The phylogeny of diving beetles (Coleoptera: Dytiscidae) and the evolution of sexual conflict

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A model of evolution based on conflicts of interest between the sexes over mating decisions is examined in relation to diving beetles (Dytiscidae). The model predicts the following evolutionary sequence: (1) cost to females of mating increases, (2) females evolve behavioural resistance to male mating attempts, (3) males evolve devices to overcome female resistance, and (4) females evolve morphological counter-adaptations to the male devices. This model is tested using species of Dytiscidae, in which (1) some species have a very long mating duration while others mate quickly, (2) females of some species resist male mating attempts by swift and erratic swimming when seized by a male, (3) males of some species possess a grasping device in the form of sucker-shaped setae on the legs used to adhere to the pronota or elytra of females prior to mating, and (4) females of some species have a modified dorsal cuticle with irregular sculpturing which appears to interfere with the male adhesive setae. The predicted order of evolution of some of these features was tested in a cladistic analysis of 52 taxa in Dytiscidae and Hygrobiidae using characters from adult and larval morphology and a portion of the gene *wingless*. The combined analysis resulted in nine most parsimonious cladograms. The consensus cladogram of these indicates that male sucker setae arose a single time in a clade of Dytiscinae. Nested within this clade are five groups with an independently derived, modified dorsal cuticle in females. This pattern of characters in Dytiscinae is consistent with the prediction implied by the model of sexual selection. The utility of *wingless* as a marker for phylogenetic analysis of diving beetles is discussed, and the resulting phylogeny is compared with previous analyses and current classification. © 2003 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2003, **79**, 359–388.

ADDITIONAL KEYWORDS: arms race – cladistics – dimorphism – secondary sexual characters – sexual selection.

INTRODUCTION

Because females in most insects invest substantially more energy in offspring than do males, they tend to be more selective in their choice of mates (Thornhill & Alcock, 1983). Although females may derive benefits from multiple matings (Eberhard & Cordero, 1995; Yasui, 1998; Arnqvist & Nilsson, 2000), there are also potential costs involved. These include time spent away from other activities (Martens & Rehfeldt, 1989), energy expended in mating (Watson & Lighton, 1994; Watson, Arnqvist & Stallmann, 1998), increased potential for exposure to diseases or parasites (Watson *et al.*, 1998), increased risk of predation (Wing, 1988;

Arnqvist, 1989; Gwynne, 1989; Magnehagen, 1991; Fairbairn, 1993; Rowe, 1994), and risk of death or injury (Daly, 1978; Le Boeuf & Mesnick, 1991). Because multiple prolonged matings may reduce overall fitness in females, they are expected to (counter intuitively) resist mating attempts under certain circumstances, while males are expected to attempt to force copulation (Parker, 1979; Alexander, Marshall & Cooley, 1997). A predicted outcome under this scenario is the evolution of behaviours and/or morphologies in males that aid in overcoming female resistance. Females are predicted to then respond by evolving counter-adaptations to overcome the male advantage. Males may then evolve to overcome the female counter-adaptation, and so on. Thus, an evolutionary 'arms race' of escalating responses and counter-responses may drive the evolution of sexually selected

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characters (Alexander *et al.*, 1997; Parker, 1979). The never-ending nature of these races presumably results in rapid divergence characterizing features related to mating (Darwin, 1871; Alexander *et al.*, 1997).

This form of sexual selection has been proposed to be operating in a number of arthropods including dung flies (Allen & Simmons, 1996; Parker, 1979), scorpionflies (Thornhill, 1980; Thornhill & Sauer, 1991), water striders (Arnqvist, 1992; Krupa, Leopold & Sih, 1990; Weigensberg & Fairbairn, 1994; Lauer, Sih & Krupa, 1996; Arnqvist, 1997) and isopods (Jormalainen & Merilaita, 1993; Jormalainen, Tuomi & Yamamura, 1994; Sparkes, Keogh & Haskins, 2000). These groups have several features in common: long mating events (which presumably increases the cost of mating to females); male grasping devices apparently used to coerce females to mate; resistance to mating behaviourally by the female and, in some putative cases, morphologically, by devices that interfere with a male's ability to grasp or copulate. Male grasping structures are fairly common in insects (see Eberhard, 1985 and references therein). However, clear evidence of female morphological counter-adaptations to male grasping devices is very rare (Eberhard, 1985; Arnqvist & Rowe, 1995; Andersen, 1997).

Certain members of the aquatic, predaceous beetle family Dytiscidae have mating behaviours and secondary sexual morphology that may have resulted from an intersexual arms race (Bergsten, 1999; Bergsten, Töyrä & Nilsson, 2001). In some taxa, such as *Dytiscus* Linnaeus (Régimbart, 1877; Blunck, 1912; Aiken, 1992) and *Rhantus* Dejean (Smith, 1973), males suddenly pounce upon any females they encounter. Upon being seized, the female often swims rapidly and erratically, apparently attempting to dislodge the male through irregular movements and by driving him into the substrate or against objects. Sometimes she successfully removes him (Aiken, 1992; K.B. Miller, unpubl. observ.). Male protarsomeres 1–3 and, to a lesser extent, mesotarsomeres 1–3, are sexually dimorphic and are broadly expanded with ventral adhesive setae (Figs 1, 3). Although such setae are not unusual for dytiscids (or beetles in general, see Stork (1980) and Stork & Evans (1976)), males of certain taxa within the subfamily Dytiscinae have very large adhesion setae with apices that are suction-cup shaped. The suction-cup setae possess a distinctive structure (Figs 2, 4) (Korschelt, 1923) and are strongly adhesive (Plateau, 1872; Aiken & Khan, 1992). Most other taxa within the family, including Cybistrini, the sister group to the other Dytiscinae (Miller, 2000), have adhesive setae with apices that do not resemble suction cups (Figs 1, 3) (Chatanay, 1911) and do not appear to be as strongly adhesive when placed on a smooth female elytron (K. B. Miller, unpubl. observ.). In the taxa examined, the sucker-shaped setae func-

tion as a grasping device which a male uses to adhere to the smooth elytra or pronotum of a female before or during mating (Plateau, 1872; Régimbart, 1877; Blunck, 1912; Aiken, 1992). In *Dytiscus*, the group studied in greatest detail (Aiken, 1992; Régimbart, 1877; Blunck, 1912), these setae are used to attach to a female's dorsal surface during an initial, very intense struggle phase. If the male is able to remain attached, he then initiates copulation attempts and holds the female in a variety of ways including using the tarsal claws or entire legs to grasp the edges of the female (Aiken, 1992; Plateau, 1872; Régimbart, 1877; Blunck, 1912).

Whereas the dorsal surfaces of males are smooth (Figs 5, 8, 11, 14, 17), the females of some species are strikingly modified. Although females of species throughout the Dytiscidae often have slightly altered dorsal surfaces, some species of Dytiscinae are strongly and conspicuously modified (Figs 6, 7, 9, 10,
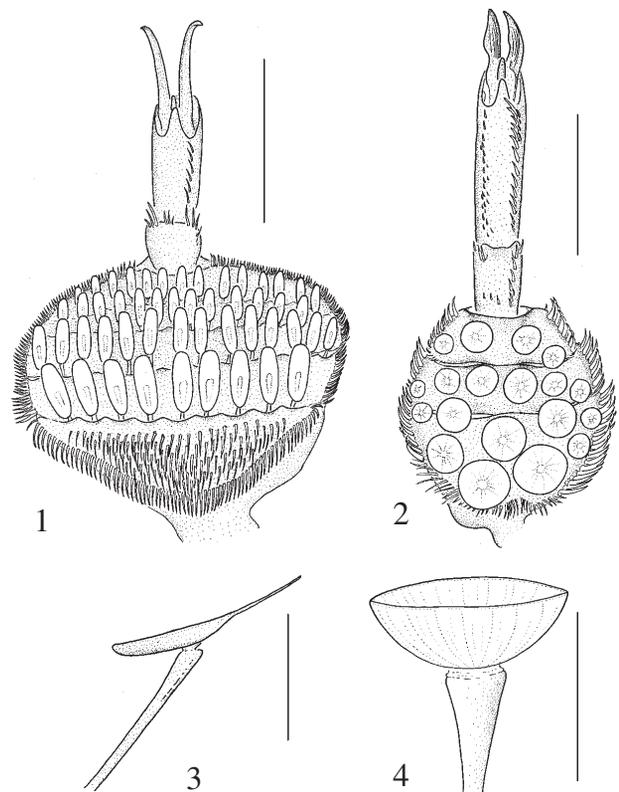

Figures 1–4. Male secondary sexual features. 1,2 = male protarsi, ventral aspect showing distribution of adhesive setae on expanded protarsomeres 1–3; 1 = *Cybister fimbriolatus*, 2 = *Hyderodes schuckardi*. 3,4 = adhesive setae, lateral aspect; 3 = *Cybister fimbriolatus*, 'asymmetric' type, 4 = *Hyderodes schuckardi*, 'symmetric' type or 'suction-cup' shaped.
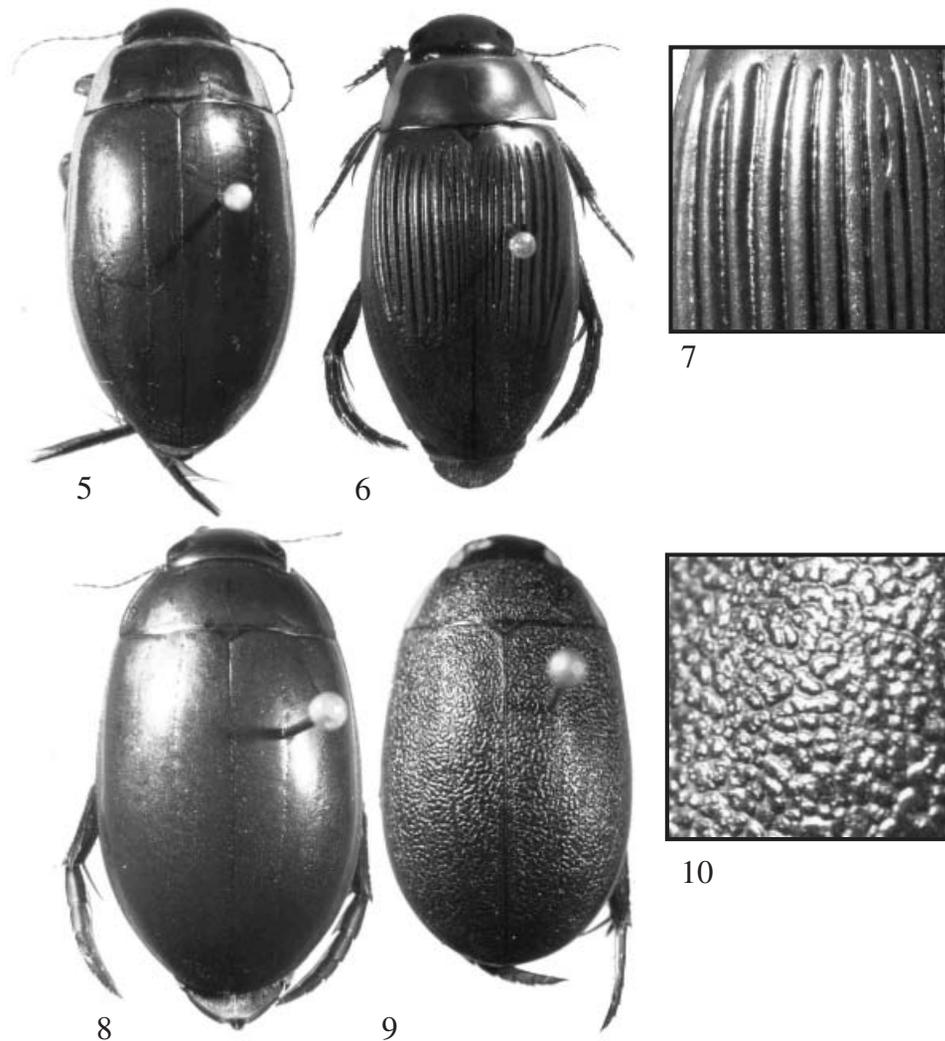
Figures 5–10. 5–7 = *Dytiscus fasciventris*; 5 = male, 6 = sexually dimorphic female, 7 = surface of dimorphic female elytron. 8–10 = *Hyderodes schuckardi*; 8 = male, 9 = sexually dimorphic female, 10 = surface of dimorphic female elytron.

