



Phylogeny and historical biogeography of Agabinae diving beetles (Coleoptera) inferred from mitochondrial DNA sequences

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Abstract

The Agabinae, with more than 350 species, is one of the most diverse lineages of diving beetles (Dytiscidae). Using the mitochondrial genes 16S rRNA and cytochrome oxidase I we present a phylogenetic analysis based on 107 species drawn mostly from the four main Holarctic genera. Two of these genera (*Ilybius* and *Ilybiusoma*) are consistently recovered as monophyletic with strong support, *Platambus* is never recovered as monophyletic, and *Agabus* is found paraphyletic with respect to several of the species groups of *Platambus*. Basal relationships among the main lineages are poorly defined, although within each of them relationships are in general robust and very consistent across the parameter space, and in agreement with previous morphological analyses. In each of the two most diverse lineages (*Ilybius* and *Agabus* including part of *Platambus*) there is a basal split between Palearctic and Nearctic clades, estimated to have occurred in the late Eocene. The Palearctic clade in turn splits into a Western Palearctic clade and a clade containing mostly Eastern Palearctic species, and assumed to be ancestrally Eastern Palearctic but with numerous transitions to a Holarctic or Nearctic distribution. These results suggest an asymmetry in the colonization routes, as there are very few cases of transcontinental range expansions originating from the Nearctic or the Western Palearctic. According to standard clock estimates, we do not find any transcontinental shift during the Pliocene, but numerous speciation events within each of the continental or subcontinental regions.

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1. Introduction

Dytiscidae diving beetles, with ca. 3800 known species (Nilsson, 2001), comprise one of the major lineages of aquatic Coleoptera. They are highly modified for living in a diversity of continental waters both as larvae and adults, showing a wide range of ecological strategies mainly reflected in different swimming behaviors and their associated morphotypes (Balke, in press; Ribera and Nilsson, 1995). Dytiscids have a world-wide distribution, with the highest species diversity in the tropical and subtropical regions (Balke, in press; Nilsson, 2001). However, a few north temperate lineages are very diverse, such as the subfamily Agabinae. The group in-

cludes more than 350 known species in 10 genera, which are mostly distributed in the Holarctic, with only a few small genera and species groups occurring in South America, sub-Saharan Africa, and the Oriental and Australian regions (Nilsson, 2000, 2001).

The Agabinae is a morphologically homogeneous group exhibiting few characters useful for systematics (Nilsson, 2000). They were traditionally considered a tribe within the subfamily Colymbetinae, and placed among the basal lineages of Dytiscidae (see, e.g., Guignot, 1933). Phylogenetic analyses based on 18S rRNA sequences confirm this basal placement, although the group does not form a monophyletic clade with Colymbetini (Ribera et al., 2002a). This is consistent with results obtained by Miller (2001) based on morphological characters, who accordingly raised Agabini to subfamily level. The Agabinae are characterized by the presence of a linear group of short, stout setae near

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the posterior ventroexternal angle of the metafemur (Brinck, 1948; Nilsson, 2000), although some species have apparently secondarily lost this character (e.g., *Hydronebrius* and some species of *Platambus*, Nilsson, 2000). Attempts to establish natural groups were made only recently, but because of the homogeneous morphology the delimitation of the Agabinae genera remains problematic (Nilsson, 2000). Two major groups of genera can be separated within the subfamily, according to the presence of a ventral setal fringe on the female metatibia and the shape of the clypeal fovea: the *Platynectes* group (four genera with a Neotropical and Oriental distribution) and the *Agabus* group (six genera with mostly Holarctic distribution). Within these genera, species groups have been defined on the base of character combinations, with no robust synapomorphies except for some subgenera of *Agabus* (Nilsson, 2000).

The Agabinae not only constitute a major element of the tree of Dytiscidae, they are also of interest to the study of species richness and historical biogeography. Species differ greatly in the overall size of their geographic ranges, with some species distributed very widely throughout much of the Palearctic, Nearctic, or both. On the other end of the spectrum, some species are limited to narrowly defined biogeographic regions, in particular those species occurring in mountain regions in the southern Palearctic (e.g., Fery and Nilsson, 1993; Guignot, 1933).

In this paper we present mtDNA data for all major lineages of Agabinae and a large number of the known species, with an emphasis on the Holarctic fauna. We also address the main geographical divisions between the Palearctic and Nearctic regions, and provide an approximate temporal framework for the evolution and the main vicariance events affecting lineages on both sides of the northern Atlantic and Pacific oceans.

2. Material and methods

2.1. Taxon sampling and DNA sequencing

The ingroup included a total of 118 individuals from 107 species in five genera of Agabinae, with representation of all major lineages (Table 1 and Appendix A). Sampling was focussed on the larger genera of the *Agabus* group *sensu* Nilsson (2000) (*Agabus*, *Ilybius*, *Platambus*, and *Ilybiosoma*) which has a predominantly Holarctic distribution. No specimens of the two smaller genera within this group (*Hydrotrupes*, one species, and *Hydronebrius*, four species) could be obtained for study. Due to the taxonomic homogeneity of the genus *Ilybiosoma*, and the difficulty in separating the currently recognized species (Larson et al., 2000), repeated specimens of the only widespread species (*I. seriatum*) were included. Voucher specimens are kept in the collections of the Natural History Museum (London).

Outgroups included 24 species of all major lineages of Dytiscidae with the exception of Hydroporinae and Laccophilinae, plus families Hygrobiidae and Amphizoidae (Appendix A). This extensive selection of outgroups was considered necessary, as the phylogenetic position of Agabinae among the basal Dytiscidae lineages is still uncertain. All trees were rooted in Amphizoidae, which clearly belongs outside Dytiscidae (Miller, 2001; Ribera et al., 2002a,b). Rooting the tree in Amphizoidae allowed the relationships among the remaining outgroups and the ingroup to vary freely, thus testing the possibly monophyly of Agabinae and the *Agabus* group of genera.

Specimens were collected in absolute ethanol, and muscular tissue used for DNA isolation via a phenol–chloroform extraction as described in Vogler et al. (1993) or by extraction columns (Quiagen). Sequences of 16S rRNA (16S) were amplified as a single fragment of ca. 500 bp, using primers 16Sa (5' ATGTTTTTGT AAACAGGCG) and 16Sb (5' CCGGTCTGAACTC AGATCATGT). A single fragment of ca. 800 bp of COI (from the middle of region E3 to the 3' end, Lund et al., 1996) was amplified using primers “Jerry” (5' CAACA TTTATTTTGATTTTTTGG) and “Pat” (5' TCCAAT GCACTAATCTGCCATATTA) (Simon et al., 1994). It was not possible to obtain PCR products for the COI fragment of five species (*A. affinis*, *I. bedeli*, *I. apicalis*, *P. glabrellus*, and *A. nannup*), and for three species (*A. affinis*, *A. lapponicus*, and *P. pictipennis*) portions of the sequence at the beginning or the end of the sequence were missing. For the 16S fragment all specimens were successfully amplified, except for a small number of base pairs at either end of the fragment in some species. All new sequences generated in the study were deposited in GenBank under Accession Nos. AY138591–AY138768. Other sequences were obtained from Ribera et al. (2001a,b, 2002b) (Appendix A).

The following PCR cycling conditions were used for DNA amplification: 1'–2' at 95 °C, 30'' at 94 °C, 30'' at 47–50 °C (depending on the melting temperatures of the primer pair used), 1'–2' at 72 °C (repeated for 35–40 cycles), and 10' at 72 °C. Amplification products were purified using a GeneClean II kit (Bio 101). Automated DNA sequencing reagents were supplied by Perkin Elmer Applied BioSystems (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit). Sequencing reactions were purified by ethanol precipitation and were electrophoresed on an ABI3700 sequencer. Sequence ambiguities were edited using the Sequencher 3.0 software package (Gene Codes Corporation).

2.2. Phylogenetic analysis

Sequences for COI were identical in length, and there were no major differences in the length of 16S

Table 1
Taxonomic coverage of the genera, subgenera, and species groups of Agabinae

Genus ^a	Subgenus	Sp. group	No. sp.	No. sp. sampled ^b
<i>Agabus</i>	<i>Acatodes</i>	<i>arcticus</i>	6	3
		<i>confinis</i>	33	8
		<i>fuscipennis</i>	5	—
		<i>japonicus</i>	12	3
		<i>lutosus</i>	3	2
		<i>obsoletus</i>	5	1(1)
		<i>raffrayi</i>	5	—
	<i>Agabus</i>	<i>aeruginosus</i>	3	—
		<i>antennatus</i>	1	1
		<i>clavicornis</i>	3	1
		<i>disintegratus</i>	3	1
		<i>falli</i>	1	—
		<i>labiatus</i>	7	2
		<i>lineatus</i>	2	1
		<i>punctulatus</i>	5	—
		<i>uliginosus</i>	5	—
		<i>Gaurodytes</i>	<i>adpressus</i>	2
	<i>affinis</i>		9	4
	<i>ambiguus</i>		5	4(2)
	<i>aubei</i>		1	1
	<i>brunneus</i>		4	4
	<i>guttatus</i>		23	11
	<i>nebulosus</i>		6	2
	<i>paludosus</i>		2	1
	<i>ragazzii</i>		5	—
	<i>striolatus</i>		1	—
	<i>taiwanensis</i>		1	—
	<i>tristis</i>		9	4
	<i>Agametrus</i>			7
<i>Andonectes</i>			1	—
<i>Hydronebrius</i>			4	—
<i>Hydrotrupes</i>			1	—
<i>Ilybiosoma</i>	<i>seriatum</i>	14	4(6)	
	<i>discicollis</i>	2	—	
<i>Ilybius</i>	<i>chalconatus</i>	19	8	
	<i>erichsoni</i>	3	2	
	<i>opacus</i>	13	6(2)	
	<i>subaeneus</i>	32	21	
<i>Leuronectes</i>			5	—
<i>Platambus</i>	<i>americanus</i>	2	—	
	<i>confusus</i>	1	—	
	<i>glabrellus</i>	2	2	
	<i>maculatus</i>	17	3	
	<i>optatus</i>	14	3	
	<i>sawadai</i>	5	—	
	<i>semenovi</i>	4	—	
	<i>semivittatus</i>	6	2	
<i>spinipes</i>	4	—		
<i>Platynectes</i>	<i>Australonectes</i>	1	—	
	<i>Gueorguievtes</i>	26	2	
	<i>Platynectes</i>	11	—	
Total			361	107(11)

^a Classification and number of described species follows Nilsson (2001).

