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Late Miocene diversification of the genus *Hydrochus* (Coleoptera, Hydrochidae) in the west Mediterranean area

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We provide a reconstruction of the phylogenetic relationships, the geographical and temporal origin, and the mode of diversification of the Mediterranean species of the aquatic beetle family Hydrochidae (Coleoptera, Hydrophiloidea). A total of ca. 3 KB of sequence data of three mitochondrial and two nuclear genes were used to reconstruct the phylogeny of 62 specimens of 21 species of *Hydrochus*, including all western Mediterranean species but one. We estimated the times of divergence using Bayesian methods and an evolutionary rate of 0.0115 substitutions/site/MY, and used an ultrametric calibrated tree to construct a Lineage Through Time (LTT) plot to test alternative models of diversification. A well resolved, well supported phylogeny showed that all western Mediterranean *Hydrochus* formed a clade, sister to a group including species with a central and eastern European distribution. The origin of the western Mediterranean clade was estimated to be at ca. 13MY, and the speciation events took place between this time and the end of the Messinian, at about 5.3MY. The LTT plot best fitted a model with a shift in the rate of diversification at ca. 8 MY, with a single speciation event (originating two Iberian endemics) subsequent to this period. We conclude that most of the western Mediterranean species of Hydrochidae, including the Ibero-Maghrebian endemics, are ancient elements likely to have remained in the same geographical area since their Miocene origin. Our results add to a growing body of evidence showing the importance of Mediterranean long-term, Tertiary refugia as both cradles and museums of diversity.

1. Introduction

The Mediterranean region is one of the world hotspots of biodiversity (Médail and Quézel, 1999; Myers et al., 2000), with a complex geological history and a rich mosaic of habitats favouring diversification (Blondel and Aronson, 1999). For many Mediterranean groups of organisms the peninsulas (Iberia, Balkans and Turkey) contain the highest diversity, with a substantial part of these species forming species radiations of restricted distributions in each of these areas (e.g. Oosterbroek and Arntzen, 1992; Crivelli and Maitland, 1995; Petit et al., 2003; Sanmartín, 2003). Many insect groups, and among them Coleoptera, follow this general pattern (see e.g. Jäch, 1993; Fery and Brancucci, 1997; Fery and Hosseinie, 1998; Löbl and Smetana, 2004 for some aquatic families), but there is a general lack of data of the origin of these species in what refers both to their temporal and geographical origin. Recent work on aquatic Coleoptera established the recent (Pleistocene) origin of most Iberian endemics of one of the families (Dytiscidae), which are in general vicariant species with widespread European distributions (Ribera, 2003; Ribera and Vogler, 2004; Ribera and Faille, 2010). Only in some cases (e.g. genus *Deronectes*) there was an older diversification within the Iberian peninsula. In a different family (Hydraenidae), although some groups of narrow range endemics show also a predominantly Pleistocene origin (e.g. *Haenydra* lineage, Ribera et al., 2011), others seem to have an older Origin, such as the *Ochthebius* (*Enicocerus*) *exsculptus* species group, with two Late Miocene Iberian endemic species (Ribera et al., 2010a). The idiosyncratic origin of different groups with similar distributions suggests that until the phylogeny and biogeography of a wide range of Mediterranean groups is investigated it would not be possible to draw general conclusions about the origin and assemblage of the Mediterranean fauna.

Among aquatic Coleoptera, one group with a predominantly Mediterranean distribution of which there is virtually no phylogenetic or biogeographic information is the family Hydrochidae (Hydrophiloidea) (Hansen, 2004). Hydrochidae includes only one accepted genus, *Hydrochus*, with a worldwide distribution and about 180 described species (Hansen, 1999; Short and Hebauer, 2006). All *Hydrochus* are aquatic, living in stagnant or slowly flowing water (Jäch, 1998). In the west Mediterranean (Iberian peninsula, Morocco and south France), the genus *Hydrochus* is represented by 12 species, 7 of them endemic to the area. In the east Mediterranean (the Balkans, Turkey, the Near East and Iran) there are six widespread species and only two described endemics from Iran and Turkey

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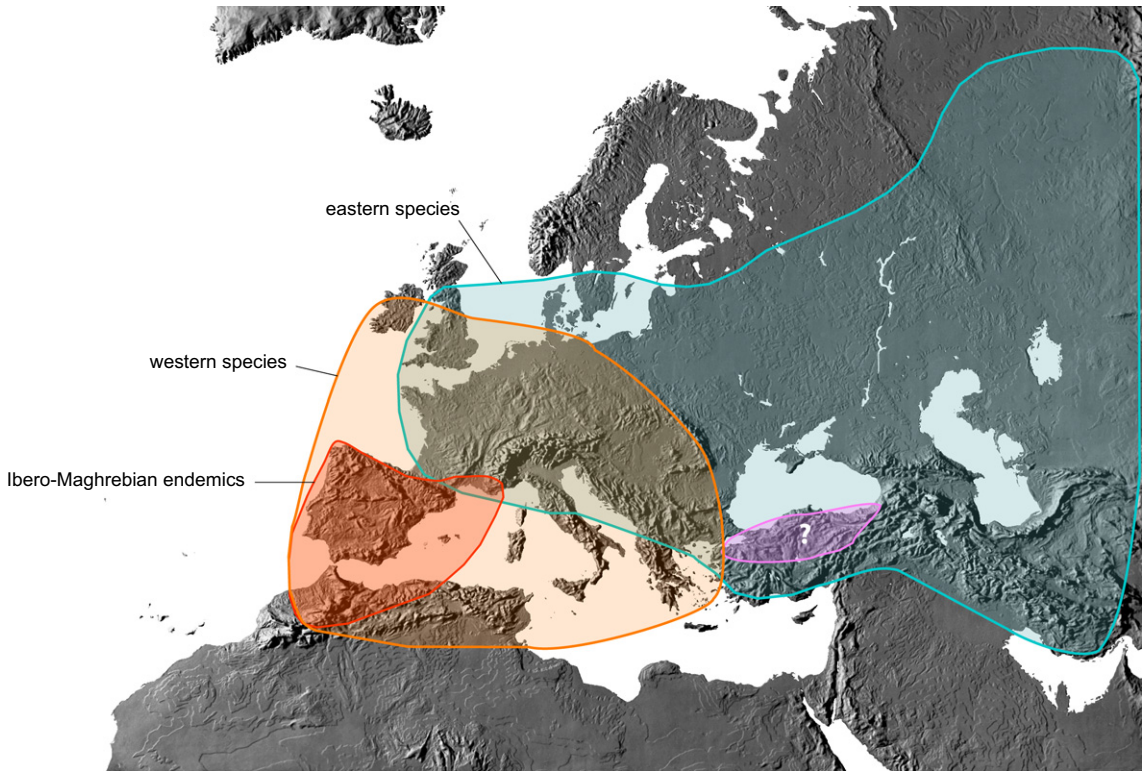