12, 13, 15, 16, 18, 19). Modifications include: longitudinally sulcate elytra in some *Dytiscus* (Figs 6, 7) (Bergsten, 1999; Roughley, 1990), strongly granulate dorsal surfaces in *Hyderodes* Hope (Figs 9, 10) and *Graphoderus zonatus* (Hoppe) (Figs 12, 13) (Nilsson, 1986; Bergsten *et al.*, 2001); irregularly rugulose pronotal and anterolateral elytral surfaces in *Hydaticus* Leach (Figs 15, 16); setose, longitudinal sulci on the elytra in some species of *Acilius* Leach (Figs 18, 19). Other female elytral modifications exist in species of dytiscines including *Thermonectus* Dejean, many Cybistrini, *Tikoloshanes* Omer-Cooper and *Notaticus* Zimmermann; these are not nearly as conspicuous as in the other taxa, though they may have been influenced by the same selective pressures. These modifications even attracted the attention of Darwin (1871),

who suggested they may function as aids to the male during mating, at least in *Dytiscus*, though it is not intuitively clear how this might work. In contrast, Bergsten *et al.* (2001) suggest that the modifications of the female dorsal surface are adapted to interfere with the male grasping device, giving them greater control over the decision to mate. To show a relationship between male setae and female dorsal modifications they present evidence of a positive correlation between the number and size of male protarsal setae and the proportion of modified females in populations of the species *Graphoderus zonatus*. In this species, as in many species of Dytiscinae with modified elytra, females are dimorphic, with some modified and others not, and the proportion of modified females appears to vary among populations (Nilsson, 1986; Roughley,
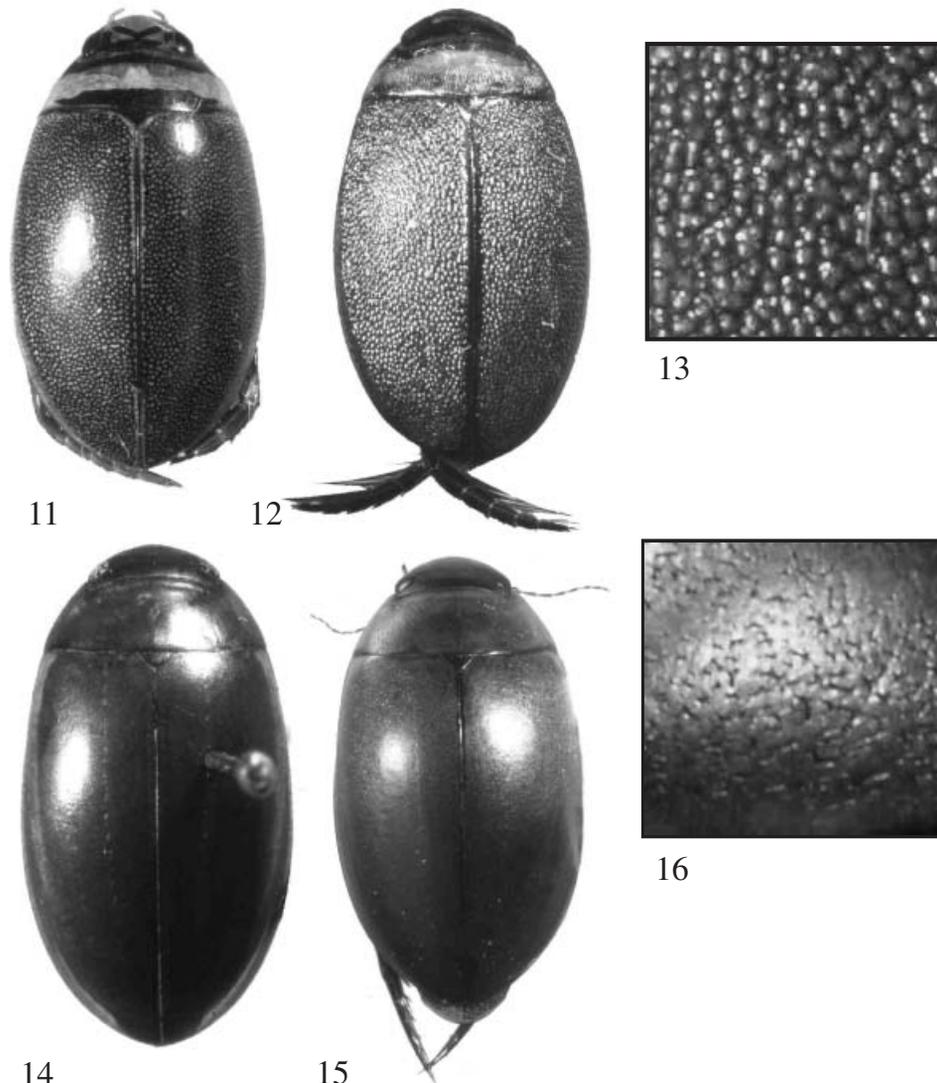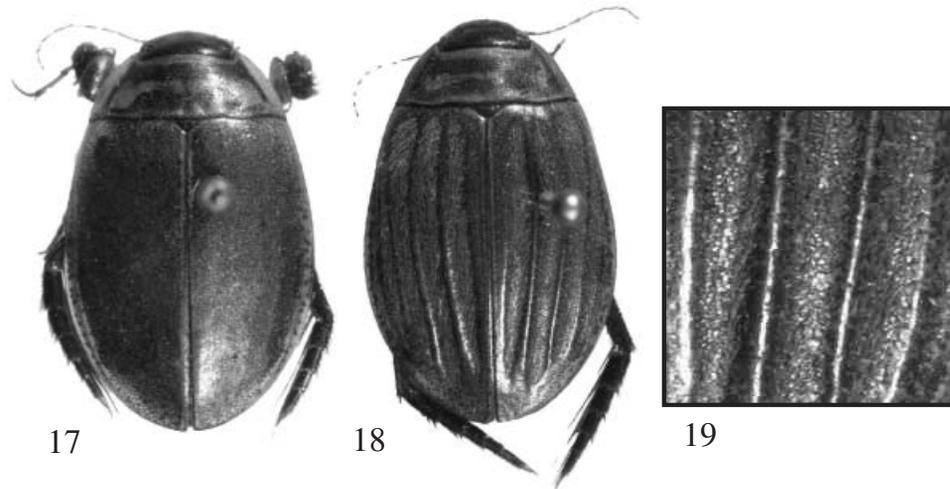
Figures 11–16. 11–13 = *Graphoderus zonatus*; 11 = male, 12 = sexually dimorphic female, 13 = surface of dimorphic female elytron. 14–16 = *Hydaticus cinctipennis*; 14 = male, 15 = sexually dimorphic female, 16 = surface of dimorphic female pronotum.

1990; Bergsten *et al.*, 2001). A positive correlation between these features of this taxon suggests a functional relationship. Presumably the granulations interfere with the ability of a male to adhere to the elytra or pronotum of a female and, therefore, his ability to coerce the female to mate. It is possible that differences in the size and number of sucker setae confer different male mating success depending on the nature of female dorsal sculpture, athough Bergsten *et al.* (2001) do not provide direct evidence of this. It makes sense that deep grooves, setae or other irregularities would render sucker-discs less adhesive to elytral or pronotal surfaces. Unfortunately, detailed observations of mating behaviour in most Dytiscidae

in general are not available, and the actual effect of irregular female surfaces on the ability of males to capture females remains only intuitive at this time.

Secondary sexual characters and traits within this model of evolution are usually studied in single species or at the population level. Only rarely are they examined within the context of the phylogeny of a group (e.g. Andersen, 1997; Soulier-Perkins, 2001). Recent studies of the phylogeny of the Dytiscinae (Miller, 2000) and Dytiscidae (Miller, 2001) provide a basis for a historical context in which to view such characters. Optimizing characters on a cladogram can tell an investigator several things about their evolution, such as whether similar features are homologous

Figures 17–19. *Acilius sulcatus;* 17 = male, 18 = sexually dimorphic female, 19 = surface of dimorphic female elytron.
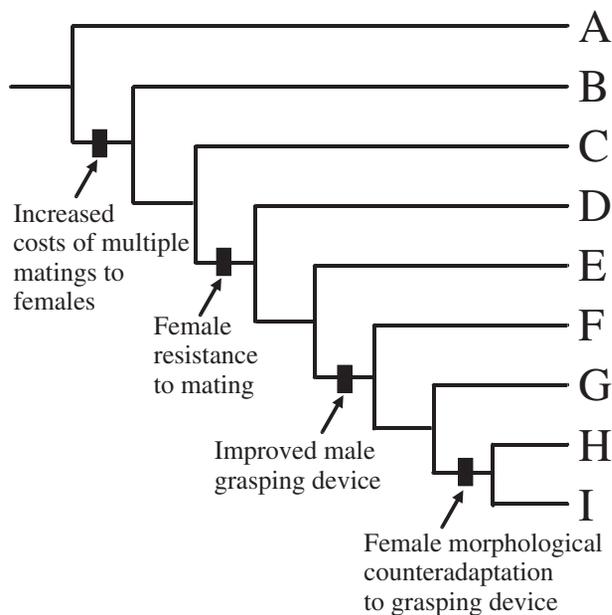

Figure 20. Expected pattern of characters associated with model of sexual selection mapped onto a hypothetical phylogeny.

examine some of the characters associated with male grasping devices and female resistance and test whether the pattern of characters in dytiscine phylogeny is consistent with that predicted by an arms race involving escalating sexual conflict. Some of the characters related to this model of evolution are predicted to evolve in the following order: (1) increased cost to females of mating (possibly resulting from prolonged mating duration), (2) female behavioural resistance to mating, (3) improved male grasping ability, and (4) a female morphological counter-adaptation to the male grasping device (Fig. 20). Behavioural studies of members of this taxon are sorely lacking. Because of this lack of data it is only practical to critically test the evolution of morphological features at this time, specifically the apparently improved male grasping devices in the form of large, sucker-shaped protarsal and mesotarsal adhesive discs and the female counter-adaptation in the form of a roughened dorsal cuticle. Nevertheless, this phylogenetic analysis will also provide a valuable context within which to study the evolution of other behavioural or morphological features as improved data are gathered.