^b In brackets, multiple specimens of the same species.

sequences (see below). Alignment was performed in a two step procedure (Phillips et al., 2000), aligning sequences in ClustalW (Higgins et al., 1992) followed by tree searches on the aligned matrix using parsimony analysis in PAUP4.0b6 (Swofford, 1999). Three different parameter settings were used in ClustalW: Alignment 1 (default values, i.e., gap cost 15, extension cost 6.66), Alignment 2 (gap cost 2, extension cost 1), and Alignment 3 (same parameters as 1, with some obvious mismatches manually corrected). PAUP searches consisted of TBR heuristic searches of 10,000 replicates, with gaps coded as a fifth character (Giribet and Wheeler, 1999). Among these parameter combinations, the preferred settings were selected according to the maximum congruence among genes, as measured with the incongruence-length difference test, ILD (Farris et al., 1994), and the modified ILD (WILD, Downton and Austin, 2002; Wheeler and Hayashi, 1998; Table 2). For the preferred alignment, analyses were repeated with gaps coded as missing characters, and after the deletion of the specimens with missing data. For all the alignments, characters were reweighted a posteriori according to the rescaled consistency index (RC) (Farris, 1969) and a complete heuristic search conducted on the best trees.

Constraint trees for determining partitioned Bremer Support values (Baker and DeSalle, 1997) were generated with Treerot (Sorenson, 1996), on both the equal weight and the reweighted trees obtained with the preferred alignment. The significance of incongruence among genes, and among the three codon positions of the COI gene, was tested with the Partition Homogeneity Test (Swofford, 1999) as implemented in PAUP.

2.3. Rate variation and biogeographical analysis

To estimate branch lengths, sequence variation of the ingroup only was fitted by maximum likelihood (ML) on the topology of the preferred parsimony tree. Optimum ML models for the genes COI and 16S, and

for the combined data, were selected using Modeltest 3.06 (Posada and Crandall, 1998). To estimate relative node ages we fitted ML branch lengths assuming a molecular clock and compared the likelihood to that obtained assuming no clock (Felsenstein, 1981). As the ML ratio was significant (see Section 3), an ultrametric tree was estimated using the non-parametric rate smoothing (NPRS) method of Sanderson (1997), as implemented in TreeEdit v1.0 (Rambaut, A. & Charleston, M.; *evolve.zoo.ox.ac.uk*). Approximate calibration of absolute time was based on the standard insect molecular clock estimate of 2% divergence per million years (Myr) for mtDNA (Brower, 1994), corresponding to a base rate (per branch) of 0.01 substitutions/site/Myr.

Geographical data on the distribution of species were obtained from Nilsson (2000, 2001) and Larson et al. (2000). The number of transitions between major biogeographical areas was reconstructed on the preferred tree using MacClade 4.0 (Maddison and Maddison, 2000), coding “Holarctic” distributions as uncertain.

3. Results

3.1. Sequence data and trees obtained under different alignment parameters

Pairwise sequence divergences among any two taxa ranged from zero for the 16S sequences of some species to 29% for the COI gene, between *Copelatus haemorrhoidalis* and *Platambus sculpturellus*. Maximum divergences within the ingroup were similar to those with the outgroups in both genes (17 and 18%, respectively, for the combined data). COI was not length variable, and sequence length for 16S ranged from 508 (*P. decempunctatus*, *P. sculpturellus*, *I. pandurus*, *I. fraterculus*, and *I. subaeneus*) to 513 nucleotides (*I. montanus*, *I. bedeli*, and *I. hozgargantae*).

Accepting that congruence among genes can be used as the criterion for the choice of the preferred

Table 2
Tree statistics and length of the aligned sequences

Alig. ^a	No. trees	Cost			ILD	WILD	CI	RI	No. Cha.	Inf. Cha.
		16S	COI	Com.						
1	112	1601	4910	6672	161	0.024	0.16	0.56	1280	530
2	144	3146	4910	8317	261	0.031	0.17	0.47	1460	654
3	>5000	1570	4910	6620	140	0.021	0.16	0.56	1284	531
3m	4434			6517			0.16	0.55	1284	522
3r	288			6711			0.16	0.55	1284	531

Alig., Alignment; Com., combined; ILD, incongruence-length difference test (Farris et al., 1994; ILD = length combined – sum length individual sets); WILD, Wheeler’s ILD (Wheeler and Hayashi, 1998; WILD = ILD/length combined); CI, consistency index; RI, retention index; No. Cha., length of the aligned sequence; Inf. Cha., number of informative characters.

^a 1, 2, 3: alignments, 1, gap cost 15, extension cost 6.66; 2, gap cost 2, extension cost 1; 3, gap cost 15, extension cost 6.66, modified by hand. m, Gaps as a missing character; r, species with missing data excluded (*A. confinis*, *I. apicalis*, *I. bedeli*, *P. glabrellus*, and *A. nannup*, see Section 2).

parameter set (Wheeler, 1995), the optimal tree was obtained from Alignment 3 (gap cost 15, extension cost 6.66, manually modified), with the lowest ILLD and WILD (Table 2). As expected, character reweighting increased the congruence among the different partitions (16S and the three codon positions of COI), as measured by partitioned Bremer support (PBS) (Table 4). In the equal weight tree, the correlation between the PBS of the COI third positions and that of the second and with the 16S gene are significantly negative ($p < 0.05$, 2 tails) (the average value of the COI second positions is negative, Table 4). The PBS values of the 16S gene are also negatively correlated with that of the second and first positions of COI, indicating that the phylogenetic signal of the data is

dominated by the COI third positions. On the contrary, on the reweighted tree all pairwise correlations among PBS (three COI positions and 16S) are significantly positive, indicating a higher level of congruence. The support of the signal is also shifted towards 16S and the first and second positions of COI (Table 4) with all partitions significantly and positively correlated with the total Bremer support value. This higher congruence among data partitions is reflected in the lack of incongruence in the Partition Homogeneity Test (not significant, $p \gg 0.05$).

In the trees obtained using the preferred Alignment 3, and after reweighting the characters a posteriori (Figs. 1 and 2; Table 3), Dytiscidae is recovered as monophyletic, with Agabinae (excluding *Platynectes*) sister to all

Table 3
Comparison of selected nodes in the phylogenetic analyses of Agabinae

No. ^a	Node	1	1R	2	2R	3	3R	3m	3mR	3r	3rR
1	Dytiscidae	1	0	1	0	0	1	1	0	1	0
	Agabinae + Colymbetinae	0	1	1	1	1	0	0	0	0	1
2	Colymbetinae + Dytiscinae	0	0	0	0	0	1	0	1	0	0
3	Agabinae excl. <i>Platynectes</i>	1	1	0	1	0	1	0	1	0	1
4	<i>Ilybiosoma</i> sister to <i>Agabus</i> s.lat.	1	1	0	0	0	1	0	0	0	1
	<i>Ilybiosoma</i> sister to <i>Ilybius</i>	0	0	0	0	0	0	0	1	0	0
	<i>Agabus</i> s.lat. sister to <i>Ilybius</i>	0	0	0	1 ^f	1 ^f	0	1 ^f	0	1	0
5	<i>Ilybiosoma</i>	1	1	1	1	1	1	1	1	1	1
6	<i>Ilybius</i>	1	1	0	1	1	1	1	1	1	1
7	<i>I. opacus</i> group	1	1	1	1	1	1	1	1	1	1
8	<i>I. chalconatus-ericsoni</i> group	1	1	1	1	1	1	1	1	1	1
9	<i>I. subaeneus</i> s.str. ^c	1	1	0	1	1	1	1	1	1	1
10	<i>Agabus</i> s.lat. ^d	1	1	0	1	0	1	1	1	1	1
11	<i>Gaurodytes</i> ^b	0	1	1	1	0	1	1 ^f	1	1	1
12	<i>A. guttatus</i> group	1	1	1	1	1	1	1	1	1	1
13	<i>Acatodes</i> + <i>A. ambiguus</i> group	1	1	1	1	1	1	1	1	1	1
	<i>A. brunneus</i> + <i>A. affinis</i> ^e groups	0	0	0	1	1	0	1	1	1	0
14	<i>A. brunneus</i> gr. sister to <i>Acatodes</i>	0	0	0	0	0	1	0	0	0	1
15	<i>A. brunneus</i> group	0	0	1	1	1	1	1	1	1	1
	<i>Platambus</i>	0	0	0	0	0	0	0	0	0	0
3	<i>P. maculatus</i> sister to Agabinae	0	0	0	0	1 ^f	1	1 ^f	0	1 ^f	0
	<i>P. maculatus</i> gr. sister to <i>Ilybius</i>	1	1	0	0	0	0	0	1	0	0

1, 2, 3: alignments (1, gap cost 15, extension cost 6.66; 2, gap cost 2, extension cost 1; 3, gap cost 15, extension cost 6.66, modified by hand); R, reweighted according to the RC; m, gaps as a missing character; r, species with missing data excluded (*A. confinis*, *I. apicalis*, *I. bedeli*, *P. glabrellus*, and *A. nannup*, see Section 2).

