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A molecular phylogeny of the cosmopolitan hyperdiverse genus *Hydraena* Kugelann (Coleoptera, Hydraenidae)

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Abstract. With almost 900 described species, *Hydraena* Kugelann (Hydraenidae) is one of the largest genera among Coleoptera. The subgeneric classification of *Hydraena* has been controversial, with 11 subgeneric names having so far been attributed to it. Some of these, *Haenydra* Rey and *Spanglerina* Perkins, have been treated as valid genera, as subgenera or as species groups. The most recent complete treatment of the genus, based on a cladistic analysis of morphological characters, recognized two major lineages, and only these were classified as subgenera: *Hydraenopsis* (mainly Gondwanan distribution), and *Hydraena* s.str. (mainly Laurasian). Here, we reconstruct the phylogeny of *Hydraena* using 212 species plus several outgroups and approximately 4 kb of sequence data from two nuclear (*SSU* and *LSU*) and four mitochondrial genes (*cox1*, *rrnL*, *trnL* and *nad1*). Data were aligned with two different strategies of multiple alignment (implemented in MAFFT and PRANK), and the phylogenies reconstructed using maximum likelihood and Bayesian methods. We estimated approximate ages of the main nodes using a relaxed molecular clock with Bayesian methods, and an *a priori* evolutionary rate of 0.01 substitutions/site/million years (Ma) plus a calibration point based on a biogeographical split. We found strong support for the monophyly of *Hydraena* and many of the clades recognized with morphological data. The following clades are considered as subgenera: *Phothydraena* Kuwert, *Spanglerina* Perkins, *Holcohydraena* Kuwert, *Hydraenopsis* Janssens and *Hydraena* s.str. The placement of three species groups, two Neotropical (*H. multispina* group, *H. paeminosa* group) and one South African/Madagascan (*H. monikae* group), is uncertain, and they are considered *incertae sedis* within *Hydraena*. The origin of the genus was estimated to be in the Lower Eocene, with many species complexes diversifying in the Pleistocene. Dispersal events seem to have played a key role in order to determine the current distribution of the species groups in the southern hemisphere (mainly in *Hydraenopsis*).

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Introduction

The genus *Hydraena* Kugelann, with almost 900 described species and hundreds of undescribed species, is the largest water beetle genus (see Appendix S1 for a complete checklist of *Hydraena* spp. with geographical distribution). Adults of *Hydraena* are small (approximately 1–3 mm long), and usually inconspicuously coloured. Most species are aquatic, living in the benthos of many different types of freshwater habitats, particularly in small streams and other bodies of running water. A few species are terrestrial, dwelling in the wet leaf litter and soil of tropical rainforests. The genus occurs on all continents except Antarctica. Many species have very restricted distributions (Jäch *et al.*, 2005; Jäch & Balke, 2008).

The concept of the genus is well defined and its monophyly is well supported by a number of morphological apomorphies (e.g. presence of a labral-mandibular interlocking device, and mentum with acute projection anteriorly; see Jäch *et al.*, 2000) and by molecular data (Ribera *et al*., 2011). Like all hyperdiverse groups, the generic/subgeneric classification of *Hydraena* has been subject to a complex history. Various authors have split *Hydraena* into several, often paraphyletic genera and subgenera (Rey, 1886; Kuwert, 1888; Seidlitz, 1888; Perkins, 1980, 1997; Berthélemy, 1986; Hansen, 1991, 1998), whereas other workers synonymized putatively monophyletic subgenera with *Hydraena* s.str. (Hansen, 1991, 1998; Perkins, 1997). Berthélemy (1986) discussed the evolution of some morphological characters of the genus, mainly the number of elytral striae and the morphology of the aedeagus. He recognized *Hydraenopsis*, *Phothydraena*, *Haenydra* and *Hadrenya* as valid subgenera. Perkins (1980, 1997) analysed in detail the exocrine secretion delivery system, a series of glandular pores and associated structures on the base of head and prothorax. Based on this system and the number of elytral striae, Perkins (1997) recognized *Haenydra*, *Hadrenya* and *Phothydraena* as valid subgenera, but synonymized *Hydraenopsis* with *Hydraena* s.str. Similarly, *Spanglerina*, described as a genus by Perkins (1980) and reduced to subgenus by Perkins (1989), was finally synonymized with *Hydraena* s.str. by Perkins (1997) due to similarities in the exocrine delivery system.

In the only global phylogeny of the genus published so far, Jäch *et al*. (2000) carried out a cladistic analysis using morphological characters of 24 representative taxa, and recognized two major lineages, which they regarded as subgenera: *Hydraenopsis*, with a hypothesized Gondwanan origin, and *Hydraena* s.str., with a Laurasian origin. Species of *Hydraenopsis* were found to share some characters of the adult head (incomplete or absent medial genal suture, short or absent pregula) and pronotum (structure of the hypomeral antennal cleaner) (Jäch *et al*., 2000). Six clades in addition to *Hydraena* s.str. (*Holcohydraena*, *H. monikae*, *H. circulata* group, *Phothydraena*, *Spanglerina*, *H. palustris* group) were not granted subgeneric status, because their relationships were not resolved satisfactorily in that analysis. Within the main lineage of *Hydraena* s.str., most of the commonly recognized species groups (or subgenera) were represented by a single species, and thus their monophyly could not be tested in the cladistic analysis, although a number of potential autapomorphies were discussed based on the examination of hundreds of additional species.

Ribera *et al*. (2011) included some representative species of *Hydraena* as outgroups for the focal clade ('*Haenydra*' lineage), but once again incomplete sampling prevented any general conclusions being drawn about the phylogeny of the entire genus.

In this complex scenario, we aimed to reconstruct the molecular phylogeny of *Hydraena* using a comprehensive sample of more than 200 species of all main lineages and a combination of nuclear and mitochondrial markers. We specifically tested the phylogenetic scenario proposed by Jäch *et al*. (2000) using morphological data, and provide here a new subgeneric classification of the genus based on the results of the sequencing and on the congruence with morphological characters. Due to the comprehensive sampling, we were also able to assess the monophyly of some of the commonly recognized species groups and to provide an approximate temporal framework for the diversification of the genus, including discussions about the main trends in the geographical distribution of the species of *Hydraena*.

Materials and methods

Taxon sampling

The complete ingroup dataset comprises 213 specimens belonging to 212 species and one subspecies of *Hydraena* (*H. gracilis balcanica*), representing most of the generally recognized species groups of the genus. Sequences of '*Haenydra*' (70 specimens belonging to 69 species and one subspecies), as well as sequences of 41 species of *Hydraena* previously used as outgroups for '*Haenydra*' (see Appendix S2), were selected from Ribera *et al*. (2011) and Trizzino *et al*. (2011). Some additional sequences were obtained from Ribera *et al*. (2010a) and Abellán & Ribera (2011). In total, sequences of 102 species of *Hydraena* were newly obtained for this work (Appendix S2). As outgroups we used a selection of seven species of different genera of Hydraenidae and Ptiliidae, the latter being generally recognized as the sister group of Hydraenidae (Caterino *et al*., 2005; Hunt *et al*., 2007) and used to root the tree (Appendix S2).

For the Palaearctic species of *Hydraena*, the taxonomy and nomenclature of Jäch (2004) have been followed, except for *H. subintegra aroensis*, which was elevated to species rank by Jäch & Díaz (2012). For non-Palaearctic species we followed the taxonomy and nomenclature of Jäch *et al*. (2000) and Perkins (2007). Seven of the 102 newly analysed specimens represent new species to be formally described elsewhere (see Appendix S2).

DNA extraction and sequencing

Specimens were collected in the field and directly preserved in 96% ethanol or in pure acetone (some specimens of the '*Haenydra*' lineage). DNA was extracted from whole specimens by a standard phenol-chloroform extraction or by DNeasy Tissue Kit columns (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. Vouchers and DNA samples of the newly analysed specimens are kept in the collections of the Museo Nacional de Ciencias Naturales (MNCN, Madrid), and in the Institute of Evolutionary Biology (CSIC-UPF, Barcelona). DNA extraction was nondestructive, to preserve voucher specimens for subsequent morphometric and morphological analyses. Usually only males were sequenced, and the male genitalia (used for the identification of the species) were dissected and mounted prior to the extraction to ensure correct identification.

