (NHM). We obtained seven gene fragments from 233 six different genes (four mitochondrial and two nuclear) in five different amplification 234 reactions (see Table S2 for primers and typical sequencing reactions): (1) 5'end of the 235 Cytochrome Oxidase Subunit 1 gene (the barcode fragment; Hebert et al., 2003, COI-236 5'); (2) 3'end of Cytochrome Oxidase Subunit 1 (COI-3'); (3) 5'end of 16S rRNA plus 237 tRNA transfer of Leucine plus 3'end of NADH subunit 1 (nad1) (16S and 238 nad1respectively); and internal fragments of the nuclear genes (4) Histone 3 (H3) and 239 (5) Wingless (Wg). Due to their lower variability, the nuclear markers were only 240 sequenced from representative specimens according to geographical and topological 241 criteria. For each amplification reaction we obtained both forward and reverse 242 sequences. In some specimens, due to difficulties in amplification, we used internal 243 primers for the COI-3' sequence, obtaining two fragments of 400 bp each (Table S2). 244 PCR products were purified by standard ethanol precipitation and sent to external 245 facilities for sequencing. DNA sequences were assembled and edited using the 246 Geneious 6 software (Biomatters Ltd, Auckland, New Zealand). Ambiguous calls in the nuclear genes were coded as "N"s. New sequences (561) have been deposited in 247 248 GenBank with accession numbers LT855666-LT856230 (Table S2). 249

250 2.3. Phylogenetic and divergence time analyses

Edited sequences were aligned with MAFFT v.6 using the G-INS algorithm and default values for other parameters (Katoh & Toh, 2008). We included sequences obtained from the literature (GenBank and BOLD databases) in some analyses to increase geographical coverage and possible genetic variation not covered by our sampling.

256 In the phylogenetic analyses we employed six partitions, corresponding to the 257 gene fragments COI-5', COI-3', 16S, nad1, H3 and Wg, and used Partition Finder 1.1.1 258 (Lanfear et al., 2012) to estimate the best-fitting models of nucleotide substitution for 259 each partition separately, using AIC (Akaike Information Criterion). We considered the 260 two fragments of the COI gene separately due to the uneven taxonomic coverage (Table 261 S1). To infer the phylogeny of the three groups, and estimate divergence dates amongst 262 species, we used Bayesian methods implemented in Beast 1.8 (Drummond et al., 2012). 263 For the analyses we included both mitochondrial and nuclear markers and implemented 264 the closest available evolutionary model to those selected by Partition Finder. We used 265 a Yule speciation model and ran two analyses to determine which clock model (strict or 266 lognormal relaxed) best fitted the data. As there are no fossils or unambiguous 267 biogeographic events that could be used to calibrate the phylogeny of the studied 268 groups, we applied published estimations of a-priori rates for the same genes in related 269 groups of beetles. For the *Platambus maculatus* complex and the genus *Oreodytes* (both 270 Dytiscidae) we used a rate of 0.013 substitutions/site/MY (SD 0.002) for protein coding 271 genes and 0.0016 substitutions/site/MY (SD 0.0002) for 16S, obtained for the related 272 family Carabidae for the same combination of mitochondrial protein coding and 273 ribosomal genes (Andújar et al., 2012). For the Hydraena gracilis complex we used a 274 rate of 0.015 and 0.006 substitutions/site/MY (SDs 0.002 and 0.0002) for the COI-275 3'+nad1 and 16S partitions respectively, obtained for the related Leiodidae (Cieslak et 276 al., 2014). Clock rates of H3 and Wg were left with uniform priors due to the absence of 277 any suitable estimations of the evolutionary rate for these nuclear genes. We executed 278 two independent analyses with the same settings, running 100 million generations 279 (saving trees every 5,000) or until analyses converged and the number of trees was 280 sufficient according to Effective Sample Size (ESS) values, as measured with Tracer 281 v1.6 (Rambaut et al., 2014). The maximum clade credibility tree of the two runs was 282 compiled with Tree Anotator v1.8 (Drummond et al., 2012) and visualized with FigTree 283 v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). For selection of the best molecular

clock we used the modified Akaike Information Criterion (AICM) with the moments
estimator (Baele et al., 2012), as implemented in Tracer v1.6, with 1,000 bootstrap
replicates.