The Dytiscinae are moderate to very large sized, predatory, aquatic beetles occurring world-wide in lentic habitats. The phylogeny of this group in particular (Miller, 2000) and the family as a whole (Miller, 2001) was explored recently. In the present study, emphasis was placed on determining relationships within the Dytiscinae and between this group and closely related taxa. Although many relationships between the members of Dytiscinae are reasonably well supported by adult morphological characters, several are less well established. Thus, a secondary objective of this project was to provide an improved hypothesis on the rela-

or homoplasious, whether certain characters are correlated historically, and in what order particular characters evolved. An analysis of the phylogeny using appropriate methods should be the starting point when exploring complex evolutionary scenarios since doing so places them within the context of their ancestry and helps to avoid errors such as misrepresenting the order or combination in which characters evolved (Coddington, 1988; Carpenter, 1989). In this paper, I

tionships between taxa from the addition of new larval and DNA data.

Nuclear protein-coding genes have increasingly been shown to provide useful DNA sequence data for elucidating relationships among a variety of insect taxa and at various levels of taxon divergence. Although often more difficult to amplify in insects, these low-copy nuclear genes appear to be more useful when compared with sequence data from other genes (Baker, Wilkinson & DeSalle, 2001). The nuclear protein-coding gene *wingless* is a member of the Wnt gene family (Schubert, Holland, Holland & Jacobs, 2000). Sequence data from this gene have been used effectively for resolving relationships in the Lepidoptera (Brower & Egan, 1997; Brower & DeSalle, 1998; Campbell, Brower & Pierce, 2000), Carabidae (Sota & Vogler, 2001) and Diopsidae (Baker, Wilkinson & DeSalle, 2001). In a comparative study of various genes for use in grouping the Diopsidae, *wingless* showed greater bootstrap support and data decisiveness and higher CIs than other genes studied (Baker *et al.*, 2001). An additional secondary goal of this study was to examine the utility of a portion of the *wingless* gene for elucidating the phylogeny of members of the family Dytiscidae. This is the first study to use *wingless* or any nuclear protein-coding gene in the family Dytiscidae.

METHODS

TAXA

Wingless was sequenced from *Hygrobia tarda* (Herbst) (Hygrobiidae) and 51 species of Dytiscidae from 27 genera, 17 tribes, and 9 subfamilies (Table 1). *Hygrobia* was used to root the cladogram since this taxon has been shown to be outside the Dytiscidae in a previous study (Miller, 2001) and has been historically regarded as the sister taxon of Dytiscidae (Beutel, 1995). Of the dytiscid subfamilies, Dytiscinae was sampled most thoroughly, and all dytiscine tribes and most of the genus-groups were included. Since this represents the first molecular study of Dytiscidae using *wingless*, a broad range of exemplars from other subfamilies was included in order to better assess the utility of the gene at various taxonomic levels.

DNA SEQUENCING

DNA was extracted using a phenol–chloroform method (Danforth, Sauquet & Packer, 1999). Primers used for amplification were LepWg1 and LepWg2a (Brower & Egan, 1997). Some taxa did not amplify well with this primer combination, and two additional primers were developed: the forward primer WgDytF1, 5′-CGY CTT CCW TCW TTC CGW

GTY ATC-3′, and the reverse primer WgDytR1, 5′-CCG TGG ATR CTG TTV GCH AGA TG-3′. In some cases, weakly amplified PCR products using LepWg1 and LepWg2a were re-amplified from gel-purified bands using nested primer combinations (i.e. LepWg1-WgDytR1 and WgDytF1-LepWg2a) to increase yield.

PCR amplifications were conducted using protocols similar to those of Palumbi (1996) with the following cycle conditions: 94°C, 1 min denaturation; 45–60°C, 1 min annealing; 72°C, 1 min extension, for 35 cycles. In some cases, but not all, amplification was improved by decreasing the denaturation temperature to 88°C after the first 10 cycles while keeping the other temperatures the same throughout. PCR products were isolated using low-melting-point agarose (FMC, Rockland, ME) at 56 V and 4°C and they were purified using the Promega (Madison, WI) Wizard PCR Preps purification kit. Three DNA fragments were amplified for many taxa. The longest (about 460 bp) was always brighter than others after gel-purification and more consistently amplified from all taxa sampled. Therefore, this fragment was selected for analysis. Automated sequencing of PCR products was done, using ABI 3700 or ABI 377 DNA Sequencers and the Wg1 or DytWg1 primers, by the Cornell BioResource Center, Cornell University, Ithaca, NY, USA.

MORPHOLOGY

Adult morphological characters used here are derived mainly from previous analyses (Miller, 2000; Miller, 2001) with a few modifications and additions (see Appendix). Larval character states were derived from examination of most taxa and published descriptions of others (see Table 1). Though used extensively in the taxonomic literature, larval characters have not been used previously in a cladistic analysis. Larvae of numerous taxa are unknown or inadequately described and these taxa were scored as unknown for all or some larval characters. Characters and states from morphology and indels (see below) are explained in the Appendix, and the matrix for the 52 taxa is presented in Table 2.

CHARACTER ANALYSIS

Simultaneous analysis
Some authors (e.g. Bull *et al.*, 1993; de Queiroz, Donoghue & Kim, 1995) have advocated conducting tests of heterogeneity on data partitions prior to deciding whether or how to combine data for simultaneous analysis. However, regardless of a perceived heterogeneity or incongruence of data sets, combining data of various types in one simultaneous analysis is the only method that is consistent with the logic of parsimony

Table 1. Taxa used in analysis including classification, GenBank accession number, collection data and source of larval character coding information

Species	Taxonomy	GenBank access no.	Collection data	Larval character derivation
Hygrobia tarda (Herbst)	Hygrobiidae	AF391997	Sardinia	Bertrand (1928, 1972)
Copelatus australiae (Clark)	Copelatinae	AF392006	Australia: NSW, roadside pool 6 km SW Braidwood 35°29'52'S 149°44'09'E 687 m, 02 November 2000	KBMC
Coptotomus lenticus Hilsenhoff	Coptotominae	AF392007	USA: New York, Tompkins Co. Ithaca. 10 September 1999	KBMC
Laccornis difformis (LeConte)	Hydroporinae Laccornini	AF392030	USA: New York, St. Lawrence Co. Macomb Tep. Fish Cr. marsh. 23 May 2000 44°28'20'N 75°33'48'W	Spangler & Gordon (1973)
Neoporus undulatus (Say)	Hydroporinae Hydroporini	AF392037	USA: New York, Tompkins Co. Ithaca. 27 April 2001	KBMC
Laccophilus maculosus Say	Laccophilinae Laccophilini	AF392031	USA: New York, Tompkins Co. Ithaca. 23 October 2000	KBMC
Agabetes acuductus (Harris)	Laccophilinae Agabetini	AF392048	USA: New York, St. Lawrence Co. Macomb Tep. Fish Cr., 23 May 2000, 44°28'20'N 75°33'48'W	Spangler & Gordon (1973)
Lancetes lanceolatus (Clark)	Lancetinae	AF392032	Australia: Victoria, Sheepwash Lagoon 10 km E Yea 37°10'16'S 145°31'44'E, 185 m 08 November 2000	KBMC
Matus bicarinatus (Say)	Matinae	AF392033	USA: New York, St. Lawrence Co. Macomb Twp. Fish Cr. Marshes. 29 May 2000	KBMC
Agabus tristis Aubé	Agabinae	AF392003	USA: Colorado, Summit Co., Bogan Flats 2784 m 39°26'42'N 106°19'54'W, 06 August 2000	
Ilybius gagates Aubé	Agabinae	AF392002	USA: New York, Tompkins Co. 10 km S Caroline, woodland pool. 10 September 1999	
Ilybius subaeneus Erichson	Agabinae	AF392029	USA: Colorado, Summit Co., Homestake Cr. ponds 2798 m, 39°24'37'N 106°25'21'W, 06 August 2000	Galewski (1966)
Colymbetes exaratus LeConte	Colymbetinae	AF392004	USA: Arizona, Apache Co. Reservoir c. 10 km W Nutrioso 24 April 2000	KBMC
Colymbetes paykulli Erichson	Colymbetinae	AF392005	USA: Colorado, Summit Co., Homestake Cr. ponds 2798 m, 39°24'37'N 106°25'21'W, 06 August 2000	Galewski (1964, 1968)
Rhantus binotatus (Harris)	Colymbetinae	AF392040	USA: Arizona, Cochise Co. Rucker Canyon, Chiracahua Mtns. 26 April 2000	KBMC
Rhantus sinuatus (LeConte)	Colymbetinae	AF392041	USA: New York, St. Lawrence Co. Macomb Tep. Fish Cr., 23 May 2000 44°28'20'N 75°33'48'W	
Neoscutopterus angustus (LeConte)	Colymbetinae	AF392035	USA: New York, Tompkins Co. McLean Bog, 26 May 2000	Hilsenhoff (1989)
Onychohydrus scutellaris (Germar)	Dytiscinae Cybistrini	AF392038	Australia: Victoria, marsh 18.6 km W Casterton 37°35'57'S 141°09'45'E, 135 m 11 November 2000	KBMC
Spencerhydrus pulchellus Sharp	Dytiscinae Cybistrini	AF392044	Australia: Western Australia, Byenup Lagoon, October 2000	
S. latecinctus Sharp	Dytiscinae Cybistrini	AF392043	Australia: Victoria, 25 km W Casterton, 6 October 2000	KBMC