For all the 109 newly analysed specimens (including the seven outgroups) we amplified and sequenced four fragments, two mitochondrial [3′-end of cytochrome *c* oxidase subunit 1 (*cox1*), and 3′-end of large ribosomal unit plus the leucine transfer plus the 5′-end of NADH dehydrogenase subunit 1 (*rrnL* + *trnL* + *nad1*)], and two nuclear [small ribosomal unit (*SSU*) and large ribosomal unit (*LSU*); see Appendix S3 for primers and Appendix S2 for the new sequences]. For 59 species the 5′-end of *cox1* (the 'barcode fragment', Hebert *et al*., 2003) was also sequenced (Appendix S2). For some specimens the 3′-end *cox1* fragment was amplified using internal primers to obtain two smaller fragments of approximately 400 bp each (Appendix S3).

Amplifications were performed with the following general cycle conditions: initial denaturation at 95°C for 5 min, followed by 33–38 cycles of denaturation at 94°C for 1 min, annealing at 47–58°C for 30 s, 1 min extension at 72°C and a last 7 min elongation step at 72°C. Reactions were performed in a 25 μL volume containing 16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8 at 25°C), 3 mM $MgCl_2$, 1 mM of each dNTP, 0.8 pmol of each primer and 1.25 units of Taq DNA polymerase. Sequences were assembled and edited with GENEIOUS 5.4 (Drummond *et al*., 2011) or by SEQUENCHER 4.7 (Gene Codes, Inc., Ann Arbor, MI, U.S.A.). A total of 327 new sequences have been deposited in GenBank (EMBL accession numbers HE970771-HE971097; see Appendix S2).

Phylogenetic analyses

For the length-variable regions, we used two alignment strategies: multiple pairwise comparisons using the online version of MAFFT v.6.8 and the G–INS–I algorithm (MF; Katoh & Toh, 2008), and multiple progressive alignment modelling the evolution of indels with PRANK (PR; Loytynoja & Goldman, 2005). Phylogenetic analyses were conducted with Bayesian analysis and an approximate maximum likelihood algorithm (ML), as these methods allow the use of a molecular-clock approach to estimate divergence times. Bayesian analyses were conducted on a combined data matrix with MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001), using six partitions corresponding to the six genes (the *rrnL* + *trnL* fragment was considered a single partition, whereas the two *cox1* fragments as two different partitions due to the large amount of missing data in the barcode fragment). The use of alternative partitions (e.g. by codon position) was not explored due to the scarcity of data for some protein-coding genes. Also, previous results with similar data and related groups show no relevant topological differences between the gene and codon partitions (Hidalgo-Galiana & Ribera, 2011; Trizzino *et al*., 2011). The best-fitting model to analyse each partition was selected by JMODELTEST (Posada, 2008) using the Akaike information criterion (AIC). The MRBAYES runs used default values and saved trees every 1000. 'Burn-in' values were established after visual examination of plots of standard deviation of the split frequencies between the two simultaneous runs. Convergence between runs was assessed with TRACER v1.5 (Drummond & Rambaut, 2007).

A ML phylogenetic reconstruction was performed with RAXML v7.0.4, using the same partition as in MRBAYES and with a GTR + G + I model. We ran 100 replicates and selected the tree with the highest likelihood. Node supports were estimated using a fast bootstrapping algorithm (1000 replicates with the CAT approximation; Stamatakis *et al*., 2008), using the same partition by genes.

We ran some additional analyses with the nuclear data only (102 species, including representatives of all main clades; Appendix S2), using RAXML and the same alignments (MF and PR) and analytical procedure as for the combined data.

Estimation of divergence times

To estimate the relative age of divergence of the lineages, we used the Bayesian relaxed phylogenetic approach implemented in BEAST v1.6.1 (Drummond & Rambaut, 2007), which allows variation in substitution rates among branches. We used an uncorrelated lognormal relaxed molecular-clock model to estimate substitution rates and the Yule process of speciation as the tree prior. We ran two independent analyses sampling each 1000 generations, and used TRACER v1.5 to determine convergence, measure the effective sample size of each parameter and calculate the mean and 95% highest posterior density interval for divergence times. The initial 10% of each run was discarded as burn-in. Results of the two runs were combined with LOGCOMBINER v1.6.2 and the consensus tree compiled with TREEANNOTATOR v1.5.4 (Drummond & Rambaut, 2007).

Because of the absence of fossil records to calibrate the trees, we used as a prior a rate of 2.0% of pairwise divergence per Ma, established for a closely related family (Leiodidae) for a combination of mitochondrial markers, including those used here (Ribera *et al*., 2010b). This rate was estimated using the combined mitochondrial protein coding and ribosomal genes, so we applied a GTR + I + G model of DNA substitution with four rate categories for the combined mitochondrial data set only, omitting the nuclear markers and pruning all the

outgroups with the exception of the closer *Adelphydraena*. Main well-supported nodes were constrained to obtain the same topology as in the phylogenetic analyses. We set as a prior rate a normal distribution with average rate of 0.01 substitutions/site/Ma and a standard deviation of 0.001. We also included a calibration point based on the separation between the Peloponnesus and mainland Greece, estimated to have occurred approximately 2.5 Ma (Simaiakis & Mylonas, 2008). This age was set as the upper limit for the split between the Greek *Hydraena vedrasi* and its sister based on molecular data, the Peloponnesus endemic *H. jaechiana* (Trizzino *et al*., 2011). Therefore, for this clade of sister species we set a timing of most recent common ancestor (tmrca) with a truncated normal distribution, with an upper value equal to 2.5, a mean of 1.5 and a standard deviation of 0.5 (Trizzino *et al*., 2011).

Results

Molecular phylogeny

The final data matrix included 220 terminals (213 ingroup and seven outgroup species; see Appendix S2) and 3558 aligned characters with MF and 3823 with PR. The difference in length corresponds mostly to the expansion of hypervariable regions of the nuclear ribosomal genes. In both cases, the selected evolutionary model was GTR + I + G for each of the mitochondrial gene partitions, whereas HKY + G was selected for the gene *SSU* and K80 + G for *LSU*. A HKY + G model was implemented in MRBAYES separately for the nuclear genes, as K80 + G cannot be implemented in MRBAYES.

The two runs of MRBAYES converged to split frequencies lower than 0.05 after 12 Ma generations in the run with MF, and 0.01 after 38 Ma generations in the run with PR. We set the burn-in fraction to 2.5 and 4.0 Ma generations in the runs with MF and PR, respectively, by estimating an optimal effective sample size of trees with TRACER v1.5 (Drummond & Rambaut, 2007).

The four topologies obtained with the two alignment methods and with Bayesian probabilities and ML were very similar, especially in the nodes with good support (Figs 1, 2; Appendix S4). Differences affected nodes with low support, basically the resolution among some of the main clades (see below).

All analyses strongly supported the monophyly of *Hydraena* (Fig. 1), and the sister relationship between *H. paeminosa* and all other *Hydraena*. The ribosomal sequences of *H. paeminosa* had several long unique insertions, resulting in a very long branch that may have introduced some artefact in their phylogenetic placement (see Discussion). The next cladogenetic event splits a group including *Phothydraena* and *Spanglerina*, and then two main clades with, on the one hand, the rest of the groups considered to be plesiomorphic and, on the other, the derived species of *Hydraena* s.str. according to Jäch *et al*. (2000). The first clade includes the *H. rugosa* and *H. circulata* groups as sisters, being in turn sisters of the South African/Madagascan species of the analyses (= *H. monikae* group) plus *Hydraenopsis*. Only in the Bayesian analysis with the two alignments were the South African/Madagascan species monophyletic (although with low support). In the ML analyses, they form a paraphyletic group, with *H. monikae* and an undescribed South African species as sister of *Hydraenopsis*, again with low support (Figs 1, 2; Appendix S4a,c).

The topology of the large clade of the remaining *Hydraena* s.str. was also very similar across methods. In all cases, the *H. palustris* group was sister to the rest, followed by the *H. bisulcata* group, and then two sister clades including, on one side, the '*Haenydra*' lineage and, on the other side, a poorly resolved clade including most of the currently recognized Palaearctic species groups (Fig. 1), which were, in general, monophyletic and well supported.

Within *Hydraenopsis*, two main clusters were detected (Fig. 1): (i) a small clade including the Chinese *H. cordiformis* plus a mixed group of species from India, China and the Philippines; and (ii) a large, poorly resolved clade, grouping an assemblage of species from all biogeographical regions. Within this latter clade, some of the species groups, previously identified by morphology, were recognizable: *H. quadricollis*, *H. scabra*, *H. jojoorculloi*, *H. miyatakei*, *H. castanea* and *H. leechi* groups (see, e.g., Jäch & Díaz, 1998; Freitag & Jäch, 2007; Fig. 2).