To test for potential topological discordances between mitochondrial and nuclear data we analysed the nuclear genes only, applying the best clock and the same settings as in the combined (mitochondrial and nuclear) analysis.

We also analysed the combined matrix (mitochondrial and nuclear) using
maximum likelihood (ML) methods. ML analyses were performed in RAxML v.7.4.2
(Stamatakis et al., 2008) as implemented in RAxML GUI v 1.3.1 (Silvestro &
Michalak, 2012). We selected the best tree out of 100 searches using the GTR+G as an
evolutionary model with the same six partitions as in the Bayesian analysis. Node
support was estimated with 1,000 bootstrap replicates.

296

297 2.4. Demographic analyses

298 To study population history for each of the three groups separately we estimated the population coalescent model that best fitted the data. We used the mitochondrial 299 300 sequences only and no outgroups. In the analysis for the *H. gracilis* complex we only 301 included specimens of *H. gracilis* and *H. anatolica*, whilst only specimens of *P.* 302 maculatus (including the "graellsi" form) and O. sanmarkii were included in the 303 analyses for *Platambus* and *Oreodytes* respectively. The datasets were divided into four 304 partitions, corresponding to each mitochondrial gene (COI-5', COI-3', 16S and nad1) 305 and the same settings used as in the topological analyses, including evolutionary models 306 selected by Partition Finder and the best molecular clock for each group. To identify the 307 best demographic model we ran four analyses including Constant Size, Exponential 308 Growth, Logistic Growth and Expansion Growth coalescence models. For model 309 selection of the best coalescent analyses we used the modified Akaike Information 310 Criterion (AICM) with the moments estimator as implemented in Tracer v1.6, with 311 1,000 bootstrap replicates. We also computed Bayesian skyline plots (Drummond et al., 312 2005) for each group, to reconstruct variation in effective population sizes through time. 313

314 3. Results

There were no length differences in protein coding genes, and the length of ribosomal genes in the ingroup ranged between 784-786 bp in the *H. gracilis* complex and 796-797 bp in the *P. maculatus* complex and the genus *Oreodytes*. The best 318 evolutionary models, as selected by Partition Finder for each individual partition (Table

S3), were implemented in the Bayesian analysis for the *H. gracilis* complex and the

320 species of the genus *Oreodytes*. In the *P. maculatus* complex, when the more complex

321 models were applied the analyses did not converge adequately for both the relaxed

322 lognormal and strict clocks, and in consequence we applied an HKY+I+G model to

ach partition.

324

325 *3.1. Hydraena gracilis complex*

The nucleotide alignment matrix showed no variability in the two nuclear genes, which were therefore excluded from subsequent analyses. The analysis using a lognormal relaxed clock did not converge adequately so we applied a strict molecular clock model.

330 The ultrametric tree obtained with the combined mitochondrial and nuclear data 331 estimated a recent origin and diversification of the *H. gracilis* complex at ca. 0.3 Ma 332 (95% confidence interval (c.i.) 0.4-0.2 Ma) (Fig. 4), with strongly supported 333 monophyly. The complex was divided into two major clades, one included the Iberian 334 H. gracilidelphis plus all specimens from the easternmost populations identified as H. 335 anatolica (eastern Anatolia, the Caucasus and Iran), and the other was formed by two 336 groups, one containing specimens of *H. anatolica* and *H. crepidoptera* from northern 337 Turkey and the other with the widespread European *H. gracilis*, including the 338 subspecies H. gracilis balcanica and two Turkish specimens of H. anatolica and H 339 graciloides. In the latter clade, specimens from the most southeasterly populations 340 occupied basal positions, with the more western and northern populations nested within 341 them (Fig. 4). The lineage with the eastern specimens of *H. anatolica* had a poorly 342 supported position between the two remaining main clades of the complex, which 343 differed between analyses. Bayesian analysis (Fig. 4) recovered the eastern lineage as 344 sister to the Iberian H. gracilidelphis, whilst ML analysis (Fig. S1) linked the eastern 345 lineage to the other clade, as sister to *H. gracilis* and the Turkish westernmost 346 populations, but always with low support.