Fig. 1. Main distribution types among the species of west Palaearctic *Hydrochus*. Ibero-Maghrebian endemics include *H. aljibensis*, *H. angusi*, *H. ibericus*, *H. interruptus*, *H. nooreinus*, *H. smaragdineus*, *H. tariqui* and the non-sampled *H. obtusicollis*; western species include *H. angustatus*, *H. flavipennis*, *H. grandicollis* and *H. nitidicollis*; and eastern species *H. crenatus*, *H. elongatus* and the non-sampled *H. farsicus*, *H. ignicollis* and *H. nodulifer*. With a question mark, uncertain distribution of *H. roberti*. See Fig. 2 for the phylogenetic relationship of the species, Appendix A for the detailed data of the studied material, and Appendix D for the detailed distributions.

(Hansen, 2004; Hidalgo-Galiana et al., 2010, Fig. 1 see Appendices D and E for the detailed distribution of the studied species), although some recent records and unpublished information from the collections of the Naturhistorisches Museums in Wien suggest that there could be several undescribed species in the area.

We provide here a reconstruction of the phylogenetic relationships, the age and the geographical origin of the western Palaearctic species of *Hydrochus* to understand their diversification and the current patterns of diversity. We use molecular data (mitochondrial and nuclear) of 16 of the 20 species present in the area, and use molecular-clock methods to estimate the age of most of the known Ibero-Maghrebian endemics.

2. Materials and methods

2.1. Background on the taxonomy of the group and taxon sampling

We include data of 16 out of 20 known species of western Palaearctic *Hydrochus* (Hansen, 2004; Appendix E), with the exception of (1) *Hydrochus obtusicollis* (Fairmaire), with a restricted distribution in north Morocco (Bennas et al., 2007), (2) *Hydrochus ignicollis* Motschulsky, with a wide European distribution, (3) *Hydrochus nodulifer* Reitter known from the Caucasus and Iran and (4) *Hydrochus farsicus* Hidalgo-Galiana, Jäch & Ribera from Iran (Hansen, 2004; Hidalgo-Galiana et al., 2010) (Appendices D and E). There are several possibly undescribed species in Turkey apparently closely related to the *Hydrochus elongatus* group (which includes *H. ignicollis* and *Hydrochus crenatus* (Fabricius)) (M.A. Jäch, personal communication, 2009). The recent record of *Hydrochus ibericus* Valladares, Díaz & Delgado from this area (Mart et al., 2009) corresponds to *H. farsicus* (U. Incekara, personal communication, 2010).

For some of the species we studied more than one specimen (Appendix A) to test for the monophyly of the currently recognised species and to detect possible intraspecific variability (specially in the case of islands or geographically isolated regions). We included as outgroups several species of *Hydrochus* from other regions of the world (Appendix A). Trees were rooted with sequences of other families of Hydrophiloidea clearly outside Hydrochidae (Bernhard et al., 2009), obtained from GenBank (Appendix A).

2.2. DNA extraction and sequencing

Specimens were killed and preserved in absolute ethanol in the field. We employed for DNA isolation a standard phenol–chloroform non-destructive extraction (voucher specimens MNCN-AH1 to MNCN-AH36) or "Charge Switch gDNA Tissue Kits" (Invitrogen, Carlsbad, USA) (voucher specimens MNCN-AH37 to MNCN-AH70, see Appendix A), following the instructions of the manufacturer. Typically only males were sequenced, and the male genitalia (or aedeagus, used for species identification) examined and preserved previous to the extraction to ensure a correct identification. Voucher specimens and DNA aliquots are deposited in the Museo Nacional de Ciencias Naturales (MNCN, Madrid) and the Institut de Biologia Evolutiva (IBE, Barcelona) (Appendix A).

Five gene fragments were amplified: three mitochondrial markers, the 3' end of the subunit 1 of the Cytochrome Oxidase (*cox1*), an internal fragment of Cytochrome b (*cob*) (both protein coding) and 12S rRNA (*rrnS*); and two fragments of nuclear ribosomal genes, the 5' end of 18s rRNA (*SSU*) and an internal fragment of 28s rRNA (*LSU*). For each fragment both forward and reverse sequences were obtained (see Table 1 for the primers used). In some specimens the *cox1* fragment was amplified using internal primers to obtain two fragments of around 400 bp each (Table 1).

General PCR cycling conditions used for DNA amplification were: 3 min at 96 °C, [30s at 94 °C, (30s- 1 min) at 47–50 °C (depending on the annealing temperatures of primer pair used),

Table 1
List of primers used for amplification and sequencing.