The nuclear markers had, in general, lower variability, and the trees resulting from the RAXML analyses had poor resolution and support (Appendix S5). Most of the main clades were, however, recovered with both alignments, including the monophyly of *Hydraena*, the monophyly of *Spanglerina* plus *Phothydraena* (with a paraphyletic *Phothydraena*), the monophyly of the *H. rugosa* plus *H. circulata* groups (with a paraphyletic *H. rugosa* group) and of *Hydraenopsis* plus the *H. monikae* group, and the monophyly of *Hydraenopsis* (Appendix S5). The clade of *Hydraena* s.str. was monophyletic with the MF alignment, but included *Phothydraena* plus *Spanglerina* with the PR alignment (Appendix S5). Within this large group of *Hydraena* s.str., the resolution was very poor due to the low variation of the sequences, but some species groups could be recognized, such as, for example, the *H. bisulcata*, *H. holdhausi* or *H. minutissima* groups (see Discussion).

Molecular clocks and diversification

For the analysis with BEAST (Fig. 3) we excluded *H. paeminosa* because of its very long branch and the possibility that its position was due to some analytical artefact. Differences between the length of the mitochondrial sequence with MF and PR were smaller than for the nuclear sequence (2297 and 2449 bp, respectively), and mostly reduced to some variable regions in the gene *rrnL*. We constrained nine basal nodes to ensure that the topologies of the main clades were congruent with those obtained in the phylogenetic analyses, most of them with high support (Fig. 1). The estimated ages were very similar for the two alignments (Table 1).

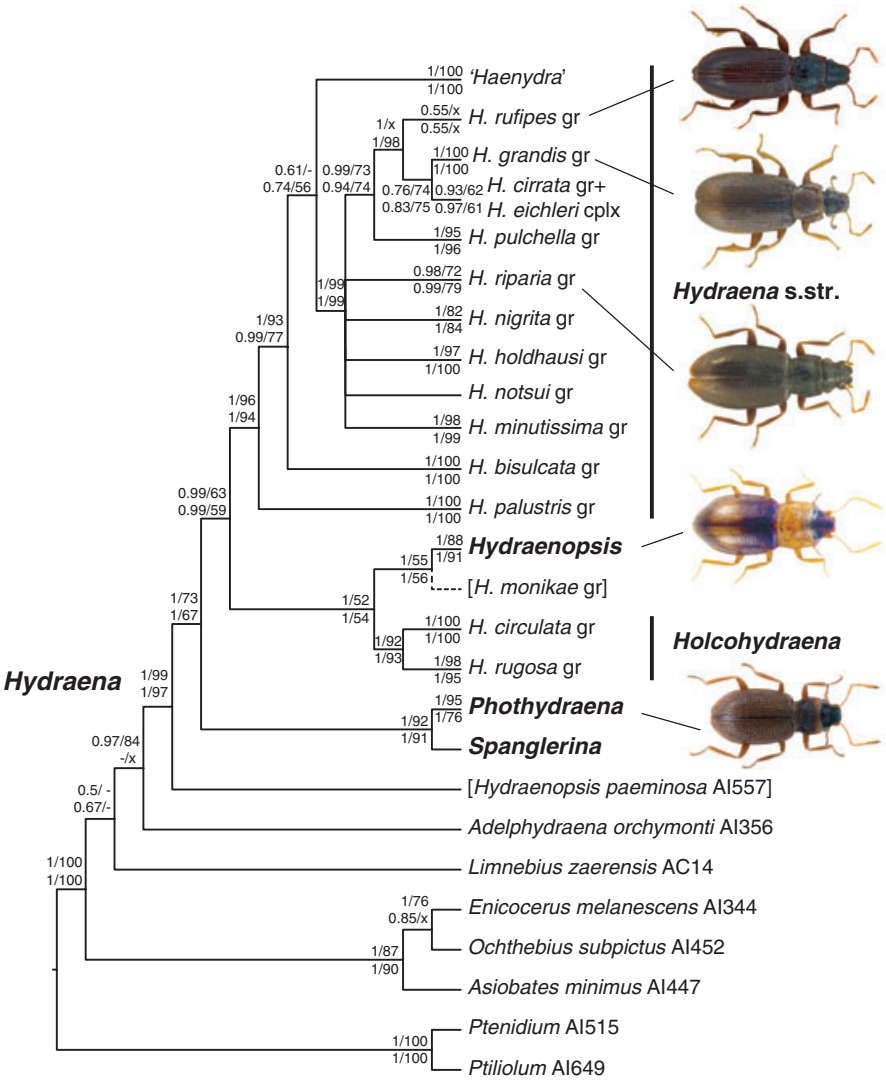

Fig. 1. Summarized cladogram of the phylogenetic relationships of the main lineages within *Hydraena*, according to the results of the different analyses. See Fig. 2 and Appendix S2 for the species included in the terminal groups and their geographical origin. Numbers in nodes, Bayesian posterior probabilities in MRBAYES (Bpp), when > 0.5/bootstrap values in RAXML (bt), when > 50%. Upper row, PR alignment, lower row, MF alignment; '–', compatible nodes (i.e. present with Bpp < 0.5; bt < 50%); 'x', incongruent nodes (see Appendix S4 for alternative topologies). In square brackets, species '*incertae sedis*' (see Appendix S1). Dotted line, species of the *H. monikae* group, not monophyletic in some analyses (Fig. 2a). Habitus photographs, from top to bottom: *H. berthelemyiana*, *H. grandis*, *H. janeceki*, *H. sanagergelyae*, *H. paganettii* (not at the same scale).

Using a calibration of 0.01 substitutions/site/Ma in combination with the constraint of the age of separation between *H. vedrasi* and *H. jaechiana* (see Materials and methods), the origin of *Hydraena* was estimated to be at approximately 43 Ma (Lower Eocene), with a wide confidence interval (as is usual in this type of analysis; Table 1). The split between *Phothydraena* + *Spanglerina* and the remaining *Hydraena* occurred approximately 34–35 Ma, whereas the separation between *Hydraena* s.str. and the remaining groups (incl. *Hydraenopsis*) occurred approximately 32 Ma. The separation between the *H. rugosa* group + *H. circulata* group and *Hydraenopsis* occurred 31–32 Ma, while the major derived *Hydraena* clades likely originated about 8–25 Ma (Miocene; Fig. 3). Finally, the majority of species groups were less than 15 Ma old, whereas most of the terminal clades of the allopatric sibling species were estimated to be less than 3 Ma old (i.e. of Plio-/Pleistocene origin).

Subgeneric classification

Our phylogenetic results support the classification of the species of *Hydraena* s.l. in five monophyletic groups, well supported and largely concordant with previously recognized