In the coalescence analyses a constant size model was preferred over
exponential growth (Table 1) (logistic and expansion models did not converge
adequately). The Bayesian Skyline Plot showed a continuous, slight increase in
population size, with a marked increase at ca. 50,000 years BP and a slight decrease
towards the present (Fig. 5A).

352

353 *3.2. Platambus maculatus complex*

354 The relaxed lognormal was significantly better than the strict molecular clock, 355 and was therefore implemented in the Bayesian analyses (Table 1). The temporal origin 356 of the *P. maculatus* complex was estimated to be in the Middle Miocene (ca. 12 Ma) 357 (Fig. 6), although the split between the two extant species (*P. maculatus* and *P.* 358 lunulatus) occurred in the Messinian (ca. 6.5 Ma, c.i. 9.3-4.1 Ma). Extant intraspecific variability dates from the Pliocene-Pleistocene boundary (2.5-3.5 Ma). The ultrametric 359 360 tree obtained from Bayesian analysis using the combined mitochondrial and nuclear 361 matrix strongly supported the monophyly of the two recognised species (Fig. 6).

362 Platambus maculatus was divided into three clades, one with specimens with a 363 predominantly western distribution (northern Spain, France and the British Isles, 364 including specimens from some Scottish lochs, these forming a monophyletic lineage); 365 a second clade with specimens from easterly populations (central Europe, Scandinavia, 366 the Balkans, Anatolia and the Middle East); and a third clade including specimens of the 367 Iberian "graellsi" form plus P. maculatus from northern Italy and the two sampled 368 specimens from one of the localities in the Pyrenees (PIR11, Fig. 6; Table S1). The 369 relationships between these three lineages were not well supported, and varied between 370 analyses. In the Bayesian analysis using only nuclear markers (Fig. S2), all specimens 371 of P. maculatus "graellsi" plus the northern Italian and PIR11 P. maculatus were 372 included within the western clade. In the ML analysis, on the contrary (Fig. S3), we 373 recovered the northern Italian specimens of *P. maculatus* as sister to all other 374 specimens, which were split into three poorly supported groups (western clade, eastern 375 clade and the "graellsi" form plus the PIR11 P.maculatus referred to above).

None of the four coalescence models converged adequately, but the Bayesian
Skyline Plot showed a nearly constant effective population size until ca. 15,000 years
BP, with a recent increase (Fig. 5B).

379

380 *3.3. European Oreodytes species*

A relaxed lognormal clock was preferred over a strict molecular clock, and was implemented in the Bayesian analyses (Table 1). Preliminary results showed the existence of two group of species, one closely related to *O. sanmarkii* (the *O. sanmarkii* group) and the other to *O. davisii* (the *O. davisii* group), the later including the European *O. septentrionalis* and *O. alpinus*. These two clades were constrained as monophyletic in subsequent Bayesian analyses without outgroups. *Oreodytes alpinus*was nested within *O. davisii* in the analysis using the combined mitochondrial and
nuclear matrix (Fig. 7), forming a clade with the eastern Palaearctic *O. mongolicus*(Brinck). In the analysis using only nuclear data, *O. alpinus* and *O. mongolicus* were

also sisters, but both sister to *O. davisii* (Fig. S4)

391 Divergence time analysis (Fig. 7) dated the separation between the O. davisii 392 and O. sanmarkii groups to the Oligocene (ca. 27 Ma, c.i. 36-20 Ma) and diversification 393 within them to the Lower Miocene (ca. 22 Ma, c.i. 30-15 Ma) for the O. sanmarkii 394 group and the Middle Miocene for the O. davisii group (ca. 13 Ma, c.i. 18-10 Ma) (Fig. 395 7). The sister species of the clades O. davisii+O. alpinus+O. mongolicus and O. 396 sanmarkii were the North American O. snoqualmie (Hatch) and O. obesus (LeConte), 397 respectively. Intraspecific variation in both O. davisii and O. sanmarkii was of 398 Pleistocene origin (1.5-2.0 Ma) (Fig. 7).