Table 1. *Continued*

Species	GenBank access no.	Collection data	Taxonomy	Larval character derivation
Megadytes fraternus Sharp	AF392034	USA: Florida, Collier County, Jane's Scenic Road & Sterwert Fahahatchee Strand S.P., 22 March 2000	Dytiscinae Cybistrini	KBMC*
Cybister puncticollis (Brullé)	AF392009	Bolivia: Dpto. Beni, Prov. Cercado 9.5 km N Trinidad 14°46′34′S 64°58′00′W, 17 June 1999	Dytiscinae Cybistrini	
C. fimbriolatus Say	AF392008	USA: Florida, Collier Co., Fahatalechee St. Preserve, mercury vapor trap, 23–22 March 2001	Dytiscinae Cybistrini	KBMC
C. tripunctatus (Olivier)	AF392010	Australia: Victoria, 10 km E Corryong, temp pond 36°10′41′S 148°02′52′E 250 m, 05 November 2000	Dytiscinae Cybistrini	Bertrand (1972)
Dytiscus fasciventris Say	AF392011	USA: New York, Tompkins Co. Ithaca. 25 September 1999	Dytiscinae Dytiscini	KBMC
D. verticalis Say	AF392012	USA: New York, Tompkins Co. Ithaca. 26 May 2000	Dytiscinae Dytiscini	KBMC
Hyderodes schuckardi (Hope)	AF392018	Australia: Victoria, roadside pool ~20 km W Cowwarr, 38°00′52′S 146°32′03′E, 07 November 2000	Dytiscinae Hyderodini	KBMC
Notaticus fasciatus Zimmermann	AF392036	Bolivia: Dpto Beni, Prov Cercado 9.5 km N Trinidad 14°46′34′S 64°58′00′W, 17 June 1999	Dytiscinae Aubehydrini	
Hydaticus (H.) seminiger (DeGeer)	AF392026	Sweden: Östergötland, Linköping	Dytiscinae Hydaticini	Galewski (1983)
H. (H.) aruspex Clark	AF392019	USA: New York, Schuyler Co., Texas Hollow SWA, 6 September 2000	Dytiscinae Hydaticini	Hilsenhoff (1993)†, Watts (1970)†
H. (H.) cinctipennis Aubé	AF392021	USA: New York, St. Lawrence Co. Macomb Tep. Fish Cr. 23 May 2000 44°28′20′N 75°33′48′W	Dytiscinae Hydaticini	
H. (Guignotites) parallelus Clark	AF392025	Australia: Victoria, roadside pool ~20 km W Cowwarr, 38°00′52′S 146°32′03′E, 07 November 2000	Dytiscinae Hydaticini	KBMC
H. (G.) subfasciatus Laporte	AF392027	Bolivia: Dpto. Sta Cruz, Prov. Chiq. 2.7 km S San Jose 17°52′20′S 60°44′28′W 333 m 27 June 1999	Dytiscinae Hydaticini	
H. (G.) fabricii (MacLeay)	AF392022	Philippines: Boracay Central, Shallow temp water w. veg., 26 September 2000	Dytiscinae Hydaticini	
H. (G.) grammicus Germar	AF392023	China: Yunnan, 2 km N of Shizong, wetland, 10 September 2000	Dytiscinae Hydaticini	Galewski (1975)
H. (G.) bowringi Clark	AF392020	Japan: Honshu, Kawasaki, Kameyama City: Mie Pref., 01 August 2000	Dytiscinae Hydaticini	
H. (G.) major Régimbart	AF392024	China: Yunnan, 4 km S Shizong waterhole in slope towards lake. 13 September 2000	Dytiscinae Hydaticini	

Species	Classification	Accession	Locality	Reference/Source
H. (Hydaticinus) xanthomelas Brullé	Dytiscinae Hydaticini	AF392028	Bolivia: Dpto. Sta Cruz, Prov. Velasco, 1.5 km SE San Ignacio 16°23'24'S 60°57'13'W 20 June 1999	KBMC
Eretes australis (Erichson)	Dytiscinae Eretini	AF392013	Australia: S. Australia, shallow roadside pool ~15 km N Kingston. 13 November 2000	
E. griseus (Fabricius)	Dytiscinae Eretini	AF392014	Hong Kong: Lantau Ishland, S of Mui Wo, Ngau Kwu Wani, artificial cement pond, 19 September 2000	Bertrand (1933, 1949), Mayet (1887), Nowrojee (1912)‡
Acilius mediatus (Say)	Dytiscinae Aciliini	AF391998	USA: New York, Tompkins Co. Pond 10 km S Caroline, 23 October 2000	KBMC§
A. semisulcatus Aubé	Dytiscinae Aciliini	AF391999	USA: New York, Tompkins Co. Pond 10 km S Caroline, 23 October 2000	KBMC
A. sulcatus (Linnaeus)	Dytiscinae Aciliini	AF392000	Sweden: Västerbotten, Umeå, Lomtjärn, 22 May 2000	Galewski (1991), Bertrand (1928, 1972), Fiori (1949)
A. sylvanus Hilsenhoff	Dytiscinae Aciliini	AF392001	USA: New York, St Lawrence Co. Macomb Twp. Fish Cr. Marsh, 26 April 2000	KBMC
Graphoderus fascicollis (Harris)	Dytiscinae Aciliini	AF392015	USA: New York, Owego Co. nr Boylston Center, 43°39'26'S 25°55'07'W 438 m 18 August 2000	†Hilsenhoff (1993)
G. liberus (Say)	Dytiscinae Aciliini	AF392016	USA: New York, Tompkins Co. Ringwood Preserve, 19 September 2000	KBMC
G. zonatus (Hoppe)	Dytiscinae Aciliini	AF392017	Sweden: Västerbotten: Umeå: Lomtjärn, 22 June 2000	Galewski (1990)
Rhantaticus congestus (Klug)	Dytiscinae Aciliini	AF392039	Vietnam: Dac Lac Yok Don Nat Park, hdqts. Rooftop c. 2 km SE Ban Don, 22 May 1997	Bertrand (1928, 1972)
Sandracottus bakewelli (Clark)	Dytiscinae Aciliini	AF392042	Australia: NT, Ormiston	
Thermonectus circumscriptus (Lat.)	Dytiscinae Aciliini	AF392045	Bolivia: Sta Cruz, Prov Chiquitos 2.7 km S San Jose 17°52'20'S 60°44'28'W, 333 m, 27 June 1999	
T. marmoratus (Hope)	Dytiscinae Aciliini	AF392046	USA: Arizona, Pima Co. stock tank, ~6 km S Arivaca 31°31'15'N 11°16'44'W, 1088 m, 29 April 2000	
T. nigrofasciatus (Aubé)	Dytiscinae Aciliini	AF392047	USA, Arizona, Cochise Co. Rucker Canyon, Chiracahua Mtns. 26 April 2000	KBMC, Hilsenhoff 1993, Wilson (1923)

*Megadytes larvae examined were collected with M. fraternus adults in Bolivia but are possibly not the same species.
†Described in part.
‡Some of these may refer to E. sticticus (Miller, in press).
§Larvae collected in association with adults.
KBMC = K.B. Miller's personal collection.

Table 2. Data set for 52 terminals and 92 morphological and *wingless* indel characters used in cladistic analysis

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Hygrobia tarda            11011100-0000000110000-100101101010000-0010000010??0011000001100000000000000-010-0-0-000010000
Acilius mediatus          01100100-0100000010011111000-0-000001001100011001100110001101002--0010210001001110211001320-100110100
A. semisulcatus           01100150-01000000010011111000-0-000001001100011001100110001101102--0010210001001110211001320-100110100
A. sulcatus               01100150-01000000010011111000-0-000001001100011001100110001101102--0010210001001110211001320-100110100
A. sylvanus               01100150-01000000010011111000-0-000001001100011001100110001101102--0010210001001110211001320-100110100
Agabetes acuductus        11001100-00000001100000-100111100000-0000000-1100100001000012110000000-010-0-0-00010000
Agabus tristis            11001101200000110000-1000-110000-0-010000-0100000-010000110000010000?????????????????200
Colymbetes exaratus       11000101100000110000-1001111100000-00100101010000010000110000010000-010-0-0-000010000
C. paykulli               11000101100000110000-1001111100000-001010010100001010000110000010000-010-0-0-000010000
Copelatus australiae      11001100-00000110000-1000-110000-00100000-01001110000010000010000-010-0-0-000010000
Coptotomus lenticus       11001100-00000110000-1001111100000-00100000-0100000010001100000100000-010-0-0-000010000
Cybister fimbriolatus     01000101000000001100001011100-10101011000110121000000$11102--00012001101000130014 1-1111110000
C. puncticollis           01000101000000001100001011100-10101011000110121000000051102--00012001101000130014 1-1111110000
C. tripunctatus           01000101000000001100001011100-10101011000110121000000051102--00012001101000130014 1-1111110000
Dytiscus fasciventris     01110111100000101100000-1110-12010001000100100100000011112--00012000000000201120-1010011000
D. verticalis             01110110000000101100000-1110-12010001000100100100000011112--00012000000000201120-1010011000
Eretes australis          01101100-10000000010000-0-00-0-0000011001001311001010010102--00012000010000111111100-100110110
E. griseus                01101100-10000000010000-0-00-0-0000011001001311001010010102--00012000010000111111100-100110110
Graphoderus fascicollis   01100100-00000000010001110000-120000010011001311001010100102--00002100010011102110310-100110100
G. liberus                01100100-00000000010001110000-120000010011001311001010100102--00002100010011102110310-100110100
G. zonatus                01100100-00000000010001110000-120000010011001311001010100102--00002100010011102110310-100110100
Hydaticus aruspex         01100140-00000100011001112000001011001110100100110101011001102--000021000000000?????2-????2110?200
H. bowringi               01100130-00010001100111001120000010100010101010110100100110101102--000210000?????????????????200
H. cinctipennis           01100130-00010001100111001120000010100010101010110100100110101102--000210000?????????????????200
H. fabricii               01100130-00010001100111001120000010100010101010110100100110101102--000210000?????????????????200
H. grammicus              01100130-00010001100111001120000010100010101010110100100110101102--000210000000001001112-0-100110000
H. major                  01100130-00010001100111001120000010100010101010110100100110101102--000210000?????????????????200
H. parallelus             01100130-00010001100111001120000010100010101010110100100110101102--000210000000001001112-0-100110000
H. seminiger              01100130-00010001100111001120000010100010101010110100100110101102--000210000000001001112-0-100110000
H. subfasciatus           01100130-00010001100111001120000010100010101010110100100110101102--000210000?????????????????200
H. xanthomelas            01100130-00010001100111001120000010100010101010110100100110101102--000210000?????????????????200
Hyderodes schuckardi      01101120-00001001001000-100111201001100100100101102--0001210000000000201120-1010011000
Ilybius gagates           11001101200000011100000-1000-110000-00100000-010000110000100000100000?????????????????200
I. subaeneus              11001101200000011000000-1000-110000-00100010-0100000010000100000100000-010-0-0-000010000
Laccophilus maculosus     11000000-00000001100000-1000-110000-01000000-010000000000150000000001001201010000000-010-0-0-000010000
```