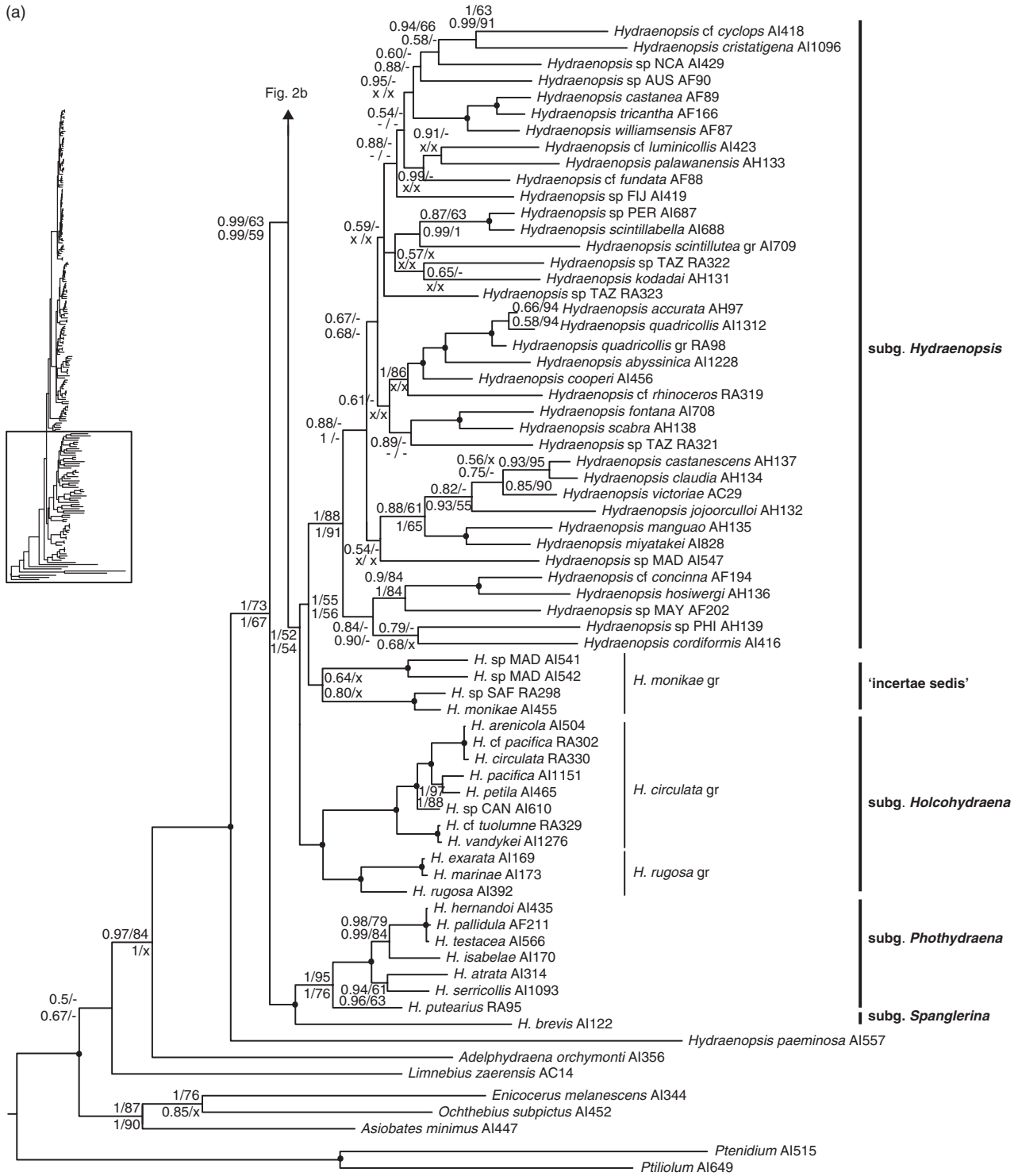

Fig. 2. Phylogram obtained by MRBAYES with the combined data and the alignment with PRANK. Numbers in nodes, Bayesian posterior probabilities in MRBAYES (Bpp), when > 0.5/bootstrap values in RAXML (bt), when > 50%. Upper row, PR alignment, lower row, MF alignment; '−', compatible nodes (i.e. Bpp < 0.5; bt < 50%); 'x', incongruent nodes (see Appendix S4 for alternative topologies). For simplicity, nodes with support values of Bpp = 1 and bt = 100 for the two alignments are indicated by a black circle, and values of Bpp = 1 and bt = 100 for one alignment are indicated by '1'.