^a Node numbers refer to Fig. 2. (In bold, preferred tree).

^b Excluding the *A. brunneus* and *A. ambiguus* groups.

^c Excluding *I. apicalis* and the *I. angustior* complex.

^d Including *Platambus* except the *P. maculatus* group.

^e Including *A. lineatus*.

^f With the inclusion of some additional taxa.

Table 4
Average partitioned Bremer support values of the shortest reweighted trees obtained with Alignment 3 (gap cost 15, extension cost 6.66, modified by hand)

	COI 1st pos.	COI 2nd pos.	COI 3rd pos.	16S rRNA	Total
Equal weight	5.44 ± 6.63	-1.59 ± 4.25	0.47 ± 1.71	3.91 ± 8.24	8.24 ± 8.39
Reweighted	0.35 ± 0.44	0.02 ± 0.24	0.01 ± 0.15	0.38 ± 1.18	0.75 ± 1.42

Cell contents: average ± SD.

other included species of Dytiscidae, and Colymbetinae and Dytiscinae as single clades. Copelatinae is not recovered as monophyletic, nor Agabinae, as the two specimens of the genus *Platynectes* are always included

among non-Agabinae Dytiscidae. Within Agabinae, the *Agabus* group of genera sensu Nilsson (2000) (i.e., *Agabus*, *Platambus*, *Ilybius*, and *Ilybiosoma*) is, however, recovered as monophyletic, although with low bootstrap

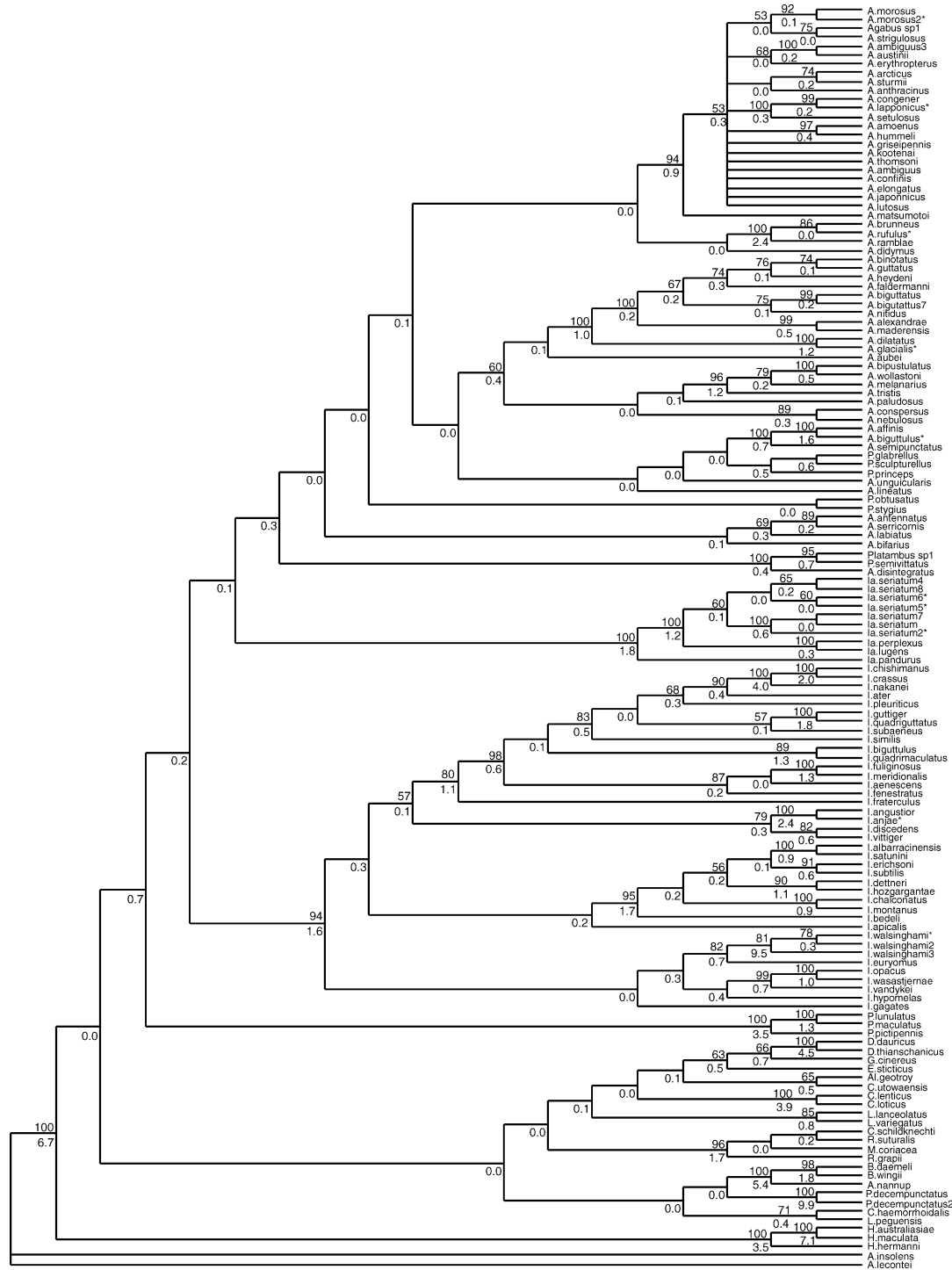


Fig. 1. Strict consensus of the 16 equally parsimonious trees obtained with the Alignment 3 (gap cost 15, extension cost 6.66, manually modified), after reweighting the characters according to the RC. Above branches, bootstrap values (only >50% shown); below branches, combined Bremer support values. *16S rRNA sequence identical to that of other species (see Appendix A). Nodes with 0.0 have positive Bremer support values lower than 0.05.

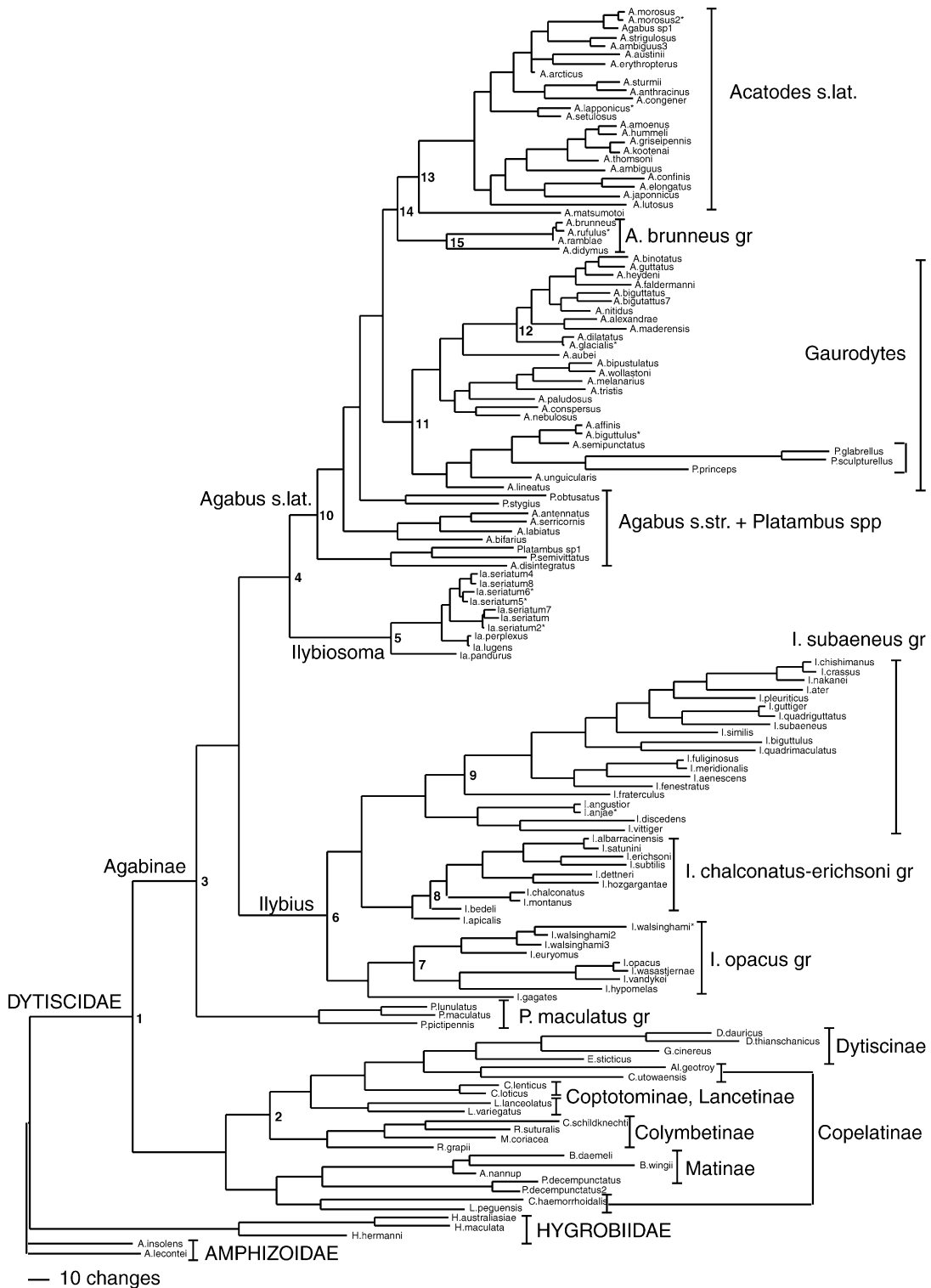


Fig. 2. Phylogram of one of the most parsimonious optimal trees obtained with the Alignment 3 (gap cost 15, extension cost 6.66, manually modified), after reweighting the characters according to the RC. Branch lengths refer to Maximum Parsimony with equally weighted characters (note the long branches of the *P. sculpturellus* clade). Names of the groups refer to Nilsson (2001), with modifications (see text). Numbers inside nodes refer to numbers in Table 3.

support. In alternative alignments Agabinae (excluded *Platynectes*) is sister to Colymbetinae, more in agreement with traditional taxonomy (Table 3).