399 We found no clear phylogeographical signal in O. davisii, irrespective of the 400 method of analysis (Bayesian or ML). In contrast, O. sanmarkii was divided into two 401 clades with a clear geographical pattern, one including specimens with an eastern 402 distribution, from Mongolia to central Europe, and the other including mainly western 403 specimens, from the Iberian Peninsula, Italy and the British Isles, although also 404 including some individuals from the Carpathians. Within the western clade, Iberian 405 populations from the Pyrenees were separated from those from Portugal and central and 406 northwestern Spain, the latter identified as the subspecies O. sanmarkii alienus. This 407 difference was more pronounced in the ML analysis (Fig. S5; although with poor 408 support) and in the analysis using only nuclear data (Fig. S4), which recovered O. s. 409 alienus as sister to remaining O. sanmarkii.

In the case of other European species of the group, we found a deep divergence
between Mongolian and European specimens of *O. septentrionalis*, estimated to have
occurred during the late Miocene (ca. 5.6 Ma, c.i. 8.3-3.5 Ma; Fig. 7).

The expansion growth coalescence model performed better than logistic or exponential ones (Table 1); the constant size model failing to converge adequately. The Bayesian Skyline Plot showed that effective population size remained constant until relatively recently, with a sharp increase ca. 10,000 years BP (Fig. 5C).

417

418 **4. Discussion**

- 419 Our results emphasise the fact that patterns of evolutionary diversification,
- 420 biogeographical history and range expansion can differ significantly, even when
- 421 comparing taxa occupying the same broad habitat type in the same region. The three
- 422 groups of water beetles examined here have all diversified during the Plio-Pleistocene,
- 423 and been subject to the same historical climatic shifts, but despite this their colonization
- 424 history and demography differ considerably.
- 425

426 4.1. Hydraena gracilis complex

427 According to our results the H. gracilis complex has a recent, Pleistocene origin, 428 with the widespread H. gracilis showing extreme genetic homogeneity throughout its 429 range. This suggests a very recent expansion, in agreement with preliminary results for 430 this and other species of the "Haenydra" lineage (Ribera et al., 2011). The existence of 431 narrow endemics around the periphery of the range of the complex, both in the far west 432 (Iberian Peninsula) and east (Anatolia, Azerbaijan and Iran) is consistent with 433 successive cycles of range expansion within the complex, followed by local extinctions, 434 most likely during the glacial periods. During such times, remaining populations would 435 have been isolated in refuges, resulting in the divergence of *H. gracilidelphis* in the 436 Iberian Peninsula and H. anatolica, H. graciloides and H. crepidoptera in Turkey and 437 the Middle East - a scenario consistent with the model for diversification of this group 438 proposed by Ribera et al. (2011).

439 On current data it was not possible fully to resolve the relationships of eastern 440 Anatolian and Iranian populations of *H. anatolica*, which may be the remnants of earlier 441 diversification cycles (as is the case for *H. gracilidelphis* in the west) or represent early 442 isolates of the most recent range expansion. The separation between western and eastern 443 populations in *H. anatolica* suggests that the Anatolian Diagonal, a mountain range 444 running from north-eastern to south-western Anatolia, acts as an effective barrier to 445 gene flow between western and eastern regions, a pattern observed in many Turkish 446 taxa (Gündüz et al., 2007). The basal position of northern and central Turkish 447 populations of the *H. gracilis* group suggests a second range expansion from these 448 areas, crossing the straits to the Balkans and expanding to western and northern Europe, 449 resulting in the current widespread European H. gracilis. This pattern of expansion has 450 been observed in other taxa, including insects (e.g. grasshoppers, Korkmaz et al., 2014) 451 suggesting that Anatolia was an important glacial refugium, which contributed to the 452 recolonization of Europe in some lineages (Ansell et al., 2011; Hewitt, 1996; Rokas et