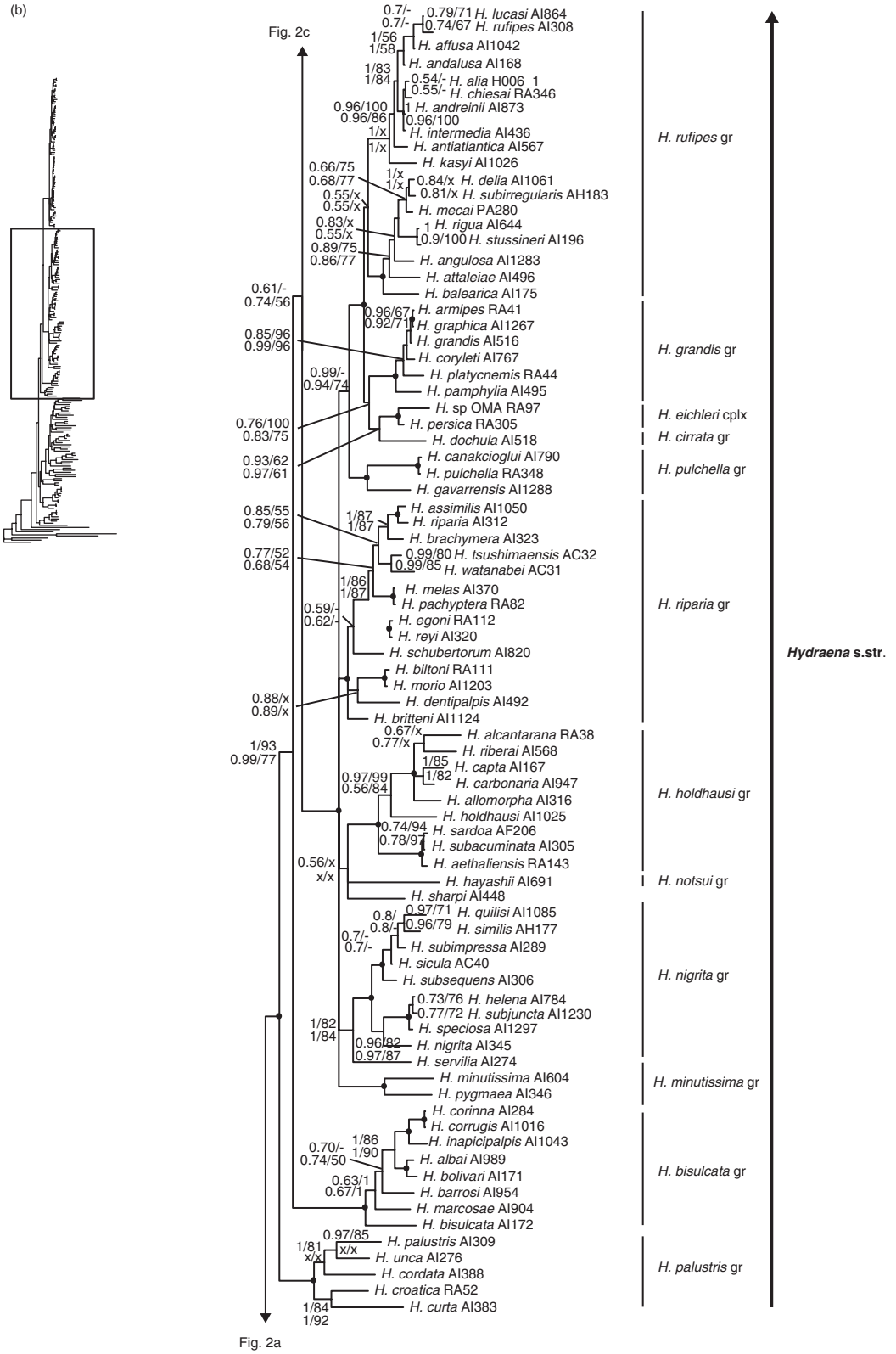

Fig. 2. Continued

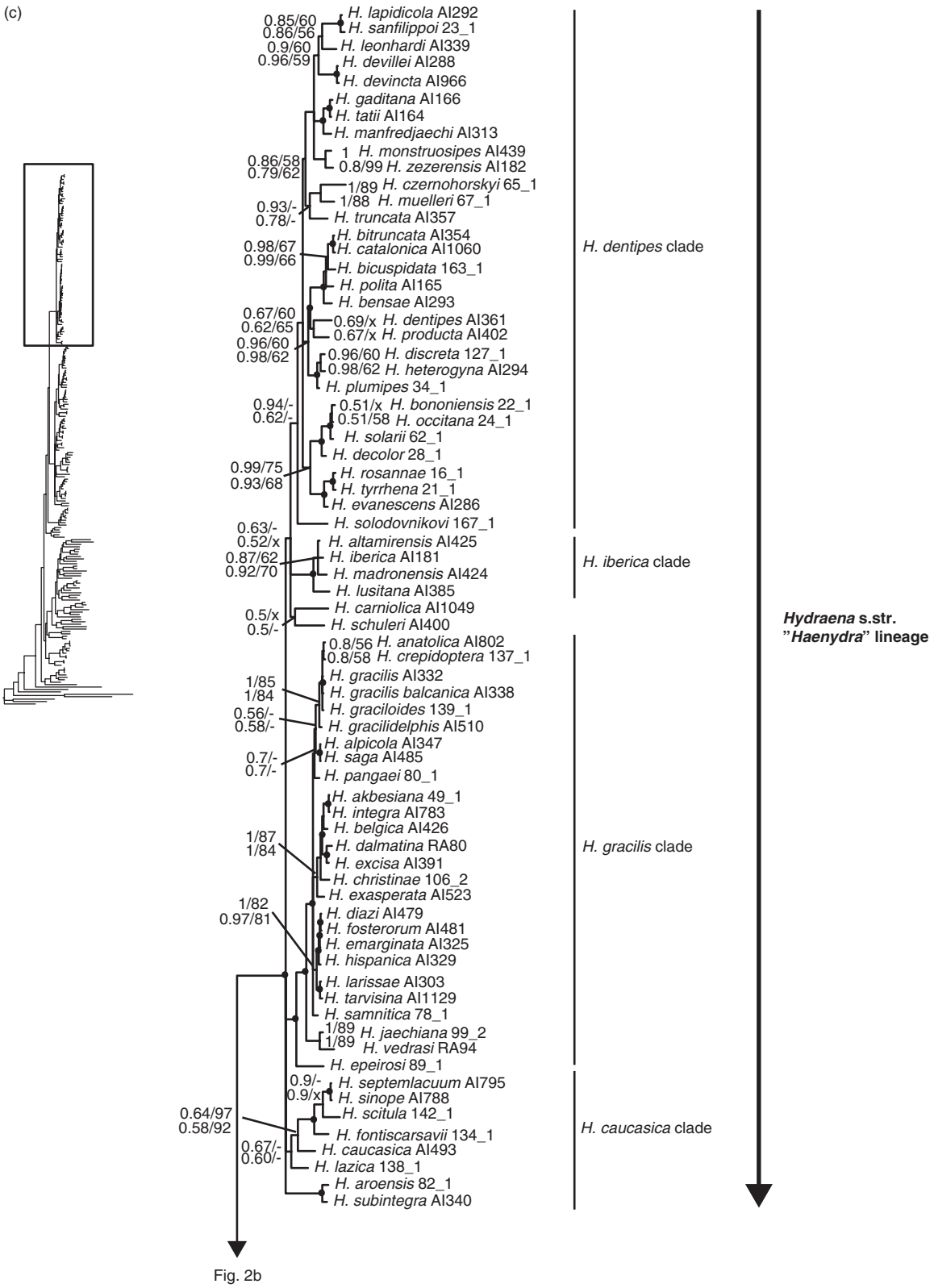

Fig. 2. Continued

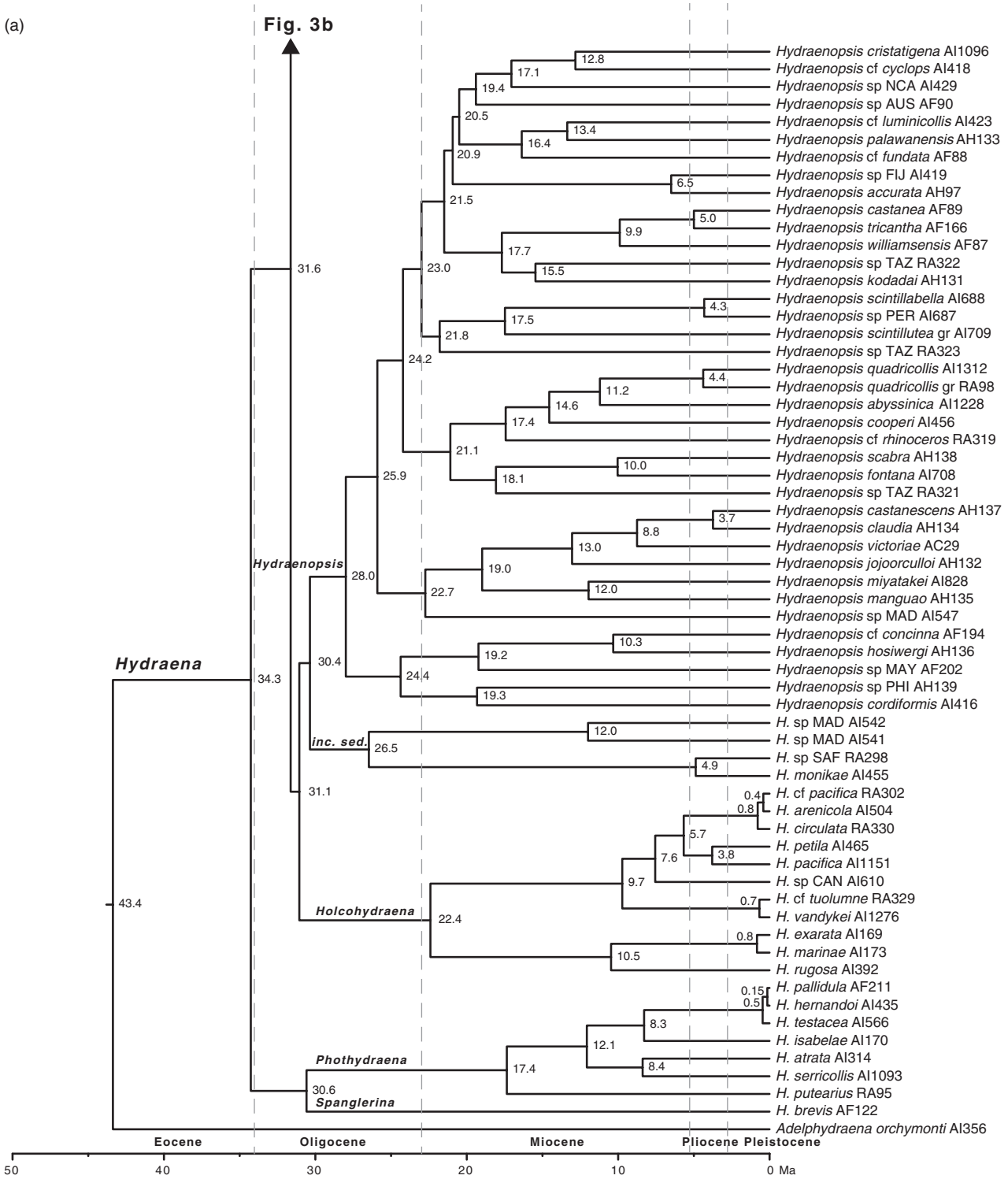

Fig. 3. Ultrametric tree obtained by BEAST with the mitochondrial sequences of the PR alignment, with a molecular rate of 2% divergence/ Ma and the constraint of the split between *H. jaechiana* and *H. vedrasi* (red circle, see Materials and methods) at 1.5 Ma. Some nodes were constrained to be congruent with those obtained with the combined dataset (black circles).