Closer inspection of the *Agabus* group reveals three major clades, corresponding to the genera *Ilybius*, *Ilybiosoma*, and *Agabus* sensu lato (s.lat.). The latter

includes the genus *Platambus*, except for the *P. maculatus* group, which is either sister to all other members of the *Agabus* group of genera or to *Ilybius* (Table 3). These three clades are very robust to alignment variations, with two of them (*Ilybius* and *Ilybiosoma*) recovered in all trees, and with high Bootstrap and Bremer support (Table 3, Fig. 1). The relationships among them are, however, less well defined. In the preferred tree *Ilybiosoma* is sister to *Agabus* s.lat. (Fig. 1), although all possible combinations of the three taxa were found (Table 3).

The relationships within *Ilybius* and *Ilybiosoma* are robust, and very similar in all trees obtained (Table 3, Fig. 1). Within *Ilybius*, the species of the *I. opacus* group plus *I. gagates* are sister to the rest, which is divided in the *I. chalconatus* plus *erichsoni* groups, and the *I. subaeneus* group (with the exception of *I. apicalis*, which is sister to the species of the *I. chalconatus*–*erichsoni* group) (Table 3, Fig. 1).

Within *Agabus* s.lat., three small clades form a basal paraphyletic series, consisting of *Platambus* (excluding the *P. maculatus* group) and the subgenus *Agabus* s.str. (Figs. 1 and 2). The remaining species of *Agabus* are divided in two large clades: the subgenus *Gaurodytes* (with the exclusion of the *A. brunneus* and *A. ambiguus* groups) and the subgenus *Acatodes* (plus the *A. brunneus* and *A. ambiguus* groups). The species of the *P. sculpturellus* group plus *P. princeps* are included within the species of the *A. affinis* group, but with no support and in a very long branch (Figs. 1 and 2), suggesting a possible artifact of analysis.

The position of the *A. brunneus* group is very variable, being in some trees sister to the *A. affinis* group (Table 3). Within *Gaurodytes*, the *A. guttatus* group is always recovered and with strong support, in most cases sister to *A. aubei*. The other species groups (*A. tristis* group and *A. nebulosus* group) are also recovered as monophyletic in all analyses. On the contrary, within *Acatodes* (plus *A. ambiguus* group) the resolution is minimal in all cases, being the only part of the preferred tree with unresolved polytomies (Fig. 1).

3.2. Rate of variation within and between clades and gene regions

The best ML model (as estimated with Modeltest, using the Hierarchical Likelihood Ratio Tests criteria) for the combined 16S and COI sequence for the ingroup only (i.e., Agabinae excluding *Platynectes*) was a complex GTR + I + G, with estimated base frequencies, among-site rate variation and a Gamma distribution shape parameter of 0.46. The ratio of the likelihood values obtained enforcing and not enforcing a molecular clock on one of the most parsimonious reweighted trees obtained with Alignment 3, and considering gaps

as a missing character, was highly significant ($p \ll 0.001$). The exclusion of *P. sculpturellus*, *P. glabrellus*, and *P. princeps*, with very long branches and in an unlikely position in the tree (Fig. 2, see above), reduced the difference considerably, although it was still highly significant. An ultrametric tree was thus estimated using the non-parametric rate smoothing (NPRS) method of Sanderson (1997) (see Section 2, Fig. 3), excluding the three species with long branches. For comparative purposes an ultrametric tree was also estimated by directly enforcing a molecular clock in PAUP.

The best ML models estimated for the COI and 16S genes separately were also complex GRT + I + G models, with Gamma shape parameters of 0.40 and 0.60, respectively. In all cases the ML ratio between the values obtained enforcing and not enforcing a molecular clock were highly significant ($p \ll 0.001$). Average length of the COI branches (as estimated with the optimal ML model for COI on the most parsimonious combined tree) was 0.071 substitutions/site, while that of the gene 16S (estimated with the optimal ML model for 16S on the most parsimonious combined tree) was only 0.015 substitutions/site. These differences are reflected in the ultrametric trees estimated with NPRS for both genes separately. On average, the branches of the COI gene were approximately twice as long after the NPRS correction (0.071 vs. 0.125), compared to a factor of four (0.015 vs. 0.055) in 16S. The increase in branch length for the combined data was similar to the value for COI (0.045–0.090). The total divergence for the ingroup (for the basal node in Fig. 3, and excluding the *P. sculpturellus* clade) for the gene 16S alone in the NPRS tree was 0.33 substitutions/site. Divergence among the three main lineages (*Ilybius*, *Ilybiosoma*, and *Agabus* s.lat.) was approximately 0.30 substitutions/site, and maximum divergence within each of these main lineages approximately 0.25 substitutions/site. For the COI gene these values were approximately doubled (0.70, 0.65, and 0.60, respectively).

Maximum divergences for the combined data in the ultrametric tree of the ingroup estimated with NPRS were ca. 0.60 substitutions/site (at the node splitting the species of the *P. maculatus* group from all others, determining the estimated age of the whole lineage), while enforcing the clock directly without rate smoothing gave a maximum divergence of 0.35 substitutions/site. Within the remaining taxa, the level of divergence was very similar in the two main monophyletic lineages (*Ilybius* and *Agabus* s.lat.), although with different absolute estimates according to the method: 0.41 (*Ilybius*) and 0.48 (*Agabus* s.lat.) substitutions/site for the NPRS, and 0.28 substitutions/site (both *Ilybius* and *Agabus* s.lat.) for the direct estimation of ML branch length.

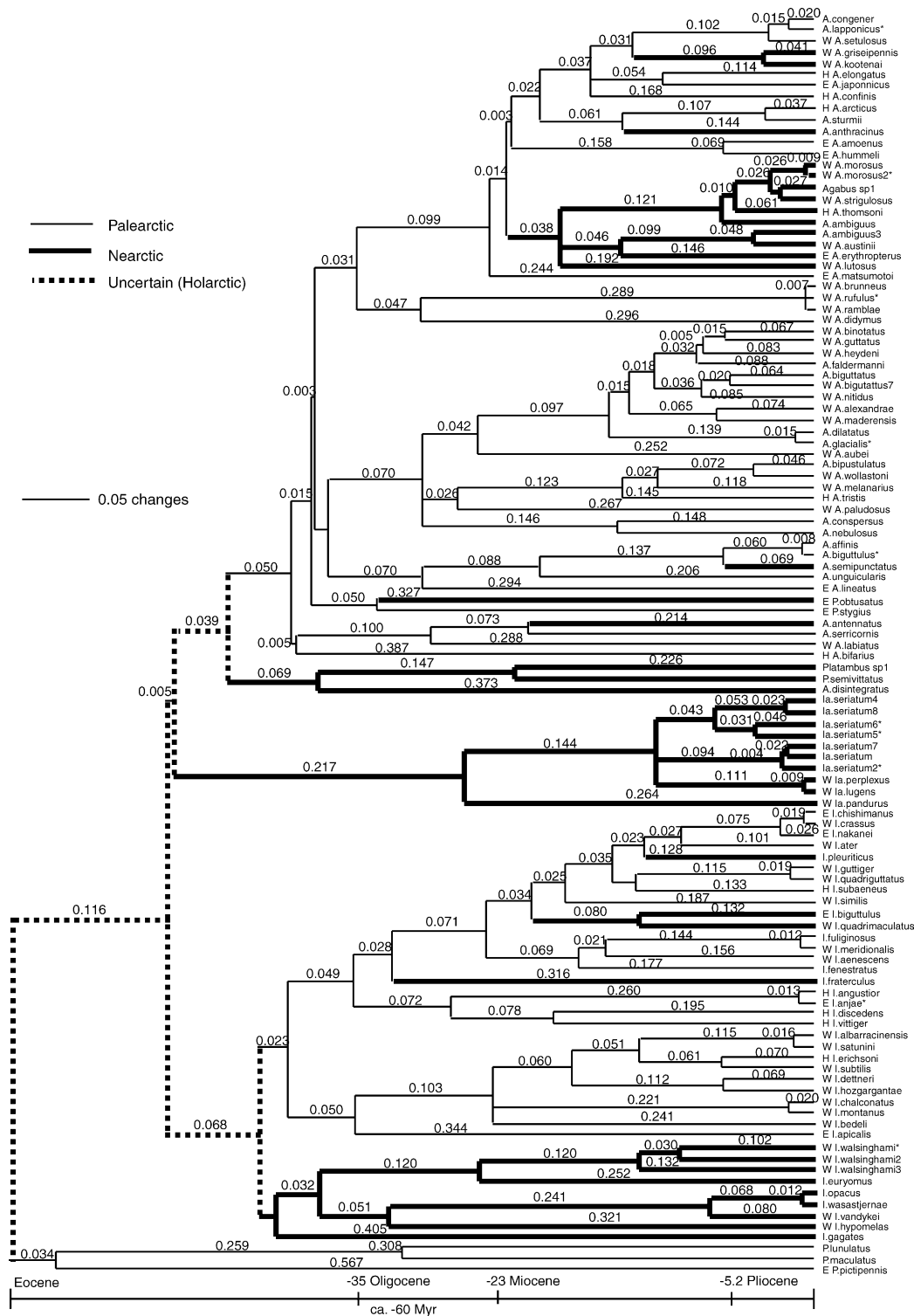


Fig. 3. Ultrametric tree obtained with the non-parametric rate smoothing method of Sanderson (1997) using the topology of one of the optimal trees of Fig. 1 (ingroup only, excluded *P. sculptureus*, *P. glabrellus*, and *P. princeps*), with initial branch lengths estimated through ML (see Section 2). Distribution of the species, E, East; W, West; H, Holarctic. Holarctic species were coded as uncertain (see Section 2). The scale was calibrated using a standard rate of ca. 0.01 substitutions/site/Myr per branch (Brower, 1994).