al., 2003). Such expansion from Anatolia to the Balkans may have been possible
through a substantial decrease in sea level during glacial periods (Aksu et al., 1999;
Ergin et al., 2007), which resulted in a large part of the Sea of Marmara becoming dry
land through which terrestrial and freshwater taxa may have dispersed.

457

458 4.2. Platambus maculatus complex

Although the precise geographic origin of the *P. maculatus* complex remains
uncertain, their closest relatives are distributed in eastern and central Asia (Nilsson,
2015; Nilsson & Hájek, 2017b). Subsequent to the western range expansion from
central Asia, one lineage differentiated in Asia Minor and Anatolia, resulting in *P.
<i>lunulatus*, and the other Europe, resulting in *P. maculatus*.

464 Range expansion in *P. maculatus* followed a clear geographical pattern, with 465 western and eastern populations falling into two well-supported clades (Fig. 6). An 466 exception here is the uncertain position of specimens from northern Italy and the Iberian 467 "graellsi" form, both of which show appreciable morphological differences in elytral 468 pattern and sculpture. Northern Italian specimens have long been recognised as amongst 469 the largest, most convex and shiny of this species (Sharp, 1882; Balfour-Browne, 1940) 470 (Figs 6, 8), whilst populations from central Iberia were originally described as a distinct 471 species (Agabus glacialis Graells, subsequently changed to A. graellsi), but then 472 reduced to a variety and finally synonymised with *P. maculatus* (Nilsson & Hájek, 473 2017a,b). They have a more reddish coloration than the typical forms of *P. maculatus*, 474 with a poorly defined colour pattern and a very dense and deep microsculpture, giving 475 their dorsal surface a dull, rough appearance (Sharp, 1882) (Figs 6, 8). Both central 476 Iberian and north Italian populations are at the periphery of the main range of the 477 species, in a situation similar to that for *H. gracilidelphis* and the Iberian *Oreodytes* (see 478 below). In *Platambus* our data are, however, inconclusive regarding the origin of these 479 forms, which could have been isolated in the Iberian and Italian Peninsulas as remnants 480 of an earlier range expansion in the complex, or result from incipient isolation during 481 the most recent expansion event.

Within the western clade, specimens from some oligotrophic Scottish lochs, also at the periphery of the species main range, formed a monophyletic lineage. This is most remarkable, as they were, together with *P. maculatus* "*graellsi*" and the northern Italian populations, the only forms highlighted in the monumental revision of Sharp (1882). Scottish loch animals were described as being the smallest of the species, flatter in 487 shape, with a duller surface and reduced yellow markings on the elytra (Sharp, 1882; 488 Balfour-Browne, 1940) (Figs 6, 8). However, three specimens from Loch Eck, with a 489 similar morphology (although with less marked differences) were not placed in this 490 clade, but amongst other lineages within the wider western clade (Fig. 6; Table S1). We 491 performed additional analyses, including sequences obtained from public databases, to 492 identify specimens with similar haplotypes to those of Scottish lochs, and found a single northern Swedish sequence which nested within the Scottish clade (obtained from 493 494 Bergsten et al., 2012). The external morphology of this specimen was within the typical 495 range of *P. maculatus* however, and other beetles from the same locality had similar 496 morphology, and COI haplotypes which nested within the western continental lineage. 497 The nuclear markers used did not have enough resolution to determine if such 498 incongruences are the result of introgression, but in any case our results suggest that 499 some Scottish populations, and perhaps others in northern Europe, could be remnants of 500 an early northward range expansion in *P. maculatus*.