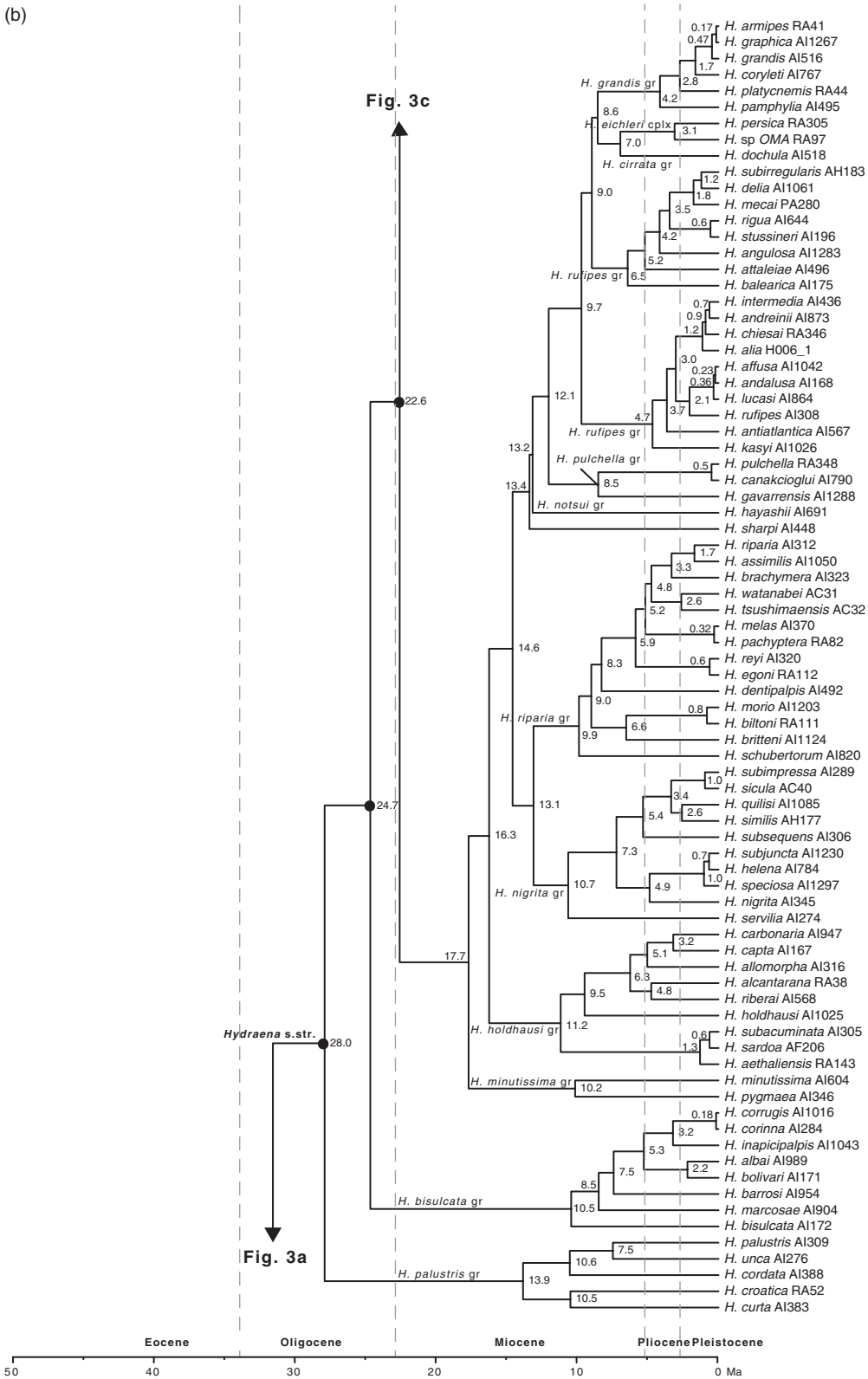

Fig. 3. Continued

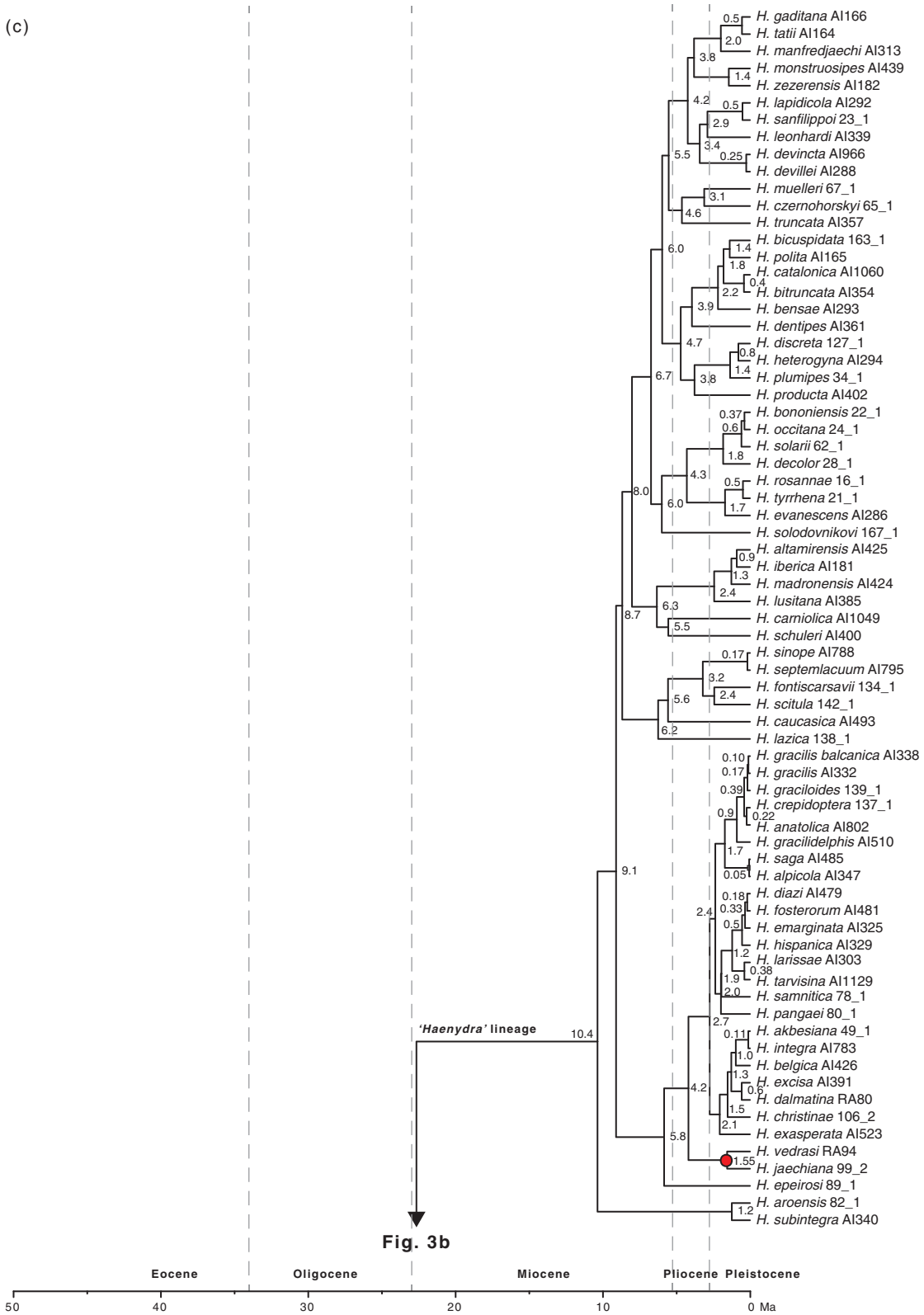

Fig. 3. Continued

Table 1. Estimated ages [Ma (95% confidence interval)] obtained in BEAST for some nodes in the phylogeny of *Hydraena* with the two alignments.

Clade	PRANK	MAFFT
Hydraena, stem group	43.4 (31–58)	43.3 (30–58)
*Hydraena**	34.3 (27–42)	34.5 (27–42)
Phothydraena + *Spanglerina**	34.2 (22–39)	29.4 (21–38)
*Phothydraena**	17.4 (11–24)	21.5 (14–29)
Hydraena excl.* (*Phothydraena* + *Spanglerina*)	31.1 (25–38)	32.1 (26–40)
*Holcohydraena**	22.4 (15–30)	22.3 (15–30)
H. rugosa group	10.5 (6–16)	10.9 (6–16)
H. circulata group	9.7 (6–13)	9.7 (6–13)
*Hydraenopsis** + *H. monikae* group	30.4 (24–37)	30.8 (24–38)
*Hydraenopsis**	28.0 (22–34)	28.2 (22–34)
Hydraena s.str.*	28.0 (22–35)	27.9 (22–35)
H. palustris group	13.9 (9–19)	14.2 (9–19)
Hydraena s.str. excl. *palustris* group*	24.7 (19–31)	25.3 (20–32)
H. bisulcata group	10.5 (7–13)	10.8 (7–14)
Hydraena s.str. excl. (*palustris* and *bisulcata* grs)*	22.6 (17–28)	23.6 (18–30)
H. minutissima + *notsui* + *holdhausi* + *nigrita* + *riparia* + *pulchella* + *cirrata* + *eichleri* +*grandis* + *rufipes* groups	17.7 (14–22)	18.5 (14–23)
H. minutissima group	10.2 (6–15)	10.7 (6–15)
H. holdhausi group	11.2 (8–15)	11.4 (8–15)
H. nigrita group	10.7 (8–14)	10.4 (8–13)
H. riparia group	9.9 (7–12)	10.1 (7–13)
H. pulchella group	8.5 (6–12)	9.1 (6–13)
H. cirrata group	7.0 (5–10)	7.0 (5–10)
H. grandis group	4.2 (3–6)	4.1 (3–6)
H. rufipes group	–	9.1 (7–12)
Haenydra lineage	10.4 (8–13)	10.6 (8–14)

Unless specified, all ages refer to crown groups (i.e. the age of the last common ancestor of all included species in the clade). Asterisks indicate nodes which monophyly was constrained in the BEAST analyses (see Fig. 3).

taxa that we reconsider here as subgenera (see Appendix S1 for a complete list of subgenera and species):

Genus *Hydraena* Kugelann, 1794. Type species: *H. riparia* Kugelann, 1794, by monotypy.