Even accounting for a slower rate of the gene 16S (as in, e.g., Gómez-Zurita et al., 2000, with 0.0038 substitutions/site/Myr instead of the standard 0.01

substitutions/site/Myr of Brower, 1994), there is an inconsistency between the estimates of the age of divergence of the gene 16S and that of the combined

data. Using this calibration in the gene 16S alone, age estimates are ca. 30% older than with the combined data.

3.3. Biogeographic separation of palearctic and nearctic lineages

The distributions of the species (Nearctic, Palearctic or Holarctic) was mapped on the preferred phylogenetic tree to assess historical vicariance and dispersal patterns. Two of the three deep clades of the Agabinae, *Ilybius* and *Agabus* s.lat., exhibit a basal separation of exclusively Nearctic and Palearctic clades (the non-Nearctic species of the third main lineage, *Ilybiosoma*, could not be obtained for study, see Section 4). Within the Nearctic clade of *Ilybius* at least two species secondarily expanded their ranges to the north Palearctic (*I. opacus* and *I. wasastjerna*, Fig. 3). Within the Palearctic lineage of *Ilybius*, further splits separate a Western Palearctic clade (*I. chalconatus-erichsoni* group), and a clade including all the species from the Eastern Palearctic, plus some Western Palearctic and (secondarily) Nearctic species (*I. subaeneus* group). Among the Palearctic clade there are at least three inferred transitions to the Nearctic, and two range extensions to Holarctic distributions. In *Agabus* s.lat. there are two basal clades with an old Nearctic–Palearctic disjunction: the species of an expanded *A. labiatus* group (including *A. antennatus* and *A. serricornis*), and the species of the *P. optatus* group. The remaining *Agabus* split in: (1) a largely Western Palearctic clade (*Gaurodytes* excluding *A. brunneus* and *A. ambiguus* groups), with only one expansion to an Holarctic range (*A. tristis*) and a single transition to the Nearctic (*A. semipunctatus*) and (2) a clade with mixed distributions, Western and Eastern Palearctic, and Nearctic (*A. brunneus* group and *Acatodes* plus *A. ambiguus* group, Fig. 3).

4. Discussion

4.1. Sequence length variability

The small difference in sequence length in 16S rRNA resulted in alternative alignments and major differences in tree topology, particularly at basal nodes defining the relationships among the main lineages. The use of a direct optimization method, such as the one implemented in the POY software (Gladstein and Wheeler, 1997; see Ribera et al., 2002a for an example in Dytiscidae) would perhaps provide a unique topology, but the method is more demanding on computation given the size of the matrix (142 taxa with ca. 1300 nucleotides) and would not overcome the fact

that these nodes lack support. The main phylogenetic and biogeographic conclusions are, however, not affected, as relationships within each of the main lineages are in general well supported and with more stable topologies.

Most nodes with robust support in the preferred tree were also present in the majority of trees obtained with other alignments, either using equally weighted or reweighted data. Nodes which were not universally recovered had minimal support in most cases, but even alternative resolutions of those nodes produced a limited range of tree topologies. Some of these poorly supported nodes could be attributed to data artifacts, including long branches, which may be the reason for the unexpected position of the *Platambus sculpturellus* group, the possible polyphyly of the genus *Platambus*, and/or the exclusion of *Platynectes* from Agabinae.

4.2. Phylogeny of agabinae

According to our results, Agabinae (with the possible exclusion of *Platynectes*) is a monophyletic lineage not sister to Colymbetinae, in agreement with previous conclusions based on morphological (Alarie et al., 2002; Miller, 2001) and molecular (Ribera et al., 2002a) data. The exclusion of *Platynectes* from Agabinae agrees with our previous results based on a different gene, nuclear 18S rRNA (Ribera et al., 2002a). Although no morphological character supports the exclusion of *Platynectes* from Agabinae, the highly plesiomorphic condition of most Agabinae precludes any definitive conclusion.

Regarding basal relationships within Agabinae, the redefinition of the genera proposed by Nilsson (2000) is largely confirmed. *Ilybiosoma* and the expanded *Ilybius* that now includes several species groups formerly in *Agabus* are recovered as monophyletic with very strong support. The genus *Agabus* is also recovered in the preferred tree, although with low support and only with the inclusion of some species groups of *Platambus*. On the contrary, the genus *Platambus* is never recovered as monophyletic. The species of the *P. maculatus* group (part of the genus “*Platambus*” in the restricted sense previous to Nilsson, 2000) are of uncertain position, either as sister to all remaining Agabinae, or sister to *Ilybius*. The other species of the genus are included within *Agabus* s.lat., or sister to it. The placement of the *P. sculpturellus* group (the former genus *Agabinus* plus *P. princeps*) remains uncertain, as their position in the preferred tree has no morphological support and is clearly affected by long branches. Alarie (1995, 1998) considered the species of *Agabinus* to be sister to *Agabus* + *Ilybius*, although the American species of *Platambus* (sensu Nilsson, 2000) were included within *Agabus*. In Miller (2001) *Agabinus* is placed in an

unresolved polytomy formed by species of *Platambus* in agreement with Nilsson (2000).

In what refers to the relationships within the three main generic groupings of Agabinae, the species groups of *Ilybius* defined by Nilsson (2000) are in general well supported. The *I. opacus* group (*sensu* Larson, 1996) is recovered as monophyletic, and sister to *I. gagates*. The relatively isolated position of *I. gagates* is supported by its deviating morphology (cf. Miller, 2001). Together with *I. larsoni* (not sampled, but morphologically very close to *I. gagates*, Fery and Nilsson, 1993), these are the only Nearctic species of the *I. chalconatus* group as defined by Fery and Nilsson (1993). The relationships within the *I. opacus* and the *I. subaeneus* groups agree with the phylogenies proposed in Larson (1996) and Ribera et al. (2001a), respectively, with only minor differences. Within the *I. chalconatus-erichsoni* group, the species included in the *I. chalconatus* subgroup by Fery and Nilsson (1993) (with the exclusion of *I. gagates*) are largely paraphyletic, although other relationships are compatible with their suggestions.

The genus *Ilybiosoma* is always recovered as monophyletic. Its position within Agabinae is however uncertain. In the preferred tree it is placed sister to *Agabus* s.lat., although with low support. This is in agreement with Miller (2001), based on morphological characters. In other trees it is sister to *Ilybius*, or basal to *Agabus* plus *Ilybius*. The study of the European species *Agabus striolatus* (which according to Nilsson, 2000 has some similarities with the species of *Ilybiosoma*), and the non-Nearctic species of the genus (from Iran and Ethiopia) could help to resolve its placement. The only previous hypothesis of the relationships among the species of *Ilybiosoma* is that of Larson (1997), based in a phenetic ordination using morphometric measures. The species groups thus defined agree with our results, although only two of them were included.

Within *Agabus*, the subgenera defined by Nilsson (2000) are not recovered as monophyletic in the molecular analysis, although the main lineages largely conform to them. The basal clades include all species of *Agabus* s.str., plus some species groups of *Platambus* (*P. optatus* and *P. semivittatus* groups) in a paraphyletic series. The first lineage is *A. disintegratus* plus the species of the *P. semivittatus* group, followed by the *A. labiatus* group plus the species with clubbed antennae (*A. antennatus* and *A. serricornis*), a relationship suggested by Angus (1984). This group is basal to *Agabus* s.lat., confirming the provisional placement of Nilsson (2000).

A possible explanation for the observed polyphyly of the species of *Platambus* (as defined in Nilsson, 2000) could be the relatively poor sampling of this genus with respect to *Agabus* and *Ilybius*, with some

potentially key groups missing (e.g., the *P. sawadai* and *P. semenowi* groups, included in the former *Platambus* in a restricted sense, Nilsson, 2000). The genus was redefined based on weak morphological characters (a combination of prosternal process with lateral bead broadly inflated posterior of procoxae and/or mesocoxa widely separated, Nilsson, 2000). Yet this type of evidence is not inferior to that defining other genera of Agabinae which do have strong support in our analysis (such as *Ilybiosoma*, only characterized by the anteroventral spiniferous punctures along entire length of the metatibia). Our results are more in agreement with the concept of *Platambus* prior to Nilsson (2000), although the lack of key taxa does not allow firm conclusions.

The subgenus *Gaurodytes* in our restricted sense (i.e., without the species of the *A. brunneus* group, of uncertain position, and those of the *A. ambiguus* group, included together with *Acatodes*, see below) has strong support, and the relationships of the main lineages within it are almost identical in all trees obtained under different parameters. The inclusion of the species of the *P. sculpturellus* clade within the *A. affinis* group is most likely an artifact of their long branch, as noted.

The relationships within the *A. affinis* group (excluded the *P. sculpturellus* clade) are in perfect agreement with Nilsson and Larson (1990). However, this group is found to be nested within *Agabus*, and not sister to the whole genus, as suggested by the same authors.

The close relationship between the *A. paludosus*, *A. nebulosus*, and *A. guttatus* groups was already suggested by Nilsson (1992a), in agreement with our results. The inclusion of the *A. tristis* group in this clade contrasts with the results of Larson (1989), who suggested that it is closely related to the species of the *A. ambiguus* group.