Gene	Primer	Sequence	Reference
cox1	Jerry (5′)	5′ CAACATTTATTTTGATTTTTTGG	Simon et al. (1994)
	Pat (3′)	5′ TCCAATGCACTAATCTGCCATATTA	Simon et al. (1994)
	Chy1 (5′)	5′ T(A/T)GTAGCCCA(T/C)TTTCATTA(T/C)GT	Ribera et al., 2010b
	Tom1 (3′)	5′ AC(A/G)TAATGAAA(A/G)TGGGCTAC(T/A)A	Ribera et al., 2010b
cob	CB3 (5′)	5′ GAGGAGCAACTGTAATTACTAA	Barraclough et al. (1999)
	CB4 (3′)	5′ AAAAGAAA(AG)TATCATTCAGGTTGAAT	Barraclough et al. (1999)
rrnS	12Sai (5′)	5′ AAACTAGGATTAGATACCCTATTAT	Simon et al. (1994)
	12Sbi (3′)	5′ AAGAGCGACGGGCGATGTGT	Simon et al. (1994)
SSU	18S 5′ (5′)	5′ GACAACCTGGTTGATCCTGCCAGT	Shull et al. (2001)
	18S b5.0	5′ TAACCGCAACAACAACTTTAAT	Shull et al. (2001)
LSU	ka (5′)	5′ ACACGGACCAAGGAGTCTAGCATG	Monaghan et al. (2007)
	kb (3′)	5′ CGTCCTGCTGTCTTAAGTTAC	Monaghan et al. (2007)

(50s- 1 min) at 72 °C] (repeated for 35–40 cycles), and 10 min at 72 °C. Sequencing was performed by the Sanger method in an external facility. The products obtained were purified by a standard ethanol precipitation. Sequencing errors/ambiguities were edited using the Sequencher 4.7 software package (Gene Codes Corporation, Ann Arbor, USA). New sequences were deposited in GenBank with accession numbers HM569373-HM569596 (Appendix A).

2.3. Phylogenetic analyses

We aligned length-variable fragments with MAFFT 5.8 on-line version (Katoh et al., 2002), shown to perform better than alternative pair-wise alignment methods (Golubchik et al., 2007), using the G-INS-i algorithm and default values for the rest of parameters.

We used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) to estimate the topology and node support. We included a combined data matrix partitioned according to two different criteria: (1) the five genes (*cox1*, *cob*, *rrnS*, *LSU* and *SSU*), and (2) a partition by codon position on the combined *cox1* and *cob* fragments, plus the mitochondrial ribosomal gene (*rrnS*) and the two nuclear ribosomal genes combined (*LSU* plus *SSU*) (i.e. a total of five partitions for each criteria). For each partition we implemented the available evolutionary model with the closest match to that selected by ModelTest 3.6 (Posada and Crandall, 1998), using Akaike weights as selection criteria. MrBayes ran 7.5×10^6 or 40×10^6 generations for the gene and codon partition respectively, using default values and saving trees each 1000 generations. "Burn-in" values were estimated by plotting the standard deviation of the split frequencies between two simultaneous runs and visually checking for convergence. The two partition strategies were compared using Bayes factors (BF) (Kass and Raftery, 1995), as computed in Tracer 1.5 (Drummond and Rambaut, 2007) using 1000 replicates. A partition was considered significantly better when the ln(BF) had an increase of 10 or more for each additional parameter (p) (i.e. a PM factor = $\Delta \ln BF/\Delta p > 10$; Pagel and Meade, 2004; Miller et al., 2009).

We also used Maximum Likelihood as implemented in the on-line version of RAxML 7.0.3 (which includes an estimation of bootstrap node support, Stamatakis et al., 2008), using GTR + G as the evolutionary model, estimated independently for each of the gene partitions. To check for possible incongruence between mitochondrial and nuclear genes we run two separate analyses in RAxML with the respective sequences, using the same conditions as for the combined dataset.

2.4. Estimation of the ages of divergence

Molecular dating was carried out with Beast v1.4.7 (Drummond and Rambaut, 2007). Beast generates ultrametric rooted trees, incorporating a time-scale if a calibration point or an *a priori* rate are specified. We excluded the multiple specimens of the same species and constrained all well supported nodes according to the results of the previous phylogenetic analyses, and employed an uncorrelated lognormal relaxed clock and a global GTR + I + G evolutionary model (Drummond and Rambaut, 2007). As there is no fossil record or an unambiguous biogeographic event that could be used to calibrate the tree we used an estimated rate of 0.0115 substitutions/site/MY for the combined mitochondrial genes. This rate is the standard pair-wise difference of 2.3% per MY (Brower, 1994), which could be different for *Hydrochus*, but agrees with estimations based on biogeographic events in some related Coleoptera groups for a mix of protein-coding and ribosomal mitochondrial genes (0.010 substitutions/site/MY, Ribera et al., 2010b; 0.013 substitutions/site/MY, Papadopoulou et al., 2010). As this rate applies to the combined mitochondrial sequence, we build a matrix excluding the nuclear genes and analysed it as a single partition. The prior rate was set as a normal distribution with average 0.0115 substitutions/site/MY and a standard deviation of 0.0005. We set a Yule speciation process (a pure birth process, with a uniform probability of speciation) as a tree prior, and made two independent runs with the same settings and combined the results after deletion of 10% of the generations as burnin with Tracer 1.5 and other applications of the Beast package (Drummond and Rambaut, 2007).

2.5. Rate and mode of diversification

We estimated the rate of diversification using the log-lineage through time approach (LTT) (Harvey et al., 1994; Nee et al., 1994). We used Genie (Pybus and Rambaut, 2002) to compile the LTT plot using the ultrametric tree obtained in Beast. LTTs represent graphically the time elapsed between successive branching events (Barraclough and Nee, 2001). The ultrametric tree contains information on the number of lineages and the molecular distance of every lineage to the root (the relative time of each node from the root node).