```
Laccornis difformis         10-01000-0010001100000-1000-11000000-0010000010010010101000000000001000-000-0-0-000010001
Lancetes lanceolatus        11001101200000110000-1001111000000-0000100-0010010100000110000000000-010-0-0-000010000
Matus bicarinatus           11001100-0001001100000-1000-11000000-0010010100000000110000000010000-010-0-0-000010000
Megadytes fraternus         01000101000000001100000-1100-10001101001101121000000211102--0001200110100130141-1111100000
Neoporus undulatus          10-01000-0010001100000-1000-11000000-0010001001001101100000001000-000-0-0-00010001
Neoscutopterus angustus     11000101100000110000-1001111000000-0010011010000010101000000001000-010-0-0-00010000
Notaticus fasciatus         01100000-0000000100011000-1201000100010010010110101102--0001200022??????????00
Onychohydrus scutellaris    01000100000000110000-1100-10000100001111000000011102--0012001101000130131-1111100000
Rhantaticus congestus       01100100-0000000100111000-12000001001301100010001102--0102100100110011021110310-100110100
Rhantus binotatus           11001101100000110000-1001110000000-00101001010001010001101000000000010-0-0-00010000
R. sinuatus                 110011011000001110000-100111110000-12000001001010000110100001000000?????????00
Sandracottus bakewelli      01100100-00000001001110000-120000010010100110011002--001021000?????????00
Spencerhydrus latecinctus   01000101000011100010100000-0-0011010001101210000000011102--0001200110100120021-1111100000
S. pulchellus               0100010100001100010100000-0-0011010001101210000000011102--0001200111?????????00
Thermenectus marmoratus     01100100000000010011110000-0-0000010011001311001011102--001021000?????????-??????00
T. circumscriptus           01100100-00000001001111000-0-0000010011001311001011102--0002100002?????????-??????00
T. nigrofasciatus           01100100-00000001001111000-0-0000010011001311001011102--0002100010111021103200-100110100
```

+ = additive characters, $ = polymorphism (= character states 4 and 5).

(Nixon & Carpenter, 1996; Kluge, 1998). In this analysis, data from two partitions, the morphology/indel data and the sequence data, were analyzed separately to determine the overall signal that may be independently present in each partition and to explore various aspects of the nucleotide data. However, the preferred topology used to infer the phylogeny is based on a simultaneous parsimony analysis of all the data.

Inclusion of characters

Some authors (e.g. Brooks & McLennan, 1991; de Queiroz, 1996) have argued that those characters of particular interest for an evolutionary hypothesis should not be included in the analysis since doing so would result in bias and/or circularity. They advocate that such characters be mapped on an already-existing tree. Since the goal of this type of analysis is to determine the homology of these characters, and the only test of homology is character congruence (de Pinna, 1991), characters of interest should logically be included in the analysis (Deleporte, 1993; de Pinna, 1996; Luckow & Bruneau, 1997). The characters of interest here, male pro- and mesotarsal adhesive setae and female cuticular modifications, are included in the parsimony analysis to test their homology and infer their order of evolution.

Alignment

Alignment of the sequences was done using MegAlign (Lasergene software, DNASTAR Inc., Madison, WI, USA). The sequences did not include introns and alignment was relatively unambiguous, but several 3-bp (or multiples of 3) indels were present in some taxa and were concentrated in one region of the sequence. The alignment of these was examined for possible alternatives. Slightly different alignments were explored for some taxa that appeared to have alternative alignments, but these did not influence the overall results of the analysis and only one alignment was utilized for the final analysis (shown in Table 3, including two taxa without gaps).

Parsimony

For parsimony analyses, matrices were created and manipulated using WinClada (Nixon, 1999–2002) and analyzed using NONA (Goloboff, 1995). Searches were conducted with the parsimony ratchet (Nixon, 1999) as implemented by WinClada using 200 iterations per replication, one replication, two trees held per iteration and about 10% of the characters sampled. The shortest trees found were then opened in NONA and the commands 'hold 20000', 'amb =', 'poly' and 'max*' were used to search for additional ambiguity islands. After this, the commands 'amb-', 'poly = ', and 'best' were used to find the unambiguously supported and fully collapsed most parsimonious trees. The com-

Table 3. Inclusive region of *wingless* containing indels for seven taxa aligned with *Hygrobia tarda* and *Dytiscus verticalis*, which lack indels, to indicate the alignment of this region used in the final analysis

	Gap1	Gap2
Hygrobia tarda	GGCAGCCAACGAGGGGACACAACGCACACAACAATATCGCGACTTCTTAATTCACAGTACT	CGAACAGCATCCACGGCACACATTCCGCATCAGAACGA
Dytiscus verticalis	GGCAGCCAACGAGAGCGGAAACAGCGCGCAGCTAATACGGCCAATTCAAACTCGCATCT	CGCATCCAGCAGCATCCACGGYGCACATTCGAATGCCGAGCGT
Notaticus fasciatus	GGCAGCCAACGCGGGGAAACAACGCACACAACGCACATGCAAATTCA---AATGCAAACTCA	CATCTTCAAACAGTATCCRCGGAGACATTCTAACTTAGAAAGA
Eretes australis	GGAAGTCAAAGA--------AATGCGCACACAAACACAGCCAACTCACACCTTGCCAACAGCG	CAACTCACACTTGCCAACAGCATCCACGAGGACACAATAACTTGGAAAGG
E. griseus	GGAAGTCAAAGA--------AATGCGCACACAAACACAGCCGTTGCCAACTCGCAC	CAACTTAGCCAACTCACGGAGACACAATAACTTGGAAAGA
Laccophilus maculosus	GGAAGTCAGCGCGGTGGAAACAACGCACAC-----AATACAAACTCCAACTCACATTT	GAGCAACAGCATACTTGGTATTCATTCGAATTCAGAAAGA
Laccornis difformis	GGCAGCCAACGTGCAGGAAACAACAACGCACATTCCAACAATGCAAATTCAAACAAT	CTCTGGAACAGCATCCATGGATCTCACACC------GAGAGA
Neoporus undulatus	GGAAGTCCAAGAGGAGGAAACAGTGCCCATTCAAATAATGCAATAATCATTAACAAACAGT	TTAACAAAATCATTAACAAACAGCATACATGGTTCACACACA------GAGCGA
Coptotomus lenticus	GGCTCCCCAACGCAACGCAATATCGCCCACGCCAAT---CCAAGTTCGAATAACCATC	CTAGCCAACAGCATACACGGATCACACACCAACTCAGAGCGA

Gaps 1 and 2 are those coded as characters 91 and 92, respectively, in Table 2.

mand 'save*' was used to save the trees and 'inters' was used to save the consensus tree. Parsimony analyses were performed on the molecular data alone, the morphology/indel data alone and all the data together. Bremer support values (Bremer, 1994) were calculated for each partition separately in NONA using the commands 'hold 10000', 'suboptimal 15' and 'bsupport 15'. Partitioned Bremer support values (Baker & DeSalle, 1997) were calculated using PAUP*4.0b2 (Swofford, 1999) using a constraint file generated by the program TreeRot (Sorenson, 1999) and 20 random additions. Bootstrap values were calculated in NONA as implemented by WinClada using 1000 replications, 10 search replicates and five starting trees per replicate without doing a max* search and saving the consensus of each replication.

Maximum likelihood

Likelihood analyses were conducted using PAUP*4.0b2 mainly to explore the possible influence of rate heterogeneity among sites, base composition biases or unequal transition/transversion rates on the results of the parsimony analysis. Initial estimates of the log likelihood were made using a parsimony tree resulting from the simultaneous analysis of all data under 20 models of nucleotide evolution (Huelsenbeck & Crandall, 1997). The models investigated include Jukes–Cantor (JC), Kimura two-parameter (K2P), Hasegawa–Kishino–Yano (HKY) and general time reversible (GTR) models. Within each model, five methods were employed to account for rate heterogeneity: none, gamma-distributed rates (Γ), the proportion of invariant sites (I), $\Gamma + I$, and site-specific rates (SSR, with each codon position being assigned a different rate).