We found *Agabus aubei* sister to the species of the *A. guttatus* group, as suggested by Balke et al. (1997). The relationships among the species of the *A. guttatus* group are in agreement with Ribera et al. (2001b). The species with a distribution centered in the West Mediterranean are sister to the species from the East (*A. dilatatus* and *A. glacialis*, not included in Ribera et al., 2001b). The Moroccan *A. alexandrae* is found to be sister to *A. maderensis*, from Madeira.

The species of the *A. brunneus* group are found to be sister to the species of the subgenus *Acatodes* plus the *A. ambiguus* group. This position is however uncertain, with low nodal support and not recovered under other alignment parameters. An alternative position for this group of species is as sister to the *A. affinis* group, and thus within the remaining species of *Gaurodytes*, a more likely relationship from a morphological point of view.

The inclusion of the *A. ambiguus* group within the clade of the species with a bifid apex of the aedeagus (i.e., *Acatodes* sensu Nilsson, 2000) was already suggested by Sharp (1882), who placed most of the species of the *A. ambiguus*, *A. arcticus*, and *A. confinis* groups in a single group (his “group 10”). This relationship was also noted by Leech (1964), based on the chaetotaxy of the male tarsomeres. These similarities were considered to be homoplasies by Larson (1994) and Nilsson (2000) on defining the subgenera of *Agabus*, who consider the absence of the subapical spine on the aedeagus as a plesiomorphic character, excluding the species of the *A. ambiguus* group from this lineage.

Within the clade [*Acatodes* + *A. ambiguus* group], the relationships are largely unresolved, although some of the species groups defined by Nilsson (2000) are partially recovered. The species of the *A. confinis* group are placed in an unresolved polytomy, and the species of the *A. japonicus* group are paraphyletic, but the species of the *A. arcticus* and *ambiguus* groups are recovered as monophyletic. According to Larson (1991) the species of the *A. confinis* group (with the exclusion of *A. elongatus*, included in a different group) do not share any synapomorphy, and the group is defined by exclusion of all other likely monophyletic groups, i.e., it is likely to be paraphyletic.

4.3. Historical biogeography of the species of *Agabinae*

The different estimates of ages from the 16S gene alone and the combined data are surprising, since in a previous study with a subset of *Agabus* analyzed here, both calibrations agreed (Ribera et al., 2001b). In that case the evolution of the combined sequence did not significantly deviate from clock-like variation and NPRS was not applied. The use of NPRS in the present case could be the reason for this discrepancy, as the correction approximately doubled the average branch lengths in COI, but increased them by a factor of four in 16S. The NPRS is thus likely to introduce deformations in the estimation of an ultrametric tree, which are more pronounced in 16S with its slower rate of evolution and greater rate heterogeneity. To alleviate this effect it seems preferable to use the combined data for estimating the age of the lineages. However, even when using the combined data, the divergence estimates from the direct enforcement of a clock in PAUP are ca. 50% lower than those obtained with NPRS, and hence these age calibrations can be no more than a very rough approximation.

According to the level of divergence estimated in the combined data using the NPRS correction, and assuming a standard clock rate of ca. 1% divergence per branch per Myr, the diversification of the main

lineages within Agabinae would have occurred in the Late Eocene. This is compatible with the fossil record, as the oldest known fossils attributed to Agabinae date from the Oligocene (see Nilsson, 2001 for a checklist of fossil taxa). The basal splits within the two main clades, *Ilybius* and *Agabus* s.lat., into Nearctic and Palearctic lineages apparently occurred concurrently (Late Eocene–Early Oligocene), strongly suggesting a vicariant separation produced by the same geological event. The study of the non-Nearctic species of *Ilybiosoma* (one Palearctic and one Ethiopian) would allow to determine if the same general split Nearctic–Palearctic is reproduced in the third main lineage of Agabinae, and at the same level of divergence.

The main Atlantic route of faunal interchange between Europe and North America, the Thulean Bridge, closed some 50 Myr ago, presumably before the radiation of the main lineages of Agabinae (Smith et al., 1994), and thus it is not likely that its closure had any effect on the evolution of the group. However, there was another Atlantic connection present until the Late Eocene (ca. 39 Myr), within the time window of the early splits within *Ilybius* and *Agabus* s.lat. This bridge connected Scandinavia with north Greenland and Eastern North America through the Canadian Arctic Archipelago (Sanmartin et al., 2001). Because of its northern position it is considered to have been suitable only for cold adapted organisms. The break-up of this land bridge in the Late Eocene could have provided the initial vicariance event, but only if the ancestral ranges of the Palearctic clades of *Agabus* and *Ilybius* were in the western Palearctic. Although our results are compatible with this assumption, the small number of Eastern Palearctic species sampled does not allow any firm conclusion.

Another possibility is an initial connection through the Eastern Palearctic by way of the early Beringian Bridges, which remained open until the Eocene–Oligocene boundary (35 Myr). The break-up of this connection was accompanied by a drastic change of global climatic conditions, and widespread vicariance events (Sanmartin et al., 2001). Both the separation of the Thulean and the Beringian bridges are compatible with the estimated date of the first vicariant split within *Ilybius* and *Agabus* s.lat., but only when the older estimation using the NPRS is accepted, or if the rate of mtDNA divergence is lower than the standard 1% per branch per Myr.

From the Mid-Jurassic (180 Myr) to late Tertiary (Oligocene, 30 Myr) the West and East Palearctic were divided by the Turgai Strait, located east of the Ural Mountains (Sanmartin et al., 2001). This separation was not complete, as the two regions were connected on both ends of the strait, more extensively

in the north. However, an additional marine transgression during the Eocene increased the isolation, which could have generally reinforced vicariance. The inferred timing of the deep diversification within the Palearctic groups of *Ilybius* and *Agabus* s.lat. is compatible with this event. Western Palearctic clades are well defined in both groups, although for the hypothetical Eastern Palearctic lineage multiple range expansions to Holarctic or Nearctic distributions would have to be postulated. The ancestral condition of the supposedly Eastern Palearctic lineage, corresponding to the *I. subaeneus* group and defined as the sister of the well supported Western Palearctic clade, is uncertain. In Ribera et al. (2001a) we proposed that the ancestral condition is Holarctic based on the distribution patterns of several basal species, but it is also possible that secondary range expansions would have eliminated information on their historical ranges.

This general pattern of a basal Nearctic–Palearctic vicariance, followed by a second vicariance within each of the main Palearctic clades between the West and the East, is in agreement with the results of more comprehensive biogeographical analyses of the Holarctic fauna (Enghoff, 1995; Sanmartin et al., 2001). The distributions within the subareas are in general strongly phylogenetically conserved and in agreement with the results of Enghoff (1995) and Sanmartin et al. (2001), as well as those for other groups of animals (e.g., rodents, Riddle, 1998; birds, Böhning-Gaese et al., 1998; Zink et al., 2000). Only within the clades including Eastern Palearctic species there is a relatively high frequency of range expansions and transitions (*I. subaeneus* group, *Agabus* subgenus *Acatodes*). This suggests a strong asymmetry in the main dispersal routes within the Holarctic, with the East Palearctic being the main source of colonizers and the Nearctic and the West Palearctic with lineages mostly restricted to their territories, also in agreement with previous biogeographical work (Enghoff, 1995; Pielou, 1979; Sanmartin et al., 2001).

Most of the observed transitions between Palearctic and Nearctic affect isolated species, and are compatible with a scenario of several subsequent connections through the Bering bridges, until the most recent (*A. semipunctatus*) at the end of the Miocene. The Nearctic clade of most recent origin (late Tertiary) is within the subgenus *Acatodes*, which includes also most of the Eastern Palearctic species. We do not infer any continental transitions during the Pliocene, except for a few apparent range expansions from Palearctic or Nearctic to Holarctic in boreal species. There is however frequent within-continent speciation; even based on our incomplete sampling we infer 22 speciation events in the last 5.2 Myr (more than 40

events if the divergence times are estimated directly with the ML enforced clock), indicating that the lack of apparent recent intercontinental vicariants is not due to the short time frame in which species separations are studied.

One of the most interesting results from the biogeographical point of view is the sister relationship of *P. obtusatus* and *P. stygius*, and their estimated level of divergence. This is compatible with the hypothesis suggested by Nilsson (1996) of a relict Tertiary distribution, even with the much lower direct estimate using ML (middle Miocene as opposed to Early Oligocene with NPRS). This type of disjunct East Palearctic–East Nearctic distribution is a common pattern among several groups of plants and animals, and is supposed to be a relict distribution of biota inhabiting temperate subtropical forests which extended throughout the Palearctic during the early Tertiary, but which later became extinct in most of their range (Wen, 1999; Wu, 1983; see Sanmartin et al., 2001 for a review).

The main biogeographical patterns obtained from our preferred tree are robust, as they do not depend on the exact resolution of basal relationships of the three main lineages within Agabinae. Most of the nodes within the main lineages are very robust, and the uncertainties affect only species poor lineages (*P. maculatus* group, *A. brunneus* group, and *P. sculpturellus* group). Most species missing from the current study can be placed with confidence into morphologically well supported groups. Their inclusion could increase the number of inferred range expansions or transitions, but is unlikely to change the arrangement of the main lineages, and their reconstructed ancestral states. Perhaps more important is the placement of a few major groups not sampled, as the species of *Ilybiosoma* from outside the Nearctic, the exclusively Ethiopian *A. ragazzii* and *A. raffrayi* species groups, the Palearctic *P. sawadai* and *P. semenowi* groups, and the genera *Hydrotrupes* and *Hydronebrius* (Nilsson, 1992b, 2001). Their phylogenetic position based on morphology is uncertain (Beutel, 1994), but their placement could potentially affect the inferred basal relationships of Agabinae.