We used the γ-statistic (Pybus and Harvey, 2000) for measuring the relative timing of the diversification, i.e. whether there is a constant diversification 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 its root than expected under a pure birth process, and vice versa (Pybus and Harvey, 2000). To test the significance of the γ-statistic we generated a null distribution of 10,000 random simulations using a pure birth process including the known missing taxa, and tested the observed γ-statistic against it (Pybus and Harvey, 2000). We also found the number of missing taxa that would be necessary to render the observed γ-statistic non-significant.

Table 2
Length of the sequenced fragments, with maximum and minimum length before and after alignment and number of informative characters, evolutionary model selected by ModelTest for the different partitions, and model implemented in MrBayes.

partition	max.	min.	aligned	informative	optimal model	implemented model
cox1	826	826	826	334	GTR+I+G	GTR+I+G
cob	358	358	358	152	Tim+I+G	GTR+I+G
rrnS	358	349	367	143	TVM+G	GTR+G
LSU	597	584	599	26	GTR+I	GTR+I
SSU	602	600	604	14	Trnef+I	GTR+I
1st codon	394	394	394	114	GTR+I+G	GTR+I+G
2nd codon	394	394	394	34	GTR+I+G	GTR+I+G
3rd codon	396	396	396	338	GTR+I+G	GTR+I+G
LSU+SSU	1190	1184	1203	40	GTR+I+G	GTR+I+G
Total	2730	750*	2754	669	GTR+I+G	GTR+I+G

* Specimen MNCN-AH4, with incomplete cox1 sequence only (see Appendix A). The rest of measures are given only for genes with the complete sequence.

We tested the adequacy of our data to different diversification models with likelihood methods. The models tested were a pure birth (Yule), a birth–death with constant diversification rate, two models with variable diversification rates (logarithmic and exponential), and a pure birth model with a shift in the diversification rate (Table 3). We checked the significance of the result with a function that generates a null distribution of the statistic and returns the probability of the observed AIC (Akaike Information Criterion) for constancy of diversification rates (as in Rabosky, 2006). All diversification tests were done using the R libraries 'ape' (Paradis et al., 2004) and 'laser' (Rabosky, 2006).

3. Results

3.1. Phylogenetic analyses

The final matrix included 66 specimens of 25 recognised species (21 of them in the genus Hydrochus, 15 of them in the ingroup W Palearctic clade) (Appendix A). There were no length differences in the protein coding genes among the studied specimens, and among the ribosomal genes length differences were mostly in the *LSU* gene (Table 2). For the combined matrix, ModelTest selected GTR + I + G as the best evolutionary model. Of the different models selected for the individual partitions some are not implemented in MrBayes, and thus we selected the most similar one with an equal or lower number of parameters (Table 2). For the partition by genes the two independent runs converged at ca. 4×10^6 generations (used as the "burn-in"), reaching a standard deviation of the split frequencies of ca. 0.006. For the partition by codons plus the nuclear and mitochondrial ribosomal genes the two runs converged at ca. 25×10^6 generations, reaching a standard deviation of the split frequencies of ca. 0.002. The runs of both partitions had enough ESS (Effective Sample Size) and a convergence diagnostic in MrBayes (PSRF, potential scale reduction factor) close to one (Ronquist and Huelsenbeck, 2003), indicating a good convergence of the MCMC chains.

The topology obtained with the two partition schemes in MrBayes was identical for the Mediterranean species, and differed only in the relative position of the species of the *Hydrochus brevis* group and the clade with the American and Australian species, which was poorly supported in both cases (Bayesian posterior probability, Bpp = 0.88 and 0.55 for the genes and codon partitions respectively, Fig. 2). The Bayes factors favoured the partition by codons, with a difference in lnBY of more than 500 units for two additional parameters (Table 3) (i.e., PM >>10).

Differences between the topologies of the two reconstruction methods used (Maximum Likelihood and Bayesian Analysis) were minimal, and affecting only three nodes: the placement of the *Hydrochus angusi* Valladares and *H. ibericus* clade (sister to the rest of clade B in RAxML, see below and Fig. 2), and the position of the Australian species and the species of the *H. brevis* group (Fig. 2). In all cases these ambiguities affected poorly supported nodes, and the two alternative topologies for the only ambiguous node among the Mediterranean species did not affect any of the results.

Table 3
Models of diversification tested. Models tested: pure birth (Yule), constant rate without extinction; birth–death (bd), constant rate with extinction; DDL, density-dependent variable rate (logarithmic); DDX, density-dependent variable rate (exponential); yule2rate (y2r), pure birth with a shift in diversification. r1, estimated speciation rate (first parameter in all models); 2nd, second parameter (extinction rate in the bd model, carrying capacity (k) in DDL, density-dependent parameter (x) in DDX, second speciation rate in y2r); 3rd, third parameter (time of shift in diversification in y2r); AIC, Akaike Information Criterion; dAIC, delta-AIC, difference in AIC scores between the model and the overall best-fit model. (a) Models when the variation within *H. grandicollis* was included, (b) models when the variation within *H. grandicollis* was not included (see Text).