After likelihoods and parameters were calculated based on the parsimony tree, branch swapping was performed using one model (see below). The initial tree used for swapping was one of the most parsimonious trees derived from analysis of data from *wingless* alone (arbitrarily chosen). The search occurred in the order: NNI, SPR(1), SPR(2), TBR(1), and TBR(2). Before each round the likelihood parameters were re-estimated based on the resulting trees from the previous round.

RESULTS

MULTIPLE COPIES OF *WINGLESS*

The sequenced fragment clustered with *wingless* sequences from Carabidae and Nymphalidae in a BLAST search of GenBank submissions. The nymphalid sequences have been proposed to be orthologous with the mouse Wnt-1 sequence (Brower & DeSalle, 1998). The orthologs of the other dytiscid copies were not identified.

Table 4. Base composition of *wingless* gene for species of Dytiscidae and Hygrobiidae used in cladistic analysis

	All positions	1st positions	2nd positions	3rd positions
T	22.4	19.4	20.9	27.0
C	23.7	20.0	19.6	31.4
A	28.5	29.4	32.3	23.6
G	25.5	31.2	27.3	17.9
P	0.999	<0.0001	<0.0001	<0.0001

P-values are based on chi-square test of heterogeneity.

BASE COMPOSITION

Overall base composition and composition by codon position are shown in Table 4. Overall there was no obvious A/T or G/C bias. Although there was no significant heterogeneity in base composition overall, each codon position did exhibit significant heterogeneity (Table 4), but again without a clear A/T or C/G bias at any position.

PARSIMONY ANALYSES

Of roughly 460 bp sequenced from the *wingless* gene, 197 bp were informative, with the majority of these from third positions (Fig. 21). The analysis of the *wingless* data resulted in two most parsimonious trees (length = 1731, CI = 24, RI = 56), the consensus of which is shown in Fig. 22. The parsimony analysis of the morphology/indels data partition resulted in 213 most parsimonious trees (length = 182, CI = 62, RI = 91), the consensus of which is shown in Fig. 23. The parsimony analysis of the combined data resulted in nine most parsimonious trees (length = 1958, CI = 27, RI = 63), the consensus of which is shown in Fig. 24.

The nine most parsimonious trees differed in rearrangements (and their combinations) of some of the more inclusive groups including subfamilies and clades of subfamilies, and within the Colymbetinae and Cybistrini clades. One of these trees (Fig. 25) was selected for additional analyses based on its placement of the Agabinae clade as sister to the clade including Lancetinae, Colymbetinae and Dytiscinae and *Megadytes* as sister to *Cybister*. This configuration is consistent with a previous analysis (Miller, 2001) which included a larger sample of taxa in these groups. Also, when only the morphology was mapped onto this tree, it was one of the three equally shortest of the nine trees. This tree is referred to hereafter as the 'preferred' most parsimonious tree. The preferred tree was the only one of the nine to exhibit this particular topology, and none of the other trees were either strongly consistent or inconsistent with previous analyses or gave any other particularly compelling reason for selection of them over any other. In any case, selection of a different most parsimonious tree would not influence conclusions about the evolutionary scenario discussed in this paper, and relationships among the clade of particular interest, the Dytiscinae, were identical in all most parsimonious trees except for some conflict in the clade including *Megadytes* and *Cybister*.

When mapped onto the preferred most parsimonious tree, second positions exhibited a smaller average number of changes overall (3.5) than did first or third positions, which was reflected in the higher consistency and retention indices, and third positions exhibited the largest average number of changes per character (9.9) of the three positions (Fig. 21).

MAXIMUM LIKELIHOOD

Log likelihood values and parameter estimates from the preferred most parsimonious tree are presented in Table 5. Log likelihood values improved in most

Figure 21. Steps on most parsimonious trees according to codon position. Numbers on top of each bar are number of informative sites (of 197 total), CI/RI for each position, and average number of steps per character for each position as mapped on preferred most parsimonious tree from simultaneous analysis (total steps from nucleotide data = 1704).
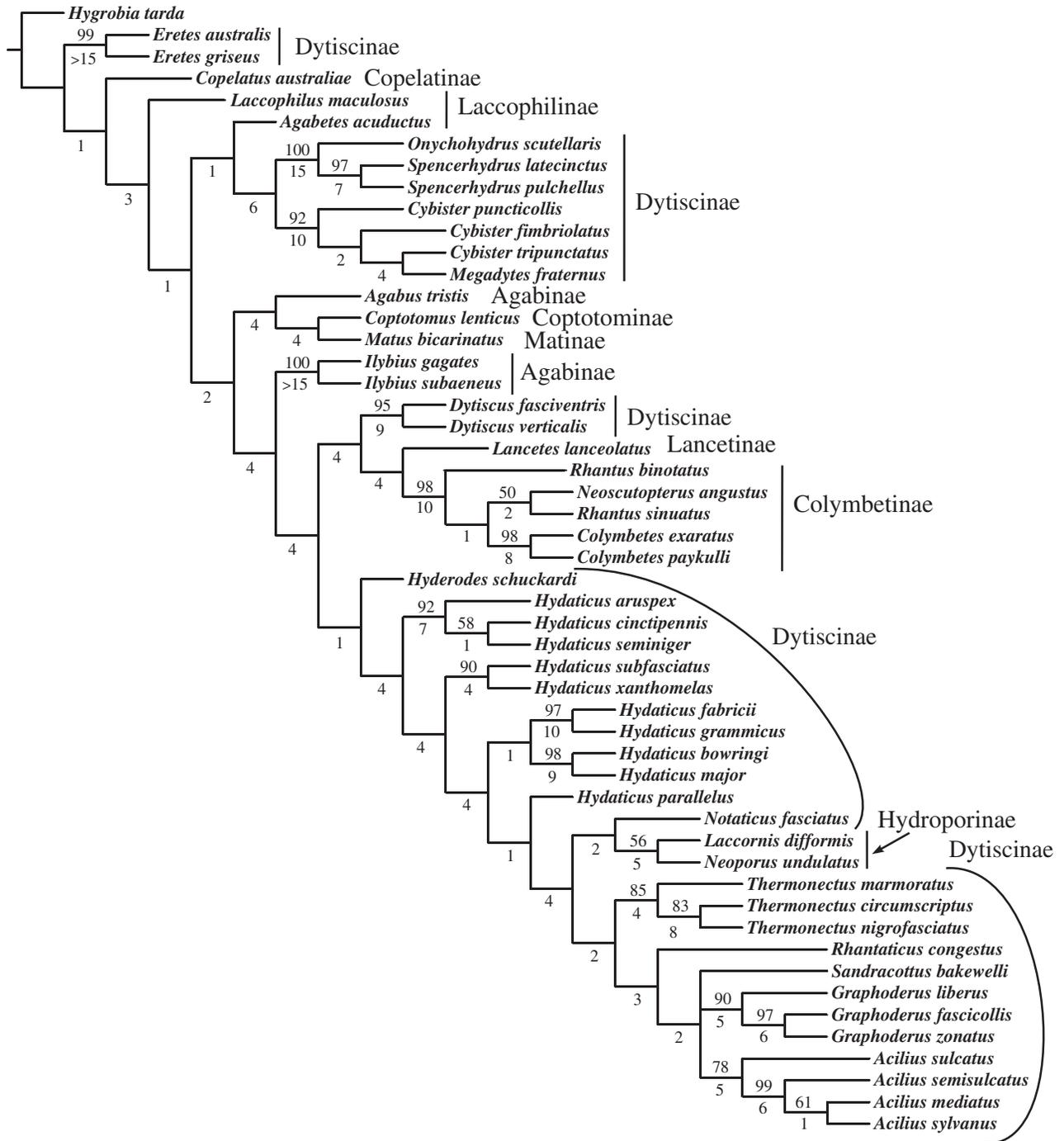
Figure 22. Consensus cladogram of two most parsimonious cladograms (length = 1731, CI = 24, RI = 56) derived from analysis of *wingless* sequence data. Numbers above branches are bootstrap values (only those greater than 50% indicated); numbers below branches are Bremer support values.

cases as additional parameters were added to the models (Table 5; Fig. 26). However, including empirical base frequencies (HKY) rather than equal base frequencies (K2P) resulted in a lower log likelihood score (Table 6; Fig. 26). As judged by likelihood ratio tests (Table 6), the GTR + I + Γ model had a significantly better log likelihood score than did the less complex models (that do not incorporate SSR) and
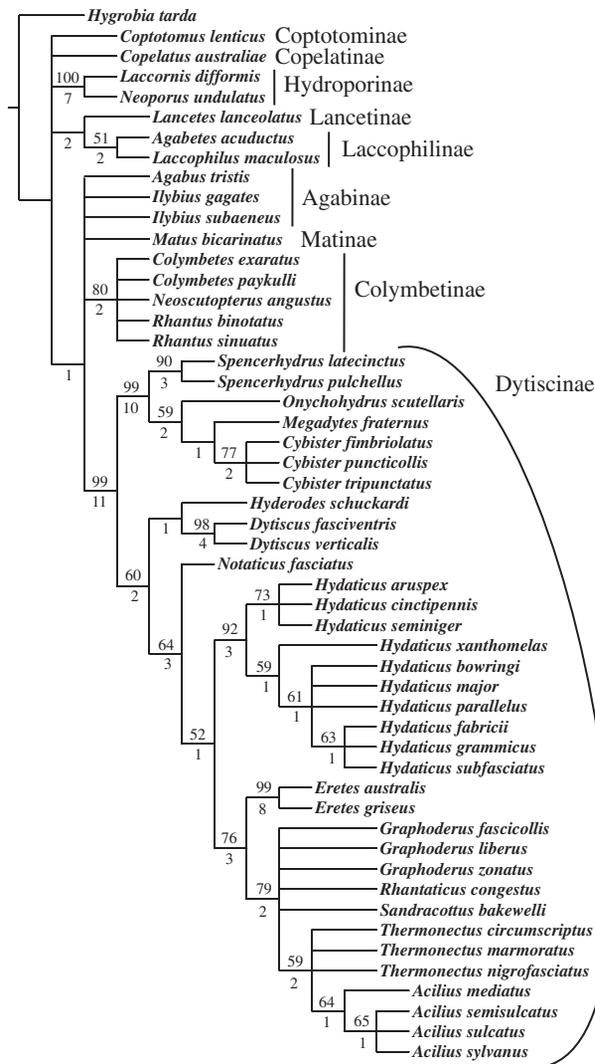
Figure 23. Consensus cladogram of 213 most parsimonious cladograms (length = 182, CI = 62, RI = 91) derived from analysis of the data partition containing morphological and *wingless* indel characters. Numbers above branches are bootstrap values (only those greater than 50% indicated); numbers below branches are Bremer support values.

in all cases a parameter accounting for rate heterogeneity significantly improved the likelihood score, suggesting that rate heterogeneity among sites may be a general feature of the data set that could influence the topology resulting from a parsimony analysis. The GTR + SSR model was used for the likelihood analysis since the model was similar to GTR + I + Γ in log likelihood score (Table 5, Fig. 26), is considerably faster to run, and is an appropriate model given the apparent heterogeneity among sites.