- 501
- 502 *4.3. European species of the genus Oreodytes*

503 Most species of the genus *Oreodytes*, including the sister species of both *O*. 504 davisii and O. sanmarkii (O. snoqualmie and O. obesus respectively) are distributed in 505 the western United States and Canada (Larson, 1990; Larson et al., 2000). The 506 geographical origin of European taxa therefore seems to have been via range expansion 507 through Beringia and Asia. In the case of O. davisii this expansion has not left any 508 apparent phylogeographical structure in the studied markers. In the combined analyses 509 (largely driven by mitochondrial data) O. alpinus and O. mongolicus were nested within 510 O. davisii, despite considerable morphological differences between these species 511 (Shaverdo & Fery, 2006; Foster & Friday, 2011). In contrast, the analysis using only 512 nuclear data clearly separated both species from O. davisii, consistent with past 513 mitochondrial introgression between populations of these closely related species who 514 are likely to have shared the same broad Pleistocene refugia. Such a situation has been 515 reported from a number of other taxa (e.g. Berthier et al., 2006; Nichols et al., 2012) 516 including aquatic beetles (Hidalgo-Galiana et al., 2014, García-Vázquez et al., 2016). 517 In O. sanmarkii there is a more clearly defined phylogeographic structure, with

standarkt there is a more clearly defined phylogeographic structure, with
extant western and eastern clades dating from the middle Pleistocene. This suggests an
early origin of European populations, with subsequent isolation of these in different
eastern and western refugia. Both *O. davisii* and *O. sanmarkii* are very cold resistant (in

the Pyrenees both can be active in winter in partly frozen streams, I. Ribera unpublished
observations), likely able to survive in relatively high northern latitudes during glacial
cycles, something which may have favoured local persistence in cryptic northern
refugia (see below).

525 Similarly to the *P. maculatus* and *H. gracilis* complexes, the only recognised 526 forms within O. davisii and O. sanmarkii are the Iberian O. davisii rhianae and O. 527 sanmarkii alienus respectively, both occurring west and south of the Ebro valley (Balke, 528 1989; Carr, 2001). In the analysis of nuclear data, and also ML analysis of the combined 529 dataset, the sequenced specimens of O. sanmarkii alienus formed a monophyletic 530 group, although in a relatively unsupported position with respect to other O. sanmarkii. 531 Irrespective of its taxonomic status it seems that O. sanmarkii alienus is relatively 532 isolated genetically from other European populations, reinforcing the pattern of 533 peripheral isolation across the ranges of widespread European taxa. Although difficult 534 to assess without molecular data, the two missing European species of *Oreodytes* in our 535 study, O. meridionalis and O. angelinii, are also most likely recent peripheral isolates in 536 Mediterranean peninsulas. Thus, O. meridionalis, from the central and southern 537 Appenines (Rocchi, 2007) was considered to be a synonymy of *O. davisii* by 538 Franciscolo (1979) due to their close external morphology. Similarly, O. angeliini, from 539 northern Greece, has only been recently recognised as a distinct species by Fery (2015), 540 having been considered within the morphological variability of O. sanmarkii by 541 previous authors.

542

543 4.4. Concluding remarks: routes of recolonization

544 Our results show that for some water beetles, as in many other groups, central 545 and northern Europe were recolonized by range expansions from peripheral refugia at 546 the end of the last glaciation. Northern areas of Iberia and Anatolia appear particularly 547 relevant for the taxa studied here, both as sources of recolonists and as cradles for 548 recent, narrow-range endemics. In addition to this classic pattern, however, we also 549 show that some widespread central and northern European species originated not around 550 the Mediterranean basin, but in central Asia, although such taxa still have peripherally 551 isolated forms in the southern Mediterranean peninsulas that in some cases possess 552 divergent haplotypes from those in central and northern Europe. Such species may have 553 colonized northern areas of the continent from cryptic refugia in central/eastern Europe 554 or western Asia during the Holocene. Of particular interest is the possibility of

peripheral refugia not only in the Mediterranean region but also in some areas in thenorth, as suggested by the Scottish form of *P. maculatus*.