Model	r1	2nd	3rd	AIC	dAIC
(a) H. grandicollis included					
Pure birth	0.120			33.66	6.33
Birth–death	0.120	0		35.66	8.33
DDL	0.340	16.945		30.08	2.75
DDX	1.036	0.991		30.47	3.14
Yule 2 rate	0.369	0.063	7.87	27.34	0
(b) H. grandicollis not included					
Pure birth	0.096			35.60	16.08
Birth–death	0.096	0		37.60	18.08
DDL	0.726	12.239		19.52	0
DDX	1.515	1.317		29.54	10.02
Yule 2 rate	0.369	0.032	7.87	24.57	5.05

3.2. Phylogeny of the Mediterranean species of Hydrochus

The monophyly of the genus *Hydrochus* was strongly supported (Bayesian posterior probability, Bpp $\geqslant$ 0.99, ML boostrap, MLb = 100%, Fig. 2), with a basal split within the sampled species of the genus separating four main well-supported lineages: (1) the central and northern European species of the *H. brevis* group; (2) the single species from Australia; (3) the American species; and (4) all remaining Palaearctic species sister to a South African species. This Palaearctic-South African clade was very well supported (Bpp = 1.0, MLb = 100%, Fig. 2, see Appendix A for the localities of the specimens). The resolution among these four lineages was poorly supported.

Within the main Palaearctic lineage, all species of *Hydrochus* with a distribution centred in the western Mediterranean region were included in a well supported clade (Bpp = 1.0, MLb = 86%, Fig. 2), sister to two species with a mostly central and northern European distribution (*H. crenatus* and *H. elongatus* (Schaller)). This west Mediterranean clade had two well-supported lineages with overlapping geographical distributions (clades A and B in Fig. 2), including both Ibero-Maghrebian endemics (*Hydrochus aljibensis* Castro & Delgado and *Hydrochus interruptus* Heyden in lineage A, and *Hydrochus tariqi* Ribera, Hernando & Aguilera, *H. nooreinus*