Branch swapping resulted in an improvement in likelihood score over that derived from the preferred parsimonious tree using the model GTR + SSR (from 7634.066 to 7564.015). The resulting topology was similar in many respects to the most parsimonious trees from the sequence data alone (Figs 22, 27). It differed from the preferred tree in several significant aspects including among other things nesting of *Agabetes* within the Cybistrini, nesting of *Coptotomus* + Hydroporinae within a large group otherwise containing only members of the Dytiscinae, and non-monophyly of the Dytiscinae (Fig. 27). Each of these results is substantially inconsistent with well-supported groupings in the simultaneous parsimony analysis (Fig. 24). The group containing *Coptotomus* + Hydroporinae was characterized by particularly long branches (Fig. 27), which could explain its anomalous placement in different methods of analysis. The likelihood analysis did place *Eretes* with *Notaticus* + Aciliini (Fig. 27), which is consistent with the strong association of these taxa in the simultaneous parsimony analysis (Fig. 24) and inconsistent with the unusual placement of *Eretes* as sister to the remaining Dytiscidae as indicated by the parsimony analysis of the *wingless* data alone (Fig. 22). Again, the branch subtending *Eretes* was comparatively long (Fig. 27), which may explain its disparate placements depending on the method of analysis. The topology resulting from the likelihood analysis of *wingless* differed from the most parsimonious trees, suggesting that application of the model was 'correcting' for certain attributes of the data set. However, its striking dissimilarity to the simultaneous analysis in several well-supported conclusions suggests that the likelihood analysis, by itself (like the parsimony analysis of the *wingless* data alone) is inadequate to persuasively infer the phylogeny of this group.

DISCUSSION

PHYLOGENY AND CLASSIFICATION OF THE DYTISCIDAE

The phylogeny of the Dytiscinae based on simultaneous parsimony analysis is congruent with previous studies (Miller, 2000; Miller, 2001) with a few exceptions. Most notably, the genus *Hydaticus* is resolved as paraphyletic in the most parsimonious trees (Fig. 24). This conclusion is unconvincing for several reasons. To begin with, the morphological characters supporting monophyly of this genus are strong, including the straight anterolateral margins of the metasternum, the type of dorsal cuticular modification present in females and the presence of a unique stridulatory device in males (consisting of a region of reticulate cells on the dorsal surface of protarsomere 2 and a series of pegs along the dorsal margin of the protibia). It is notable that the species
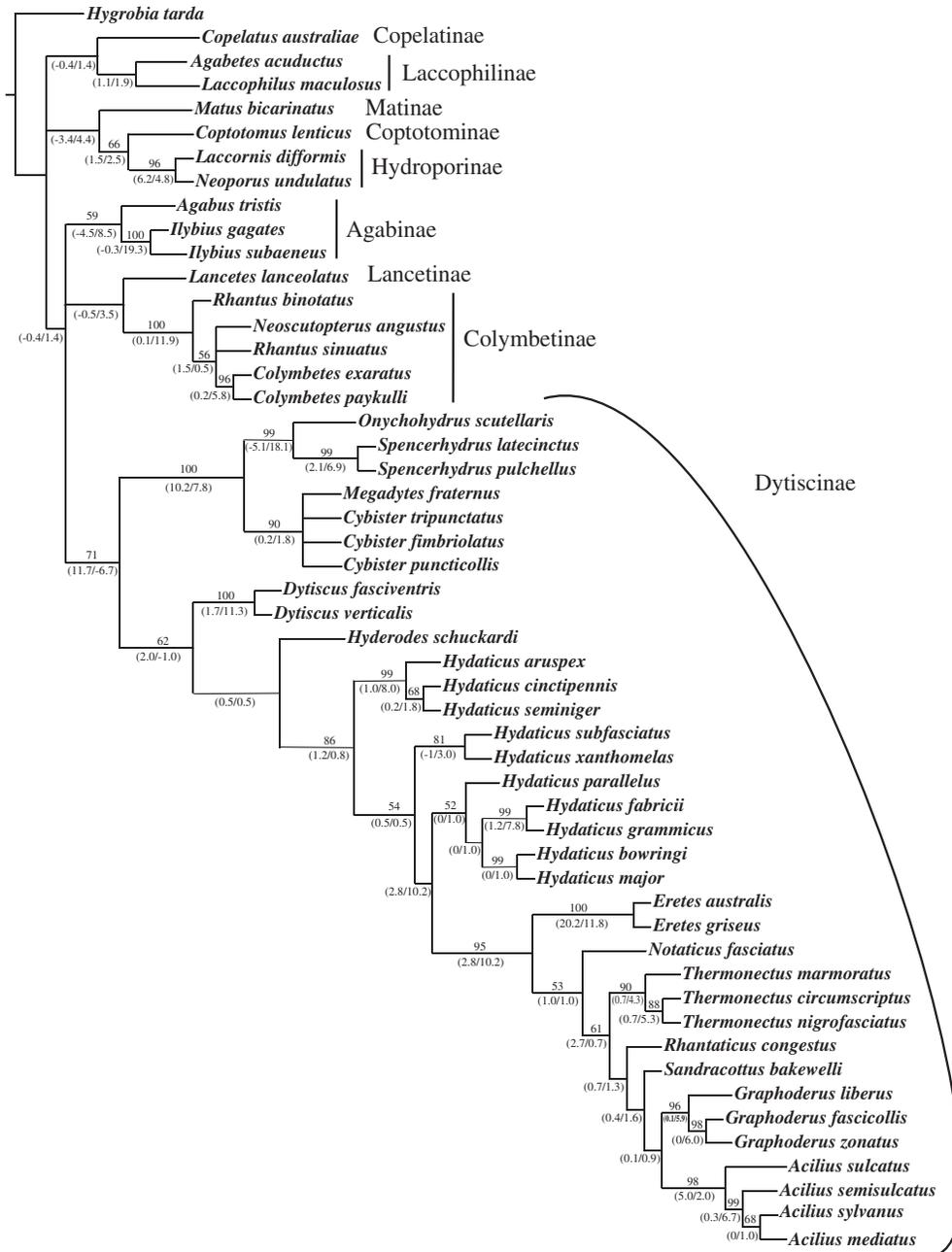
Figure 24. Consensus cladogram of nine most parsimonious cladograms (length = 1958, CI = 27, RI = 63) derived from simultaneous analysis of all data. Numbers above branches are bootstrap values (only those greater than 50% indicated); numbers below branches are partitioned Bremer support values (morphology and indels/*wingless*).

H. jucundus Reiche lacks the stridulatory device (Larson, 1996), but in other characters it strongly resembles members of *Hydaticus* (*Guignotites*) (K.B. Miller, unpubl. observ.). I suspect that its stridulatory device was secondarily lost. *Hydaticus* is monophyletic in the consensus of the morphology/indel trees (Fig. 23). In addition, the support values of the branches in the most parsimonious tree subtending these groups are lower than those of other groups similarly supported by morphology. Finally, when the monophyly of the genus is constrained in an analysis of all data, the resulting topology is only a single step longer than it is without the constraint while the remaining topology is relatively little changed.
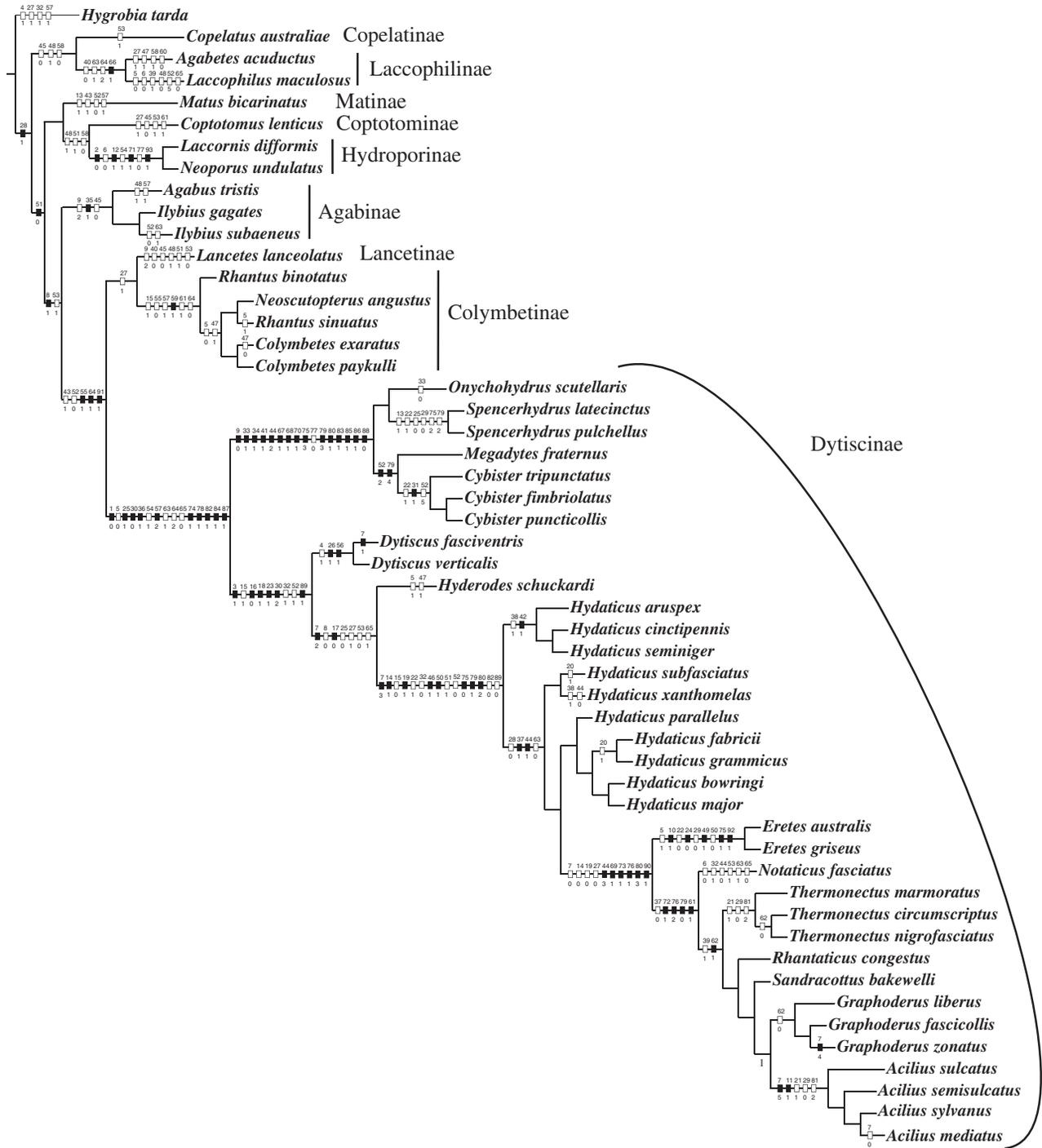
Figure 25. Preferred most parsimonious cladogram derived from simultaneous analysis of all data. Morphological and *wingless* indel characters mapped using 'fast' optimization (as implemented in WinClada). Numbers above hashmarks are character numbers, numbers below hashmarks are character state numbers. Solid hashmarks indicate unique character state transformations, open hashmarks indicate homoplasious character state transformations and reversals.

Therefore, I regard the conclusion of paraphyly of *Hydaticus* with skepticism. Monophyly of *Hydaticus s.s.* is well supported, as is a relationship between *H.* (*Hydaticinus*) *xanthomelas* (Brullé) (the only member of the subgenus) with members of *Hydaticus* (*Guignotites*) (Fig. 24).

Other interesting results from this analysis include monophyly of several genera within Aciliini (Fig. 24).

Table 5. Likelihood scores and parameter estimates for models of evolution examined in likelihood analysis of *wingless* data and the preferred most parsimonious tree derived from the simultaneous analysis of all data

Model	−ln likelihood	Estimated ti/tv ratio	Proportion invariant sites (I)	Gamma shape parameter (α)	R-matrix						Estimated relative rates		
					A-C	A-G	A-T	C-G	C-T	G-T	1st	2nd	3rd
JC	9249.096												
JC + Γ	8017.486			0.220									
JC + I	8245.672		0.491										
JC + I + Γ	7998.842		0.466	1.162									
JC + SSR	8055.288										0.243	0.116	2.631
K2P	8915.837	1.872											
K2P + Γ	7630.643	2.457		0.226									
K2P + I	7882.422	2.138	0.491										
K2P + I + Γ	7593.254	2.701	0.463	0.901									
K2P + SSR	7682.642	2.266									0.227	0.108	2.655
HKY	8939.590	1.869											
KKY + Γ	7636.235	2.622		0.227									
HKY + I	7893.767	2.221	0.491										
HKY + I + Γ	7598.181	2.894	0.462	0.878									
HKY + SSR	7695.289	2.363									0.223	0.106	2.660
GTR	8796.519				1.393	2.363	1.271	0.549	6.242	1.000			
GTR + Γ	7601.200			0.226	1.121	3.416	1.089	0.408	5.778	1.000			
GTR + I	7831.299		0.491		1.112	2.834	1.110	0.409	5.264	1.000			
GTR + I + Γ	7561.635		0.458	0.928	1.270	3.533	1.018	0.428	6.923	1.000			
GTR + SSR	7634.066				1.222	3.192	1.195	0.416	5.811	1.000	0.238	0.112	2.639

Models are Jukes–Cantor (JC), Kimura 2-parameter (K2P), Hasegawa–Kishino–Yano (HKY), and general time reversible (GTR) models incorporating among-site rate heterogeneity (Γ), proportion of invariant sites (I) and site-specific rate variation by 1st, 2nd and 3rd positions (SSR).

Figure 26. –Ln likelihood scores derived from the preferred most parsimonious tree from simultaneous analysis of all data for 20 models of nucleotide evolution. Models are Jukes–Cantor (JC), Kimura 2-parameter (K2P), Hasegawa–Kishino–Yano (HKY), and general time reversible (GTR) models incorporating among-site rate heterogeneity (G) (Γ in the main text), proportion of invariant sites (I) and site-specific rate variation by 1st, 2nd and 3rd positions (SSR).

Table 6. Comparisons of various likelihood models in log likelihood ratio tests

	Model	d.f.	$-2 \log \Lambda$	P
JC	K2P	1	666.518	<0.0001
K2P	HKY	3	–47.505	NS
K2P	K2P + Γ	4	2570.388	<0.0001
HKY	GTR	4	286.142	<0.0001
GTR	GTR + Γ	4	2390.638	<0.0001
GTR	GTR + I	1	1930.439	<0.0001
GTR + I	GTR + I + Γ	4	539.329	<0.0001

Models are Jukes–Cantor (JC), Kimura 2-parameter (K2P), Hasegawa–Kishino–Yano (HKY), and general time reversible (GTR) models incorporating among-site rate heterogeneity (Γ), proportion of invariant sites (I).

Graphoderus (including the unusual *G. liberus*) is strongly supported by molecular characters, but not by morphology (Fig. 23). A difference in the topology from previous analyses is the placement of *Notaticus* Zimmermann as sister to Aciliini (Fig. 24), rather than as sister to Hydaticini + (Eretini + Aciliini) (Miller, 2000). The larva of *Notaticus* is currently unknown, and characters from this semaphoront could shed light on the relationships of this taxon and improve the hypothesis of its relationships within the subfamily.

Outside the subfamily, most relationships remain somewhat unconvincing as indicated by the low Bremer and bootstrap values and lack of resolution (Fig. 24). However, all subfamilies hypothesized as monophyletic in a previous analysis (Miller, 2001) are