557 Glacial refugia during cold episodes in Europe were not restricted to the three 558 southern peninsulas, as shown by multiple examples from the Carpathians (Willis et al., 559 2000; Deffontaine et al., 2005; Kotlík et al., 2006; Sommer & Nadachowski, 2006), and 560 other areas in north and central Europe (Kullman, 1998; Bilton et al., 1998; Stewart & 561 Lister, 2001). Quaternary deposits suggest that a woodland zone existed in the southern 562 foothills of the Carpathian mountains and in sheltered valleys at mid elevations, even 563 during the LGM (Lozek, 2006; Willis et al., 2000). More easterly areas could have also 564 been involved in the colonization of Europe from Asia, including the Caucasus 565 (Massilani et al., 2016). Other potential refugia may have been situated in the Urals, the 566 northern slopes of the Altai, or the Crimean Peninsula (Grichuk, 1984; Hewitt, 1999; 567 Soffer 1990) as well parts of the Ukraine and European Russia (Tarnowska et al., 2016).

568 In the case of the species studied here, as with other European lotic water beetles 569 (García-Vázquez & Ribera, 2016), interglacial range expansions did not result in the 570 mixing and homogenisation of gene pools, but instead drove the isolation and 571 differentiation of populations at range edges. All the running water beetle lineages 572 studied here are relatively weak dispersers compared to most standing water relatives. 573 All P. maculatus examined by Jackson (1952, 1956) had reduced flight muscles and 574 most studied specimens of O. sanmarkii have reduced flight muscles, although there is 575 at least one record of a specimen with these fully developed (Foster et al., 2016). O. 576 davisii and H. gracilis are known to fly (Jäch, 1997; Foster et al., 2016) although have 577 been recorded doing so relatively rarely. It is interesting, however, that these two 578 species are the most genetically homogeneous of the species studied here. This 579 relatively poor dispersal ability beggs the question as to how some species are able to 580 expand their ranges to continental scales? The relative genetic homogeneity of 581 widespread species, with genetic differentiation only in peripheral isolates, together 582 with our coalescence data, suggest rapid range expansions over short temporal windows 583 which may have provided optimal ecological conditions for movement between habitat 584 patches. Recently deglaciated areas are likely to have supported a high density of lotic 585 environments, something which may have facilitated the expansion of these beetles. In 586 the Massif Central, Ponel et al. (2016) found an increase in fossil remains of lotic water 587 beetle species immediately after the Last Glacial, but also following the Younger Dryas. 588 This abundance of lotic species was associated with an increase in stream flow resulting

- 589 from snow melt during the rapid warming following these two cold periods. When soil 590 formation and sedimentation transformed the landscape, the abundance of lotic species 591 decreased in parallel with an increase in lentic taxa (Ponel et al., 2016). If the same 592 habitat succession happened at larger geographical scales across the continent, it would 593 have facilitated rapid range expansions in lotic species living close to the margins of 594 deglaciated areas, but only for a short time period. As conditions changed, many 595 populations are likely to have become locally extinct, precipitating the genetic isolation 596 of the remainder and in some their eventual speciation (Ribera et al., 2011). The 597 accumulation of narrowly endemic species in lotic lineages may result from successive 598 cycles of range expansion with subsequent speciation and local extinction in glaciated 599 areas over multiple Pleistocene glacial cycles.
- 600

601 Acknowledgements

602 We thank all the collectors in Table S1 for allowing us to study their material, and Ana 603 Izquierdo (MNCN) and Rocío Alonso (IBE) for laboratory work. We also thank J. 604 Bergsten for providing voucher specimens of Swedish P. maculatus for study, U. 605 Schmidt and L. Borowiec for some of the habitus photographs, and two anonymous 606 Referees for comments. DG-V had a FPI PhD grant from the Spanish Government. This 607 work was partially funded by projects CGL2010-15755 and CGL2013-48950-C2-1-P 608 (AEI/FEDER, UE) to IR, and the "Secretaria d'Universitats i Recerca del Departament 609 d'Economia i Coneixement de la Generalitat de Catalunya'' (project SGR1532).