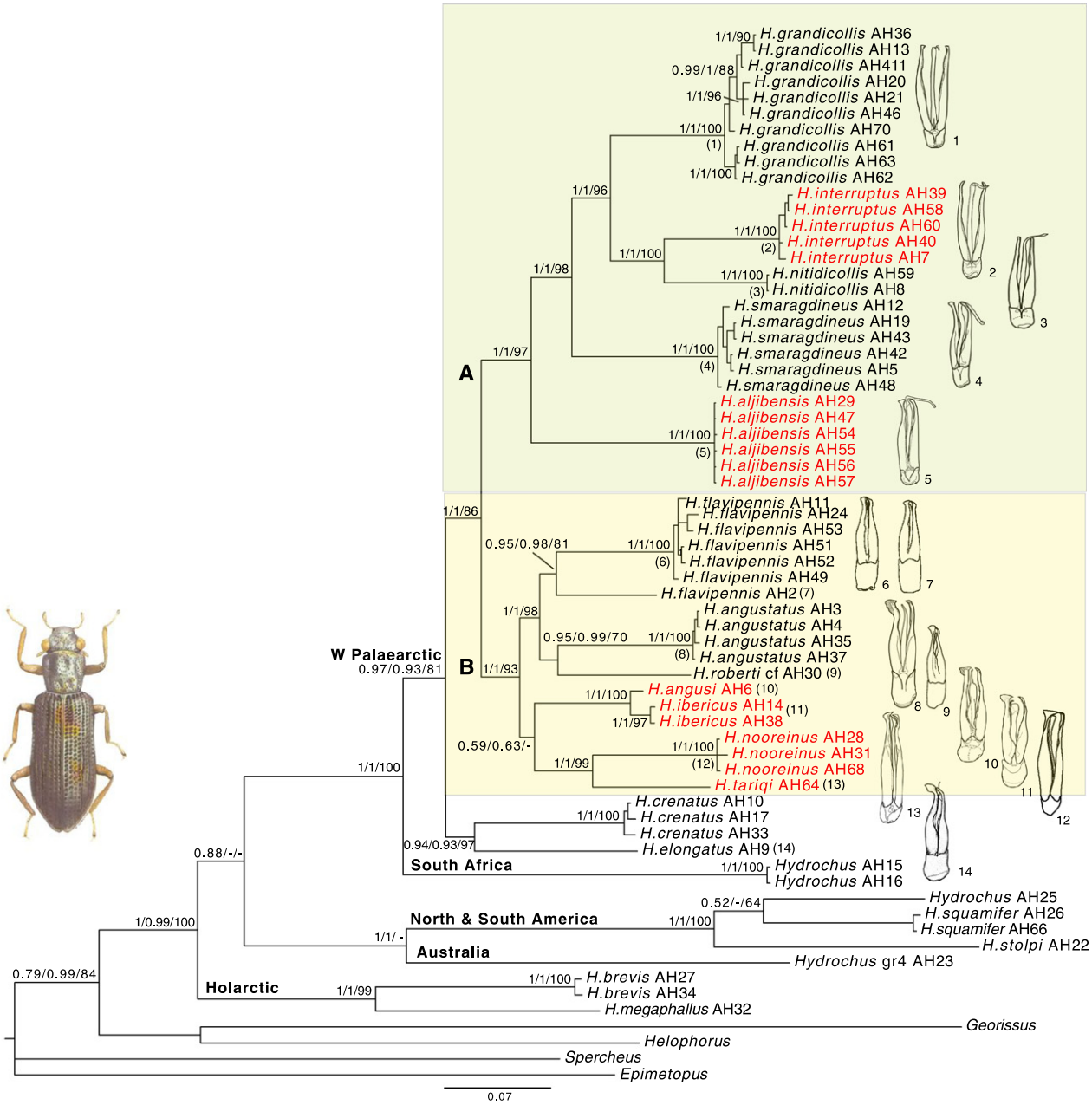

Fig. 2. Phylogram obtained with MrBayes with the combined nuclear and mitochondrial sequence and a partition by gene. Numbers above the branches, Bayesian posterior probabilities of the partition by gene/Bayesian posterior probabilities of the partition by codon/Bootstrap support values in RAxML. In red, Ibero-Maghrebian endemics. Outline drawings, male genitalia (numbers correspond to those below branches), not at the same scale. Habitus, *Hydrochus tariqi* (from Ribera et al., 1999). See Appendix A for the detailed precedence of the sequenced specimens and Fig. 1 for the general distribution of the species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Berge Henegouwen & Sáinz-Cantero, *H. angusi* and *H. ibericus* in clade B), as well as widely distributed species (Fig. 1; see Appendix D for the detailed distribution of the species). In clade B there were three well supported nodes with poorly supported relationships between them, two including Ibero-Maghrebian endemics and the third a group of species including the widely distributed *Hydrochus angustatus* Germar, a specimen provisionally identified as *Hydrochus roberti* Shatrovskij from Turkey (previously known only from the Caucasus, Shatrovskij, 1993) and the *Hydrochus flavipennis* complex. All species in clade A are present in the Iberian peninsula and the Maghreb, with two endemics of this area forming a basal paraphyletic series (*Hydrochus aljibensis* and *Hydrochus smaragdineus* Fairmaire). There are two species extending their distribution towards central and northern Europe and/or the eastern Mediterranean (*Hydrochus nitidicollis* Mulsant and *Hydrochus grandicollis* Kiesenwetter), together with the Iberian *H. interruptus*, nested within them (Figs. 1 and 2; Appendix D).

The ML analyses using only the nuclear genes recovered some of the nodes (the main Palaearctic clade, the west Mediterranean clade) but contributed little to the resolution of the west Mediterranean species on their own (Appendix B). The ML tree obtained with the mitochondrial sequence was very similar to that obtained with the combined sequence (Appendix C), except for the basal relationships among the western Palaearctic lineage.

All currently recognised species were monophyletic with strong support and generally low intraspecific variation, with the only exception of *H. flavipennis* Küster, with two lineages with a very deep divergence supported exclusively by the mitochondrial genes

(Fig. 2 and Appendix B). One of these lineages included specimens from central Spain, and the second specimens from Morocco, Spain and Tunisia (Appendix A). *H. grandicollis* presented also a strong geographical structure, with good support for the respective monophyly of specimens from Morocco, Spain and Sicily (Figs. 1 and 2; Appendix A). The variation within other species (e.g. *H. interruptus*, *H. smaragdineus*, Fig. 2) had no obvious geographical structure.

Our results are in good correspondence with informal groupings made according to morphology, and in particular to the structure and shape of the male genital organ (the aedeagus), traditionally used for species identification in the genus (e.g. Angus, 1976; see Fig. 2). Although without a formal analyses it is not possible to obtain firm conclusions, the strong asymmetry of the parameres of the male genitalia and the presence of a flagellum at the apex of the median lobe seems to be the plesiomorphic condition for the main Palaearctic clade, not present in the species of the *H. brevis* group and all the non-Palaearctic species. The European species of the *H. brevis* group (i.e. *H. brevis* (Herbst) and *Hydrochus megaphallus* Berge Henegouwen) form a very distinct lineage, most likely including some Nearctic species with a very similar external morphology and structure of the aedeagus (see e.g. Smetana, 1988). Within the main Palearctic clade, the species of the *H. elongatus* group maintain the plesiomorphic condition (flagellum plus asymmetry of the parameres), but share a characteristic apical expansion of the left paramere (Angus, 1976; Hansen, 1987). Species of clade B maintain the strong asymmetry, but the flagellum is lost or very reduced. Species of clade A seem to have secondarily developed more symmetrical parameres, and developed a longer flagellum (see the outline of the male genitalia of the studied species in Fig. 2).

There are only four recognised western Palaearctic species not included in our phylogeny. Of these, only *H. obtusicollis* occurs in the western Mediterranean: it is a rare species endemic to north Morocco likely to be related to *H. angustatus* according to the morphology of the aedeagus (Bennas et al., 2007). The aedeagus of two eastern European species (*H. ignicollis*, *H. nodulifer*) is clearly similar to that of *H. elongatus* (Hansen, 1987; Shatrovskij, 1993). Finally, the aedeagus of the Iranian *H. farsicus* and that of some of the undescribed species from Turkey in the collections of the NMW (M.A. Jäch, personal communication, 2009), although with less clear affinities, share some characters of the species in the *H. elongatus* group (Hidalgo-Galiana et al., 2010).

3.3. Rate of diversification and molecular dating

Using a standard mitochondrial rate (2.3% per MY) in Beast the split between the western Mediterranean clade and the species of the *H. elongatus* group was estimated to have occurred around Mid Miocene (ca. 14MY, Fig. 3). The diversification of the western Mediterranean clade was dated at ca. 13MY, and the speciation events took place between this time and the end of the Messinian, at about 5.3MY, with the only exception of the separation between *H. ibericus* and *H. angusi*, estimated to have originated during the lower Pleistocene (ca. 1.5MY, Fig. 3).

We restricted the analyses of diversification to the clade including the western Mediterranean species (i.e. nodes A and B in Fig. 2). We included the two lineages of *H. flavipennis*, which most likely represent distinct species estimated to have originated at more than 8 MY. Due to the uncertainty in the taxonomic status of the geographical variants within what is currently known as *H. grandicollis*, we did two set of analyses, one including four specimens, one from each of the four main geographical areas (Fig. 3, Appendix A), and another with a single specimen, i.e. not considering the geographic variation. The LTT plot (Fig. 4), reflecting the temporal pattern of diversification, showed a steady initial increase in lineages, a plateau (stasis), and a final increase mainly corresponding to haplotype diversification within *H. grandicollis*. The γ-statistic rejected the null hypothesis of a constant birth