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Appendix A. Studied material and sequence accession numbers

No.	Species ^a	Sp. group	Dist.	Country	Collector	16S rRNA	COI
Ingroup							
AGABINAE							
<i>Agabus Acatodes</i>							
1	<i>A. anthracinus</i>	<i>arcticus</i>	N	US (Washington)	I. Ribera and A. Cieslak	AY138602	AY138693
2	<i>A. arcticus</i>	<i>arcticus</i>	H	Sweden	A.N. Nilsson	AY138603	AY138694
3	<i>A. sturmi</i>	<i>arcticus</i>	P	Sweden	A.N. Nilsson	AY138633	AY138723
4	<i>A. confinis</i>	<i>confinis</i>	H	Sweden	A.N. Nilsson	AY138608	–
5	<i>A. congener</i> (8) ^b	<i>confinis</i>	P	Moravia	D. Boukal	AY138609	AY138699
6	<i>A. elongatus</i>	<i>confinis</i>	H	Sweden	A.N. Nilsson	AY138612	AY138702
7	<i>A. kootenai</i>	<i>confinis</i>	WN	Canada (Alberta)	I. Ribera and A. Cieslak	AY138594	AY138685
8	<i>A. lapponicus</i> (5)	<i>confinis</i>	P	Sweden	A.N. Nilsson	AY138619	AY138709
9	<i>A. matsumotoi</i>	<i>confinis</i>	EP	Japan (Hokkaido)	J. Bergsten	AY138622	AY138712
10	<i>A. setulosus</i>	<i>confinis</i>	WP	Sweden	A.N. Nilsson	AY138630	AY138720
11	<i>A. thomsoni</i>	<i>confinis</i>	H	Canada (BC)	I. Ribera and A. Cieslak	AY138595	AY138686
12	<i>A. amoenus</i>	<i>japonicus</i>	EP	Russia (Astrakhan)	A.N. Nilsson	AY138600	AY138691
13	<i>A. hummeli</i>	<i>japonicus</i>	EP	China (Yunnan)	J. Bergsten	AY138616	AY138706
14	<i>A. japonicus</i>	<i>japonicus</i>	EP	Kuriles Is.	N. Minakawa	AY138617	AY138707
15	<i>A. lutosus</i>	<i>lutosus</i>	WN	US (California)	A. Cognato	AY138620	AY138710
16	<i>A. lutosus/griseipennis</i>	<i>lutosus</i>	WN	Canada (BC)	I. Ribera and A. Cieslak	AY138593	AY138684
17	<i>A. morosus</i> (18)	<i>obsoletus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138591	AY138682
18	<i>A. morosus2</i> (17)	<i>obsoletus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138592	AY138683
<i>Agabus (Agabus)</i>							
19	<i>A. antennatus</i>	<i>antennatus</i>	N	Canada (Alberta)	I. Ribera and A. Cieslak	AY138601	AY138692
20	<i>A. serricornis</i>	<i>clavicornis</i>	P	Sweden	A.N. Nilsson	AY138629	AY138719
21	<i>A. disintegratus</i>	<i>disintegratus</i>	N	US (California)	W. Shephard	AY071769	AY071795
22	<i>A. labiatus</i>	<i>labiatus</i>	WP	UK	I. Ribera	AY138618	AY138708
23	<i>A. bifarius</i>	<i>labiatus</i>	H	Canada (Alberta)	I. Ribera and A. Cieslak	AY138605	AY138696
24	<i>A. lineatus</i>	<i>lineatus</i>	EP	Russia (Volgograd)	A.N. Nilsson	AY138611	AY138701
<i>Agabus (Gaurodytes)</i>							
25	<i>A. affinis</i> (26)	<i>affinis</i>	P	Sweden	A.N. Nilsson	AY138598	AY138689
26	<i>A. biguttulus</i> (25)	<i>affinis</i>	P	Sweden	A.N. Nilsson	AY138606	AY138697
27	<i>A. semipunctatus</i>	<i>affinis</i>	N	Canada (Ontario)	Y. Alarie	AY138628	AY138718
28	<i>A. unguicularis</i>	<i>affinis</i>	P	UK	I. Ribera	AY138635	AY138725
29	<i>A. ambiguus</i>	<i>ambiguus</i>	N	Canada (Ontario)	Y. Alarie	AY138599	AY138690
30	<i>A. ambiguus3</i>	<i>ambiguus</i>	N	Canada (BC)	I. Ribera and A. Cieslak	AY138596	AY138687
31	<i>A. austinii</i>	<i>ambiguus</i>	WN	Canada (BC)	I. Ribera and A. Cieslak	AY138604	AY138695
32	<i>A. erythropterus</i>	<i>ambiguus</i>	EN	US (New York)	K.B. Miller	AY138613	AY138703
33	<i>Agabus</i> sp.	<i>ambiguus</i>	N?	US (Washington)	I. Ribera and A. Cieslak	AY138597	AY138688
34	<i>A. strigulosus</i>	<i>ambiguus</i>	WN	Canada (BC)	I. Ribera and A. Cieslak	AY138632	AY138722
35	<i>A. aubei</i>	<i>aubei</i>	WP	Corsica	I. Ribera and A. Cieslak	AY039265	AY039277

Appendix A (continued)

No.	Species ^a	Sp. group	Dist.	Country	Collector	16S rRNA	COI
36	<i>A. brunneus</i>	<i>brunneus</i>	WP	Spain	A. Millan	AY138607	AY138698
37	<i>A. didymus</i>	<i>brunneus</i>	WP	Spain	I. Ribera	AF309274	AF309331
38	<i>A. ramblae</i> (39)	<i>brunneus</i>	WP	Morocco	I. Ribera	AY138626	AY138716
39	<i>A. rufulus</i> (38)	<i>brunneus</i>	WP	Corsica	I. Ribera and A. Cieslak	AY138627	AY138717
40	<i>A. alexandrae</i>	<i>guttatus</i>	WP	Morocco	I. Ribera	AY039257	AY039266
41	<i>A. biguttatus</i>	<i>guttatus</i>	P	Spain	I. Ribera	AY039258	AY039267
42	<i>A. biguttatus</i> 7	<i>guttatus</i>	WP	Canary Is. (Gomera)	S. Ericsson	AY039259	AY039270
43	<i>A. binotatus</i>	<i>guttatus</i>	WP	Corsica	I. Ribera and A. Cieslak	AY039260	AY039271
44	<i>A. dilatatus</i> (46)	<i>guttatus</i>	P	Cyprus	K.W. Miller	AY039261	AY039272
45	<i>A. faldermanni</i>	<i>guttatus</i>	P	Iran	D.T. Bilton	AY138614	AY138704
46	<i>A. glacialis</i> (44)	<i>guttatus</i>	P	Iran	D.T. Bilton	AY138615	AY138705
47	<i>A. guttatus</i>	<i>guttatus</i>	WP	France	H. Fery	AY039262	AY039273
48	<i>A. heydeni</i>	<i>guttatus</i>	WP	Spain	H. Fery	AY039263	AY039274
49	<i>A. maderensis</i>	<i>guttatus</i>	WP	Madeira	D.T. Bilton	AY138621	AY138711
50	<i>A. nitidus</i>	<i>guttatus</i>	WP	Spain	I. Ribera	AY039264	AY039276
51	<i>A. conspersus</i>	<i>nebulosus</i>	P	Spain	I. Ribera	AY138610	AY138700
52	<i>A. nebulosus</i>	<i>nebulosus</i>	P	UK	I. Ribera	AY138624	AY138714
53	<i>A. paludosus</i>	<i>paludosus</i>	WP	Spain	I. Ribera	AY138625	AY138715
54	<i>A. bipustulatus</i>	<i>tristis</i>	P	Spain	I. Ribera	AF309275	AF309332
55	<i>A. melanarius</i>	<i>tristis</i>	WP	UK	J. Denton	AY138623	AY138713
56	<i>A. tristis</i>	<i>tristis</i>	N,EP	Canada (BC)	I. Ribera and A. Cieslak	AY138634	AY138724
57	<i>A. wollastoni</i> <i>Ilybiosoma</i>	<i>tristis</i>	WP	Madeira	M. Drotz	AY138636	AY138726
58	<i>I. lugens/perplexus</i>	<i>seriatum</i>	WN	US (California)	A. Cognato	AF309271	AF309328
59	<i>I. pandurus</i>	<i>seriatum</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138648	AY138736
60	<i>I. perplexus</i>	<i>seriatum</i>	WN	US (Washington)	I. Ribera and A. Cieslak	AY138650	AY138738
61	<i>I. seriatum</i> (62)	<i>seriatum</i>	N	US (New York)	K.B. Miller	AF309272	AF309329
62	<i>I. seriatum</i> 2 (61)	<i>seriatum</i>	N	Canada (BC)	I. Ribera and A. Cieslak	AY138655	AY138743
63	<i>I. seriatum</i> 4	<i>seriatum</i>	N	Canada (Alberta)	I. Ribera and A. Cieslak	AY138649	AY138737
64	<i>I. seriatum</i> 5 (65)	<i>seriatum</i>	N	US (California)	I. Ribera and A. Cieslak	AY138652	AY138740
65	<i>I. seriatum</i> 6 (64)	<i>seriatum</i>	N	US (California)	I. Ribera and A. Cieslak	AY138651	AY138739
66	<i>I. seriatum</i> 7	<i>seriatum</i>	N	US (New York)	C. Hernando	AY138653	AY138741
67	<i>I. seriatum</i> 8 (64)	<i>seriatum</i>	N	US (California)	Y. Alarie	AY138654	AY138742
	<i>Ilybius</i>						
68	<i>I. albarracinensis</i>	<i>chalconatus</i>	WP	Portugal	I. Ribera	AF309277	AF309334
69	<i>I. bedeli</i>	<i>chalconatus</i>	WP	Tunisia	I. Ribera and A. Cieslak	AY138658	–
70	<i>I. chalconatus</i>	<i>chalconatus</i>	WP	Morocco	P. Aguilera	AF309278	AF309335
71	<i>I. dettneri</i>	<i>chalconatus</i>	WP	Spain	D.T. Bilton	AY138659	AY138746
72	<i>I. gagates</i>	<i>chalconatus</i>	N	US (New Jersey)	C. Hernando	AY138663	AY138750
73	<i>I. hozgargantae</i>	<i>chalconatus</i>	WP	Spain	I. Ribera	AY138664	AY138751
74	<i>I. montanus</i>	<i>chalconatus</i>	WP	UK	I. Ribera	AY138666	AY138753
75	<i>I. satunini</i>	<i>chalconatus</i>	WP	Russia (Volgograd)	A.N. Nilsson	AY138670	AY138757
76	<i>I. erichsoni</i>	<i>erichsoni</i>	H	Sweden	A.N. Nilsson	AY138661	AY138748
77	<i>I. subtilis</i>	<i>erichsoni</i>	WP	Sweden	A.N. Nilsson	AF309276	AF309333
78	<i>I. euryomus</i>	<i>opacus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138662	AY138749
79	<i>I. hypomelas</i>	<i>opacus</i>	WN	Canada (BC)	I. Ribera and A. Cieslak	AY138665	AY138752