1. Subgenus *Phothydraena* Kuwert, 1888. Type species *Hydraena testacea* Curtis, 1830, by monotypy. Originally described as subgenus, in the more recent literature treated as subgenus (e.g. Berthélemy, 1986; Perkins, 1997) or as species group of *Hydraena* s.str. (Jäch *et al*., 2000). It currently includes nine species of western Palaearctic distribution.
2. Subgenus *Spanglerina* Perkins, 1980. Type species *Spanglerina ingens* Perkins, 1980, by original designation. Originally described as genus, later downgraded to subgenus (Perkins, 1989) and subsequently synonymized with *Hydraena* s.str. (Perkins, 1997). Currently it includes four species distributed in Central America (Perkins, 1980).
3. Subgenus *Holcohydraena* Kuwert, 1888 (synonym: *Taenhydraena* Kuwert, 1888). Type species *Hydraena rugosa* Mulsant, 1844, designated by Berthélemy (1986). Originally described as subgenus, it was synonymized with *Hydraena* s.str. by Berthélemy (1986). The 24 species are distributed in the western Palaearctic (*H. rugosa* group, three species) and in the Nearctic (*H. circulata* group, 21 species).
4. *Hydraenopsis* Janssens, 1972. Type species *Hydraenopsis vietnamensis* Janssens, 1972, by original designation. Originally described as a genus, it was considered as a subgenus by several authors (e.g. Jäch, 1986; Perkins, 1989; Jäch *et al.*, 2000). Perkins (1997) synonymized it with *Hydraena* s.str. However, our phylogenetic concept of *Hydraenopsis* agrees with Jäch *et al.* (2000), but excludes *H. paeminosa*. Currently, this subgenus includes 458 species, mostly distributed in the southern hemisphere, but occurring also in the Nearctic and in the southern and eastern Palaearctic (Appendix S1).
5. *Hydraena* s.str. Kugelann, 1794 (synonyms: *Hadrenya* Rey, 1886; *Haenydra* Rey, 1886; *Hoplydraena* Kuwert, 1888). According to our analyses, this subgenus corresponds to the concept of *Hydraena* s.str. published by Jäch *et al*. (2000) with the exclusion of the species of *Holcohydraena*, *Phothydraena* and the *H. monikae* group. In accordance with Jäch *et al*. (2000), *Haenydra* and *Hadrenya* remain synonyms, as their consideration as subgenera would render *Hydraena* s.str. paraphyletic. Since numerous species (especially from the eastern Palaearctic Region) were not available for sequencing, we have not been able to further divide the subgenus or to draw final conclusions about species groups. Currently, *Hydraena* s.str. includes 385 species living in the Palaearctic Region and the northern margin of the Oriental Realm.

Three species groups, all of them plesiomorphic, have not been included in any of these subgenera: the Neotropical *H. multispina* and *H. paeminosa* groups (Perkins, 2011b) and the South African/Madagascan *H. monikae* group (includes one described species and several undescribed ones). Unfortunately, no species of the *H. multispina* group could be obtained for sequencing. Due to the uncertainty of the phylogenetic position of these three species groups, plus the lack of available subgeneric names, we prefer to provisionally consider them as '*incertae sedis*' within the genus *Hydraena*.

Discussion

Phylogeny and biogeography of Hydraena s.l.

We obtained a very robust phylogeny of *Hydraena* s.l. despite minor differences in the alignment and reconstruction method. Differences were restricted to poorly supported nodes and generally affected the internal phylogeny of the main clades, not the backbone of the tree, which was remarkably stable. According to our results, *Hydraena* is monophyletic and originated in the lower Eocene. The incomplete sampling

of some of the lineages of the genus, and in particular *Hydraenopsis*, does not allow a formal reconstruction of its geographical origin. It is interesting to note, however, that two of the main clades have a sister relationship between western Palaearctic and Nearctic or Neotropical species (*Phothydraena* plus *Spanglerina*, and the two species groups of *Holcohydraena*: *H. rugosa* and *H. circulata*; see Fig. 1). These disjunct distribution patterns cannot be explained by the tectonic opening of the Atlantic (which was fully completed by the Early Eocene; Hallam, 1994). *Holcohydraena* probably dispersed across the Bering Strait during the Miocene.

The position of *H. paeminosa* as sister of the remaining species of *Hydraena* is surprising but very consistent across methods. Morphologically, it combines plesiomorphic (gonocoxite divided) and highly derived (aedeagal morphology) characters. The lack of data of other species of the *H. paeminosa* group and the probably related *H. multispina* group (Perkins, 2011b) does not allow final conclusions about its phylogenetic position. As noted earlier, the long branch of the species, due to some long insertions in the nuclear ribosomal genes, may have introduced some artefacts in the analyses.

The subgenus *Phothydraena* is characterized by several unambiguous autapomorphies in the structure of the elytral punctures and in the presence of two pairs of glabrous metaventral plaques (Berthélemy, 1986; Jäch *et al*., 2000).

The only species of *Spanglerina* included in the study, *Hydraena brevis*, was in all analyses sister of *Phothydraena*. The species of *Spanglerina* share with *Phothydraena* the presence of a hyaline membrane on the pronotum (present also in *H. paeminosa*; see Jäch *et al*., 2000) and the very wide pseudepipleura. Synapomorphies of the species of *Spanglerina* are the strongly arched fronto-clypeal suture, the anteriorly and posteriorly strongly attenuate pronotum, and a derived aedeagus (Perkins, 1980).

The species of *Holcohydraena* share some plesiomorphic genital characters, and the absence of conspicuous male secondary sexual characters. It should be noted that no unequivocal morphological synapomorphies between the two species groups of this subgenus (*H. rugosa* group and *H. circulata* group) have so far been identified (Jäch *et al*., 2000). Further studies are thus required to better characterize this subgenus. On the other hand, a separate subgeneric status for the *H. circulata* group could be taken into consideration. However, since there is no published name for this group available, we refrain from a formal description.

The position of the South African/Madagascan species of this analysis (*H. monikae* group) is not well established in our phylogenies. They seem to be related to *Hydraenopsis*, but whether they are monophyletic or paraphyletic still remains questionable. We have studied the only formally described species of this group (*H. monikae*) plus three undescribed species, but the inclusion of more of the many undescribed species of the area (P. Perkins, personal communication) may contribute to the clarification of their phylogenetic position. As noted by Jäch *et al*. (2000), *H. monikae* shares some plesiomorphic characters with species belonging to *Phothydraena*, *Holcohydraena* and some *Hydraenopsis* (e.g. presence of an intercoxal cavity on abdominal sternites II and III).

Hydraenopsis is, together with *Hydraena* s.str., the most diverse clade of the genus. The analysed dataset comprises just a small representation of the 459 described species (Janssens, 1972; Jäch *et al*., 2000; Perkins, 1980, 2007, 2011a,b; see Appendix S1 for an updated checklist). Notwithstanding this, our sampling included species from a wide geographic range (Australia & Pacific, South America, Asia, Africa), representing many of the morphologically identified species groups. All the analyses grouped the sampled *Hydraenopsis* spp. into a well supported monophyletic group, with several morphological synapomorphies in the structure of the gena and the gula (Jäch *et al*., 2000).

The relatively recent origin for the diversification within the genus *Hydraena* estimated by BEAST excludes the hypothesis that the current distribution of *Hydraenopsis* was driven by vicariance as a consequence of the fragmentation of Gondwana (which was completed by the Late Cretaceous, as noted earlier). Our data suggest instead that *Hydraenopsis* probably started to diversify $\sim$ 28 Ma in the Oriental Region and that the current geographical distribution is due to dispersal events to the Australian, African and American continents. The Neotropical species have a rather derived position within *Hydraenopsis* (Fig. 2A), suggesting a relatively recent colonization from either Africa or eastern Asia. The presence of *Hydraenopsis* in some remote Pacific Islands (e.g. Fiji; Appendix S1) demonstrates their ability for long-distance transoceanic dispersal. The colonization of South America from Africa by dispersal has been hypothesized for reptiles and plants, thus indicating that dispersal may have been more important than traditionally assumed (de Queiroz, 2005; Rowe *et al*., 2010; Oaks, 2011; Townsend *et al*., 2011).

Within *Hydraenopsis*, the phylogeny remains largely unresolved, probably because of the limited number of analysed species. The sister relationships of a clade of Oriental species, including *H. cordiformis*, with the rest of the subgenus agrees with the morphological analyses of Jäch *et al*. (2000) but is not generally well supported. Within the subgenus, some of the previously defined species groups could be recognized (e.g. *H. quadricollis*, *H. scabra*, *H. jojoorculloi*, *H. miyatakei*, *H. castanea* and *H. leechi* groups), but there are no clear geographical patterns and at this stage it seems impossible to define the composition of these lineages with any confidence.

Hydraena s.str. includes the majority of the Palaearctic species of the genus (except for *Phothydraena*, *Holcohydraena* and several species of *Hydraenopsis*). Within this subgenus, several monophyletic species groups were recognized.

The *Hydraena palustris* group includes a small number of European/North African species (Figs 1, 2B). This clade was, in all analyses, sister to the rest of *Hydraena* s.str. Species of the *H. palustris* group share a single, presumably plesiomorphic character (i.e. the structure of the intercoxal cavity) with *H. monikae*, *Holcohydraena* and *Phothydraena* (Jäch *et al*., 2000). The species of the *H. palustris* group are characterized by a similar habitus, by the anteriorly strongly emarginate pronotum, by the absence of conspicuous

secondary sexual characters and by rather simple male genitalia, resembling those of other plesiomorphic groups of *Hydraena*.