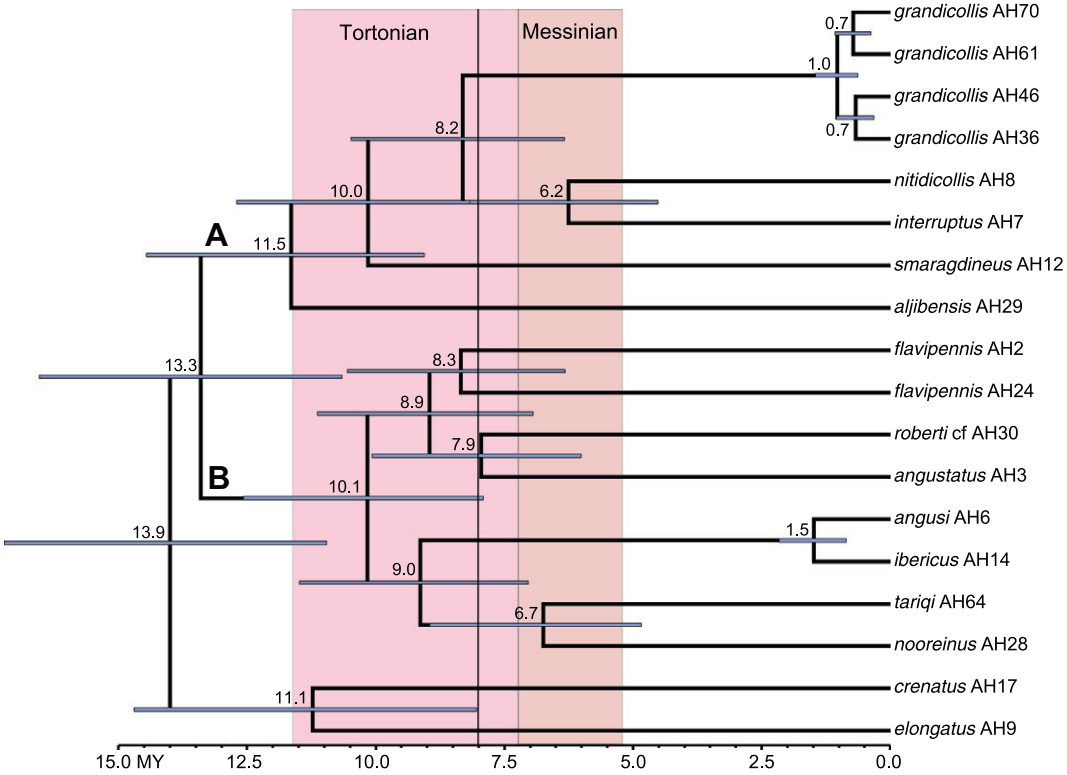

Fig. 3. Ultrametric tree obtained with Beast, using the mitochondrial sequence only and calibrated with a rate of 0.0115 substitutions/site/MY. Numbers in nodes, estimated age (MY); node bars, 95% confidence intervals of the age estimate. The vertical line at ca. 8 MY marks the estimated inflexion in the speciation rate.

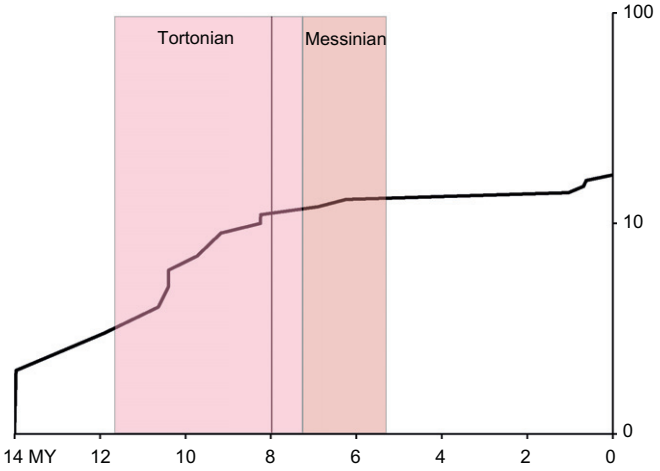

Fig. 4. Lineage Through Time plot (LTT) obtained from the tree in Fig. 3. The vertical line at ca. 8 MY marks the estimated inflexion in the speciation rate in the Yule 2 rate model (see text and Table 3). Vertical axis, logarithm of the number of lineages.

and death model, with the nodes significantly shifted towards the origin both when the variation within *H. grandicollis* was included ($\gamma = -1.84$, $p < 0.02$) or excluded ($\gamma = -3.26$, $p < 0.0001$). For both tests we considered only one missing species (*H. obtusicollis*, Appendix E), but estimated how many species would be necessary to render the γ-statistic not significant. When the variation within *H. grandicollis* was included, the number of missing species had to be increased from 1 to 10 (i.e. from 6% of missing species to 62%), and when not included, to 120 (i.e. from 8% to >900%).

The model selected in the test of diversification was in both cases a rate variable model, logistic (DDL) when the variation within *H. grandicollis* was not included, and a pure birth with a shift in the rate of diversification (yule2rate) when included (Table 3). For the logistic model the estimated carrying capacity was ca. 13 species (parameter $k = 12.24$), and the shift in the diversification rate for the Yule 2 rate model was estimated to have occurred at around 8 MY (Table 3). In both cases the best constant rate model was a pure birth, but they were significantly worst than the best variable rate models, as measured with the null distribution of the differences in the Akaike information criteria (dAICrc, $p < 0.0005$ and $p < 0.02$ when not including and including variation within *H. grandicollis* respectively).