Appendix A (continued)

No.	Species ^a	Sp. group	Dist.	Country	Collector	16S rRNA	COI
80	<i>I. opacus</i>	<i>opacus</i>	H	Sweden	A.N. Nilsson	AY138668	AY138755
81	<i>I. vandykei</i>	<i>opacus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138671	AY138758
82	<i>I. walsinghamsi</i> (83)	<i>opacus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138656	AY138744
83	<i>I. walsinghamsi</i> 2 (82)	<i>opacus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138660	AY138747
84	<i>I. walsinghamsi</i> 3	<i>opacus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138657	AY138745
85	<i>I. wasastjerna</i> e	<i>opacus</i>	H	Sweden	A.N. Nilsson	AY138672	AY138759
86	<i>I. aenescens</i>	<i>subaeneus</i>	WP	Finland	T. Berendok	AF309294	AF309350
87	<i>I. angustior</i>	<i>subaeneus</i>	H	Sweden	A.N. Nilsson	AF309289	AF309345
88	<i>I. anjae</i>	<i>subaeneus</i>	EP	Kuriles Is.	N. Minakawa	AF309295	AF309351
89	<i>I. apicalis</i>	<i>subaeneus</i>	EP	Kuriles Is.	N. Minakawa	AF309279	–
90	<i>I. ater</i>	<i>subaeneus</i>	WP	UK	I. Ribera	AF309287	AF309343
91	<i>I. biguttulus</i>	<i>subaeneus</i>	EN	US (New York)	K.B. Miller	AF309282	AF309338
92	<i>I. chishimanus</i>	<i>subaeneus</i>	EP	Kuriles Is.	N. Minakawa	AF309288	AF309344
93	<i>I. crassus</i>	<i>subaeneus</i>	WP	Sweden	A.N. Nilsson	AF309297	AF309353
94	<i>I. discedens</i>	<i>subaeneus</i>	H	Kuriles Is.	N. Minakawa	AF309296	AF309352
95	<i>I. fenestratus</i>	<i>subaeneus</i>	P	Sweden	A.N. Nilsson	AF309291	AF309347
96	<i>I. fraterculus</i>	<i>subaeneus</i>	N	Canada (Alberta)	I. Ribera and A. Cieslak	AF309281	AF309337
97	<i>I. fuliginosus</i>	<i>subaeneus</i>	P	UK	I. Ribera	AF309293	AF309349
98	<i>I. guttiger</i>	<i>subaeneus</i>	WP	Finland	T. Berendok	AF309285	AF309341
99	<i>I. meridionalis</i>	<i>subaeneus</i>	WP	Corsica	I. Ribera and A. Cieslak	AF309292	AF309348
100	<i>I. nakanei</i>	<i>subaeneus</i>	EP	Japan (Hokkaido)	J. Bergsten	AY138667	AY138754
101	<i>I. pleuriticus</i>	<i>subaeneus</i>	N	US (Vermont)	C. Hernando	AY138669	AY138756
102	<i>I. quadriguttatus</i>	<i>subaeneus</i>	WP	UK	I. Ribera	AF309283	AF309339
103	<i>I. quadrimaculatus</i>	<i>subaeneus</i>	WN	Canada (BC)	I. Ribera and A. Cieslak	AF309290	AF309346
104	<i>I. similis</i>	<i>subaeneus</i>	WP	Sweden	J. Bergsten	AF309284	AF309340
105	<i>I. subaeneus</i>	<i>subaeneus</i>	H	Sweden	A.N. Nilsson	AF309286	AF309342
106	<i>I. vittiger</i>	<i>subaeneus</i>	WN/WP	Sweden	A.N. Nilsson	AF309280	AF309336
107	<i>Platambus</i> <i>P. glabrellus</i>	<i>glabrellus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138637	–
108	<i>P. sculpturellus</i>	<i>glabrellus</i>	WN	US (California)	I. Ribera and A. Cieslak	AY138638	AY138727
109	<i>P. lunulatus</i>	<i>maculatus</i>	P	Iran	H. Fery and Elmi	AY138674	AY138761
110	<i>P. maculatus</i>	<i>maculatus</i>	P	Spain	I. Ribera	AF309273	AF309330
111	<i>P. pictipennis</i>	<i>maculatus</i>	EP	Japan	J. Bergsten	AY138676	AY138763
112	<i>P. obtusatus</i>	<i>optatus</i>	EN	US (Pennsylvania)	C. Hernando	AY138675	AY138762
113	<i>P. princeps</i>	<i>optatus</i>	EP	China (Yunnan)	J. Bergsten	AY138677	AY138764
114	<i>P. stygius</i>	<i>optatus</i>	EP	Japan (Hokkaido)	J. Bergsten	AY138679	AY138766
115	<i>P. semivittatus</i>	<i>semivittatus</i>	N	US (New York)	K.B. Miller	AY138678	AY138765
116	<i>Platambus</i> sp.		N?	US (Maryland)	W.E. Steiner and J.M. Swearingen	AY138631	AY138721
	<i>Platynectes</i> (<i>Gueorguievtes</i>)						
117	<i>P. decempunctatus</i>			Australia	C.H.S. Watts	AY138680	AY138767
118	<i>P. decempunctatus</i> gr			New Guinea	C. Vaamonte	AY138681	AY138768
	Outgroup COLYMBETINAE						
119	<i>Colymbetes schildknechti</i>			Spain	I. Ribera	AF428197	AF428236
120	<i>Meladema coriacea</i>			France	P. Ponel	AF428189	AF428209
121	<i>Rhantus grapii</i>			UK	I. Ribera	AF428195	AF428234

Appendix A (continued)

No.	Species ^a	Sp. group	Dist.	Country	Collector	16S rRNA	COI
122	<i>R. suturalis</i> COPELATINAE			Spain	I. Ribera	AF428196	AF428235
123	<i>Aglymbus geotroi</i>			Oman	Sattmann et al.	AY138639	AY138728
124	<i>Copelatus haemorrhoidalis</i>			UK	I. Ribera	AY071774	AY071800
125	<i>C. utowaensis</i>			New Guinea	M. Balke	AY071775	AY071801
126	<i>Lacconectus peguensis</i> COPTOTOMINAE			Myanmar	H. Schillhammer	AY071785	AY071811
127	<i>Coptotomus lenticus</i>			US (New York)	K.B. Miller	AY071776	AY071802
128	<i>C. loticus</i>			US (Georgia)	C. Hernando	AY138643	AY138731
	DYTISCINAE						
129	<i>Graphoderus cinereus</i>			Spain	I. Ribera	AY138647	AY138735
130	<i>Dytiscus dauricus</i>			Japan (Hokkaido)	J. Bergsten	AY138644	AY138732
131	<i>D. thianschanicus</i>			Russia (Volgograd)	A.N. Nilsson	AY138645	AY138733
132	<i>Eretes sticticus</i> LANCETINAE			Iran	H. Fery	AY138646	AY138734
133	<i>Lancetes lanceolatus</i>			Australia	C.H.S. Watts	AY138673	AY138760
134	<i>L. varius</i> MATINAE			Chile	I. Ribera	AY071784	AY071810
135	<i>Allomatus nannup</i>			Australia	L. Hendrich	AY138640	–
136	<i>Batrachomatus wingii</i>			Australia	L. Hendrich	AY138642	AY138730
137	<i>B. daemeli</i> AMPHIZOIDAE			Australia	C.H.S. Watts	AY138641	AY138729
138	<i>Amphizoa insolens</i>			US (California)	A. Cognato	AY071770	AY071796
139	<i>A. lecontei</i>			US (California)	NHM	AY071771	AY071797
	HYGROBIIDAE						
140	<i>Hygrobia australiasiae</i>			Australia	C.H.S. Watts	AY071779	AY071805
141	<i>H. hermanni</i>			Spain	I. Ribera	AY071780	AY071806
142	<i>H. maculata</i>			Australia	D. Norton	AY071781	AY071807

Dist., distribution (W, West; E, East; N, Nearctic; P, Palearctic; H, Holarctic).

^a Classification follows Nilsson (2001).

^b In brackets, species numbers with identical 16S rRNA sequences.

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