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847

849	Figure legends
850	
851	Figure 1. Distribution of studied species of the Hydraena gracilis complex. White
852	circles, sampled localities.
853	
854	Figure 2. Distribution of studied species of the <i>Platambus maculatus</i> complex. White
855	circles, sampled localities.
856	
857	Figure 3. Distribution of Oreodytes sanmarkii and Oreodytes davisii in the Western
858	Palearctic. Coloured circles, sampled localities for each species, including also
859	Oreodytes alpinus and Oreodytes septentrionalis. Localities from Mongolia and Siberia
860	for O. sanmarkii and O. alpinus respectively are not show.
861	
862	Figure 4. Phylogenetic tree of the Hydraena gracilis complex. Ultrametric tree obtained
863	with BEAST with combined nuclear and mitochondrial sequences and a partition by
864	gene. Numbers on nodes represent Bayesian posterior probabilities higher than 0.5. See
865	Table S1 for details of specimens and localities. Habitus photograph, H. gracilis (Lech
866	Borowiec).
867	
868	Figure 5. Coalescence Skyline plots of A) H. gracilis; B) P. maculatus; C) Oreodytes
869	sanmarkii. Blue lines represent 95% highest probability density; horizontal axis - time
870	before present (Ma); vertical axis - effective population size (NeT).
871	
872	Figure 6. Phylogenetic tree of the <i>Platambus maculatus</i> complex. Ultrametric tree
873	obtained with BEAST with combined nuclear and mitochondrial sequences and a
874	partition by gene. Numbers on nodes represent Bayesian posterior probabilities higher
875	than 0.5. See Table S1 for details of specimens and localities. Habitus photographs,
876	from base to tip, north Italian from, P. maculatus "graellsi", north-Scottish form, and
877	typical P. maculatus (dotted lines mark the corresponding specimens).
878	
879	Figure 7. Phylogenetic tree for studied Oreodytes species. Ultrametric tree obtained
880	with BEAST with combined nuclear and mitochondrial sequences and a partition by
881	gene. Numbers on nodes represent Bayesian posterior probabilities higher than 0.5. See

- Table S1 for details of specimens and localities. Habitus photographs, O. sanmarkii (L.
- 883 Borowiec) and O. davisii (U. Schmidt).
- 884
- **Figure 8.** Habitus of (A) *Platambus maculatus*, standard form (specimen voucher
- 886 MNCN-AH71); (B) form "graellsi" (voucher MNCN-AI733); (C) specimen from north
- 887 Italy (voucher MNCN-AH191); (D) specimen from the Scottish Lochs (voucher IBE-
- AI975). See Table S1 for details on the specimens, and Figs 6, S2 and S3 for the
- 889 phylogenetic relationships of the specimens.
- 890

- 891 **Table 1.** Clock and coalescent demographic model comparisons for each group,
- 892 including AICM values and standard errors (SE). Best AICM value for each pair shown
- in bold; models that failed to converge adequately in brackets or represented by a dash.
- 894 Differences < 2 units were not considered significant.
- 895

group	clock	AICM	SE	coalescence	AICM	SE
H. gracilis	relaxed	[9076.7]	$[\pm 0.11]$	constant	6705.3	± 0.05
	strict	9385.8	± 0.24	exponential	6717.2	± 0.24
				expansion	[6708.5]	±0.26]
				logistic	-	-
P. maculatus	relaxed	18568.9	± 0.52	constant	-	-
	strict	18596.7	± 0.44	exponential	[11173.9]	$\pm 19.20]$
				expansion	[9903.9]	± 65.07]
				logistic	-	-
O. sanmarkii	relaxed	23948.4	± 0.87	constant	-	-
	strict	23952.8	± 0.56	exponential	8204.6	± 0.01
				expansion	8200.2	±0.15
				logistic	8202.3	±0.13