4. Discussion

4.1. Hydrochus phylogeny

We obtained a robust phylogeny for the western Palaearctic species of *Hydrochus*, with very similar results for the two methods used and strong support for most internal nodes. All species currently found in the Iberian peninsula and Morocco formed a monophyletic clade sister to the species of the *H. elongatus* group, with a distribution centred in the eastern Mediterranean with extensions to central and northern Europe in some cases (Hansen, 1999; Fig. 1; Appendices D and E). *Hydrochus roberti*, so far only recorded from the Caucasus and Turkey, would be the only species of the "western clade" not present in the Iberian peninsula or Morocco. However, from our results it is clear that the name *H. flavipennis* has been used for what it is actually a complex of species with uncertain distributions. This complexity is apparent from the variety of morphologies of the aedeagus found as Quaternary fossils in Britain (Angus, 1976), strongly suggesting the presence of several species.

There are no known species restricted to Italy or the Balkans (Appendix E), contrary to what happens with other groups with predominantly Mediterranean distributions (Myers et al., 2000; Murienne et al., 2010). The period estimated for the divergence of these two main clades (eastern and western Mediterranean) was the mid Miocene (ca. 14 MY), a time in which the Italian peninsula was mostly submerged or partly merged with what would form the Balkan and Anatolian peninsulas, and there was no land connection between the north and south sides of the Mediterranean (Dercourt et al., 1985; Bruch et al., 2007). The geographic scenario of the mid Miocene Mediterranean would thus support the hypothesis of a vicariant split between two main lineages, one in the west centred in the Iberian peninsula and the second in the east including Anatolia plus the Middle east and the Balkans, in agreement with the general pattern described by Oosterbroek and Arntzen (1992) for a diversity of groups.

4.2. Diversification of the Mediterranean Hydrochus

We included in our study all the western Mediterranean species of *Hydrochus* with the sole exception of *H. obtusicollis*. The LTT plot of this lineage can thus be considered an accurate representation of the diversification history of the current species, showing no net speciation in the west Mediterranean clade since the Messinian other than the split between *H. ibericus* and *H. angusi* and the geographical variation within *H. grandicollis*, if this is considered to be the sign of incipient speciation despite the apparent lack of morphological differences (see below). The preferred diversification model adjusted to the LTT plot reflected this fact, clearly rejecting a constant diversification in front of variable rate models. The level of missing taxa necessary to cancel this effect, as measured with the γ -statistic, is unrealistically high, with 10 species when the variation within *H. grandicollis* was considered (i.e. an increase of more than 60% of the known fauna of the genus in the west Mediterranean), and more than 100 when not included (i.e. an increase of more than 900%).

The change in diversification rate was estimated to have occurred at ca. 8MY. According to our estimations, the species diversification of the genus in the Mediterranean took place during the mid to late Miocene (ca. 13-5 MY). The shoreline reconstructions on this period based on coral deposits (see e.g. Braga et al., 2003; Jolivet et al., 2006) reflect a succession of islands of different sizes in the Ibero-Maghrebian area due to strong tectonic activity and sea level changes. This could have favoured multiple vicariant events originating most of the extant W Mediterranean species of *Hydrochus*. On the contrary, the east side of the Mediterranean formed a continuous emerged mass of land for most of the Miocene (Blondel and Aronson, 1999; Jolivet et al., 2006; Popov et al., 2006; Barrier and Vrielynck, 2008), apparently offering less opportunities for diversification, although the incomplete representation of species of this clade in our phylogeny does not allow a detailed comparison. The apparent lack of speciation since the end of the Miocene (Messinian) in the western Mediterranean clade could be associated to the decreased opportunities for vicariant isolation with the coalescence of the Baetic cordilleras and the formation of the Straits of Gibraltar (Braga et al., 2003; Jolivet et al., 2006). In any case, the age estimation establish a clear pre-Pleistocene origin for most of the extant western Palaearctic species of *Hydrochus*, including the Iberian and the Ibero-Maghrebian endemics. This will agree with age estimates of some endemics of mountains systems in central and north Iberia (e.g. *Ochthebius* subgenus *Enicocerus*, Ribera et al., 2010a), or the ancient origin of the species of some clades of other Mediterranean arthropods (e.g. Murienne et al., 2010), but is in sharp contrast with estimations for other aquatic Coleoptera (e.g. Dytiscidae, Ribera and Vogler, 2004; Ribera and Faille, 2010; or other groups of Hydraenidae, Ribera et al.,

2011), where most of the endemic species are of more recent, Pleistocene origin.

In contrast to their ancient origin, the species for which enough material was studied did not show a strong geographical structure, with the exception of *H. grandicollis*, with monophyletic divergent lineages in Sicily, Morocco, the Iberian peninsula and Slovenia. There are no apparent differences in the morphology of the aedeagus among the populations of *H. grandicollis*, and the nuclear markers used were not variable enough to show differences among them, although they may be the only case of recent speciation within the clade of western Mediterranean *Hydrochus*. The case of *H. flavipennis* is likely a problem of an unrevised taxonomy, with clearly different but unrecognised species (see above). The general pattern of large inter- but relatively low intra-lineage divergence suggests the existence of short coalescent times due to reduced population size and/or a high rate of population extinction (Charlesworth, 2009). This potential high extinction rate is, however, unlikely to be the reason for the observed decrease in diversification rates in the LTT plot, which has a well defined transition point (Quental and Marshall, 2009).

We have shown the existence of an ancient element of the Mediterranean fauna, likely to have persisted in the area through the Pliocene and Pleistocene epochs. The contribution of the glacial cycles in shaping the current diversity patterns in the Mediterranean has been widely recognised (Hewitt, 2000; Petit et al., 2003; Schmitt, 2007; Médail and Diadema, 2009), but the presence of old Miocene species is of special relevance both for the possibilities they offer to help to understand the origin of the Mediterranean fauna and their intrinsic evolutionary distinctiveness. In this sense, the view of some southern Mediterranean areas as ''cumulative refugia'', both cradles and museums of biodiversity (Médail and Diadema, 2009; Tzedakis, 2009) seems to be fully applicable also to at least some groups of arthropods.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.01.018.

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