The rest of the large and poorly resolved *Hydraena* clade includes three main lineages: *H. bisulcata* group, *H. riparia* and related groups, and the '*Haenydra*' lineage.

The *H. bisulcata* group includes eight species with south-western European/northeastern African distribution. In previous analyses it was found to be sister to '*Haenydra*' (Ribera *et al*., 2011; Trizzino *et al*., 2011), although in general with low support. It must also be noted that our dataset did not include any species of the Chinese *H. armipalpis* group, which may be closely related to '*Haenydra*' (Jäch *et al*., 2000). Therefore, the phylogenetic placement of this large clade within the subgenus *Hydraena* could not be settled satisfactorily.

Within the lineage including *H. riparia* (plus related species groups), species with close morphological affinities (e.g. aedeagal similarities) were usually placed together with strong support. Some of these clades correspond to traditionally recognized species groups, such as the *H. riparia*, *H. nigrita*, *H. minutissima*, *H. grandis*, *H. cirrata*, *H. holdhausi* or *H. rufipes* groups (Figs 1, 2A; e.g. Jäch, 1988; Berthélemy *et al*., 1991; Jäch & Skale, 2009). However, the composition of these groups and the relationships between them are still uncertain, with low support for some nodes and alternative topologies.

The *H. riparia* and *H. nigrita* groups were recovered in a single monophyletic clade by some analyses, but paraphyletic in others. The *H. riparia* group includes species usually larger in size and with more marked secondary sexual characters (especially in the maxillary palps), while the species of the *H. nigrita* group are usually smaller, with less conspicuous secondary sexual characters.

Species of the *H. grandis* group share a very large body size (sometimes being more than 3 mm long), marked secondary sexual characters and very complex male genitalia. According to molecular data, this clade is monophyletic, well supported and sister to the few analysed species of the *H. cirrata* group + *H. eichleri* complex (sensu Jäch & Kasapoğlu, 2006) (Fig. 2B). The split between the *H. grandis* group and the *H. cirrata* group + *H. eichleri* complex occurred around 8–9 Ma, whereas the majority of species arose more recently, during the Pleistocene glacial cycles (Fig. 3).

The *H. rufipes* group was not monophyletic in some analyses (Fig. 2B; Appendix S4). Further morphological studies are required to clarify the relationships within this group. In all analyses, *H. pulchella* and its allies were placed in the same clade as the *H. grandis* and *H. rufipes* groups, although generally with low support (Fig. 2B).

The *H. minutissima* group was considered as a valid subgenus or even as a genus (*Hadrenya*) by several authors (Berthélemy, 1986; Hansen, 1991; Perkins, 1997). In agreement with Jäch *et al*. (2000), the analyses of the molecular data placed the species of this group within the clade of *H. riparia* and related groups. The *H. minutissima* group was hypothesized to be related to '*Haenydra*' by Perkins (1997), which could not be confirmed in our analyses.

Finally, the *H. holdhausi* group (Berthélemy *et al*., 1991) is monophyletic and well supported, with the Thyrrhenian species (*H. subacuminata* from Corsica, *H. sardoa* from Sardinia, and *H. aethaliensis* from Elba) being sisters of the rest (Fig. 2B). The molecular-clock estimations suggested that the current distribution of the species of this group could not be due to vicariance as a consequence of the fragmentation and rotation of the Sardinian micro-plate from the Iberian Peninsula, which occurred some 33–25 Ma ago (Schettino & Turco, 2006). Our estimations suggest that the *H. holdhausi* group originated at the end of the Miocene, with subsequent dispersals during the Tortonian and Messinian (Fig. 3), a biogeographical scenario similar to that recently proposed for the origin and radiation of Corso-Sardinian members of the '*Haenydra*' lineage (Trizzino *et al*., 2011).

Some aspects of the phylogeny and diversification of the '*Haenydra*' lineage were discussed in detail by Ribera *et al*. (2011) and Trizzino *et al*. (2011). This large derived group originated approx. 9–10 Ma in the Tortonian (Fig. 3). There are four main clades – the *H. iberica*, *H. dentipes*, *H. caucasica* and *H. gracilis* clades – which, based on our calibration, split at about 7–8 Ma (Fig. 2C).

The *H. iberica* clade comprises a group of four species, all endemic to the Iberian Peninsula. According to DNA data this group originated in the late Miocene, although it diversified more recently, in the Plio-/Pleistocene.

Within the *H. gracilis* clade, which includes more than 25 species with an articulated aedeagal distal lobe (Trizzino, 2011; Trizzino *et al*., 2011), our analyses recognized three monophyletic main groups (Fig. 2C), corresponding to the *H. gracilis*, *H. excisa* and *H. emarginata* complexes. With the exception of *H. gracilis* and *H. excisa*, both widely distributed in Europe, the species of this clade are usually highly endemic. Some of the Greek species of the *H. gracilis* lineage were placed in an isolated position, as sister to the rest of the clade: *H. jaechiana* (Peloponnesus), *H. vedrasi* (Balkan Peninsula) and *H. epeirosi* (Greece, including Peloponnesus) (Audisio *et al*., 1996).

The *H. caucasica* clade, corresponding to the *H. caucasica* group (sensu Trizzino, 2011), includes the easternmost species of '*Haenydra*'. These species are characterized by a relatively homogeneous habitus and by male genitalic features. Their relationships with the rest of '*Haenydra*' are not well established (Fig. 2C; Trizzino *et al*., 2011).

The *H. dentipes* clade includes a series of well supported subclades, corresponding to species complexes, which can be recognized based on their morphology and distribution: *H. dentipes*, *H. lapidicola*, *H. truncata*, and *H. evanescens* complexes (Ribera *et al*., 2011; Trizzino *et al*., 2011).

The *H. dentipes* complex includes ten species with a mostly western Palaearctic distribution. With the exception of two relatively isolated species (*H. dentipes* and *H. producta*), the *H. dentipes* complex is split into two monophyletic and allopatric clades including *H. heterogyna* and allies and *H. polita* and allies (Ribera *et al*., 2011; Trizzino *et al*., 2011). According to our calibration, these two clades had a common origin at the end of the Messinian, although

the relatives of *H. heterogyna* diversified more recently, around 1.4 Ma, whereas for *H. polita* and allies, the origin was estimated in the cold Plio-/Pleistocene transition, approximately 2.2 Ma.

The *Hydraena lapidicola* complex includes a dozen species (Fig. 2C), almost all exhibiting restricted and allopatric geographic ranges. All species are characterized by distinct male secondary sexual characters (e.g. concerning maxillary palps and/or legs) and relatively large body sizes. There are five monophyletic groups, which, in general, correspond to previously morphologically identified species complexes, including (i) *H. hungarica*, (ii) *H. lapidicola*, (iii) *H. devillei*, (iv) *H. tatii*, and (v) *H. monstruosipes* and relatives (Trizzino, 2011).

The *Hydraena truncata* clade includes three species: *H. truncata*, widespread in the western Palaearctic from Portugal to Ukraine, as well as *H. muelleri* and *H. czernohorskyi*, both endemic to an area comprising southern Austria, northeastern Italy, northwestern Slovenia and northern Croatia. These three species, seemingly well differentiated in external morphology (Trizzino, 2011), are of pre-Pleistocene origin.

The *Hydraena evanescens* clade includes seven species characterized by a circum-Tyrrhenian distribution. Molecular data suggest a split of this clade into two subclades corresponding to *H. decolor* and relatives (Audisio & De Biase, 1995), on one side, and the Corso-Sardinian *H. evanescens* and its two sister species on the other (Audisio *et al*., 2009).

Finally, *H. solodovnikovi*, from southwestern Russia, has an isolated and uncertain position within the *H. dentipes* clade.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2012.00654.x

Appendix S1. Checklist of the known species of the genus *Hydraena* Kugelann, 1794 (as of July 2012), including data on geographical distribution.

Appendix S2. List of studied material, with voucher number, locality, collector and accession numbers. In bold, sequences newly included in this study.

Appendix S3. List of primers used for sequencing.

Appendix S4. Phylogenetic trees obtained with (a) RAXML with PRANK alignment; (b) MRBAYES and MAFFT alignment; (c) RAXML with MAFFT alignment. Numbers at nodes, Bayesian posterior probability values (b) or bootstrap values (a, c).

Appendix S5. Phylogenetic trees obtained with the nuclear markers only and (a) RAXML with PRANK alignment; (b) RAXML with MAFFT alignment.

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