monophyletic here also, some with high branch support. Other important conclusions include extraction of Matinae and Agabinae from Colymbetinae (corroborating Miller, 2001), Lancetinae resolved as sister to Colymbetinae instead of as sister to Dytiscinae (Miller, 2001), monophyly of *Ilybius* (*sensu* Nilsson, 2000) and relative placement of Agabinae with Lancetinae, Colymbetinae and Dytiscinae (Fig. 24).

THE *WINGLESS* GENE IN DYTISCIDAE

Brower & DeSalle (1998) suggest that this short fragment of *wingless* alone can fully resolve relationships in the Heliconiinae, but that the analysis would be improved by combining additional molecular or morphological data, a conclusion reinforced by Baker *et al*. (2001). Here, data from the gene *wingless* appear to be useful for improved hypotheses of relationships among the Dytiscidae when combined with morphological data. This *wingless* data set is relatively small (about 460 bp and 197 informative characters), and by itself leads to some unusual groupings such as the nesting of the Hydroporinae within the Dytiscinae, the sister group relationship between the Eretini and the remaining Dytiscidae and the non-monophyly of the Dytiscinae (Fig. 22). However, several groups, such as *Ilybius*, *Colymbetes* and *Graphoderus*, are not strongly supported by the morphological characters used in this analysis, yet are well supported by the molecular data (Figs 22, 24). In addition to providing phylogenetic resolution where little or none exists in the morphological data set, the molecular data increases the support for various groups that exhibit morphological synapomorphies, such as the clade,
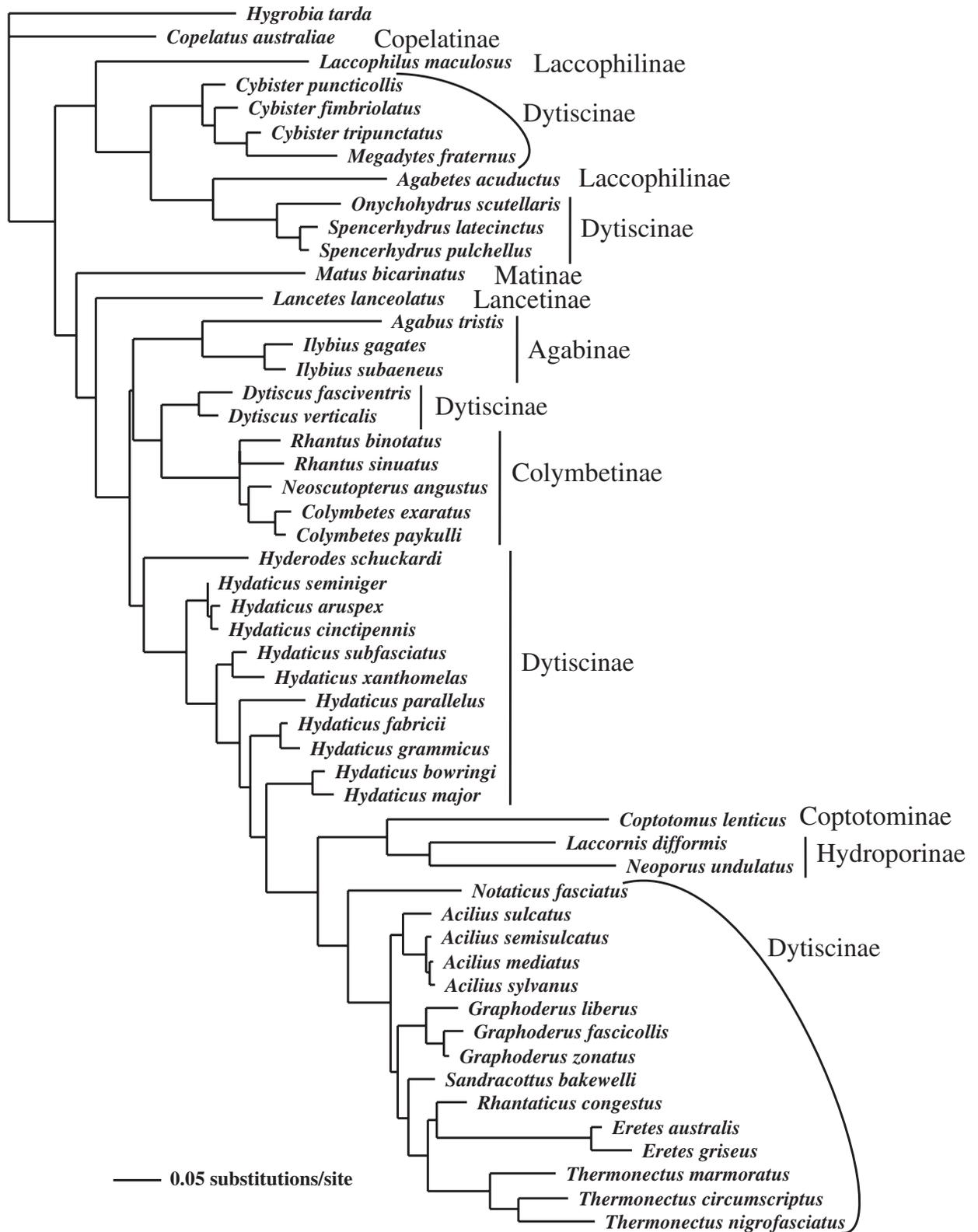
Figure 27. Topology resulting from maximum likelihood analysis of *wingless* sequence data based on the GTR + SSR (general time reversible + site-specific rate) model (–ln likelihood = 7564.01532). Lengths of branches are proportional to character changes.

Hydaticini + (Eretini + (Aubehydrini + Aciliini) (Fig. 24). *Wingless* appears to provide the greatest support for 'apical' or less inclusive clades (Fig. 22) suggesting that within the Dytiscidae the gene may be most useful for grouping taxa at the tribal level and below. Unfortunately, for the most inclusive groupings, such as subfamilies, neither this portion of *wingless* nor morphology convincingly resolves relationships, suggesting that additional data from an expanded sample of *wingless*, other genes, or systems of morphology are necessary, or that some relationships among dytiscid taxa may be inherently difficult to resolve.

<center>EVOLUTION OF SECONDARY SEXUAL CHARACTERS IN THE DYTISCINAE</center>

The phylogeny of dytiscines presented here (Figs 25, 28, one of nine equally parsimonious solutions, see above) indicates that the sexually dimorphic characters evolved as predicted by the arms-race model of sexual selection. These characters are expected to have evolved in the following order: first, sucker-shaped male adhesive setae, and second, irregularly modified dorsal cuticular structure in females (Fig. 20).

The enlarged, circular sucker-setae on the male pro- and mesotarsi evolved a single time in the common ancestor of the Dytiscinae excluding the Cybistrini (Fig. 28) (Miller, 2000; Miller, 2001). Nested within the clade of taxa with male sucker-shaped setae are five groups in which females have a dramatically modified dorsal cuticle (Fig. 28). This order of appearance is consistent with the model of sexual conflict (Fig. 20). The dorsal cuticular structures of the females in several taxa appear to be similar, but non-homologous, responses to the similar pressures upon females by increased male control over mating decisions. That there appear to be several independent origins of similar features in females in this clade lends relatively greater credence to this conclusion than would only a single instance. The results suggest that the modification in *Hydaticus* (Figs 15, 16) arose once and was then lost (Fig. 28) if this group is indeed paraphyletic (but see above for reasons to doubt this conclusion). A modified female cuticle is also optimized as lost in *Acilius mediatus* (Fig. 28).

Behavioural characters (e.g. prolonged mating and female behavioural resistance) were not included in the study because of lack of good empirical information about mating in dytiscids. However, there have been at least two published accounts of dytiscid mating behaviour and additional anecdotal observations were made during this study. Females of both *Rhantus binotatus* (Smith, 1973) and *Dytiscus alaskanus* (Aiken, 1992) exhibit intense struggling upon being seized by a male. In these two species mating duration is quite long (in the order of several hours in *D. alaskanus* (Aiken, 1992)). Anecdotal observations of species of *Acilius* (*A. sylvanus*, *A. semisulcatus* and *A. mediatus*) and *Graphoderus liberus* indicate that these species also exhibit long mating duration and apparent behavioural resistance to mating since females swim extremely erratically upon being adhered to by a male with his prolegs (K. B. Miller, unpubl. observ.). In contrast, mating duration in *Ilybius gagates* and *Neoporus undulatus* is short and females do not appear to resist male mating attempts at all (K. B. Miller, unpubl. observ.). If these character states are optimized on the preferred most parsimonious tree (Fig. 25) based on these few observed genera, prolonged mating and female resistance optimize as preceding male sucker-shaped adhesive setae just as predicted by the model of sexual conflict evolution. Future more carefully documented observations of mating in a broader sampling of taxa will allow this aspect of antagonistic evolution in dytiscids to be more critically tested.

CONCLUSIONS AND FUTURE STUDIES

The evidence from the phylogeny is consistent with the hypothesis that intersexual conflict over mating decisions occurs within the Dytiscinae (Bergsten *et al.*, 2001). Although relatively little is known of the mating behaviours of these beetles, their phylogeny provides an important context within which to explore their evolution and associated morphologies in the future. Female counter-adaptations to male grasping devices are very rarely described (Arnqvist & Rowe, 1995; Andersen, 1997), but in dytiscines there appears to be a particularly good example of just such a suite of female features which evolved repeatedly several times in the same clade apparently in response to a unique male grasping device.

Although an arms-race scenario is one explanation for the evolution of this particular pattern of characters, other factors may also be involved in the evolution of dytiscine mating systems. In particular, it is not clear to what extent sexual conflict and female choice are interacting among these taxa to produce the pattern of behaviours and morphology. As Eberhard (2001) has pointed out, it may be difficult to discriminate between these two theories of mating evolution. The general absence of clear female morphological responses to male grasping devices in most arthropods studied (Eberhard, 1985) suggests that sexual conflict scenarios may not be as widespread as it seems. Morphology that appears to benefit a male by allowing him to coerce a resistant female may instead be a means of stimulating her to mate since in many species a female will not allow access to her genitalia
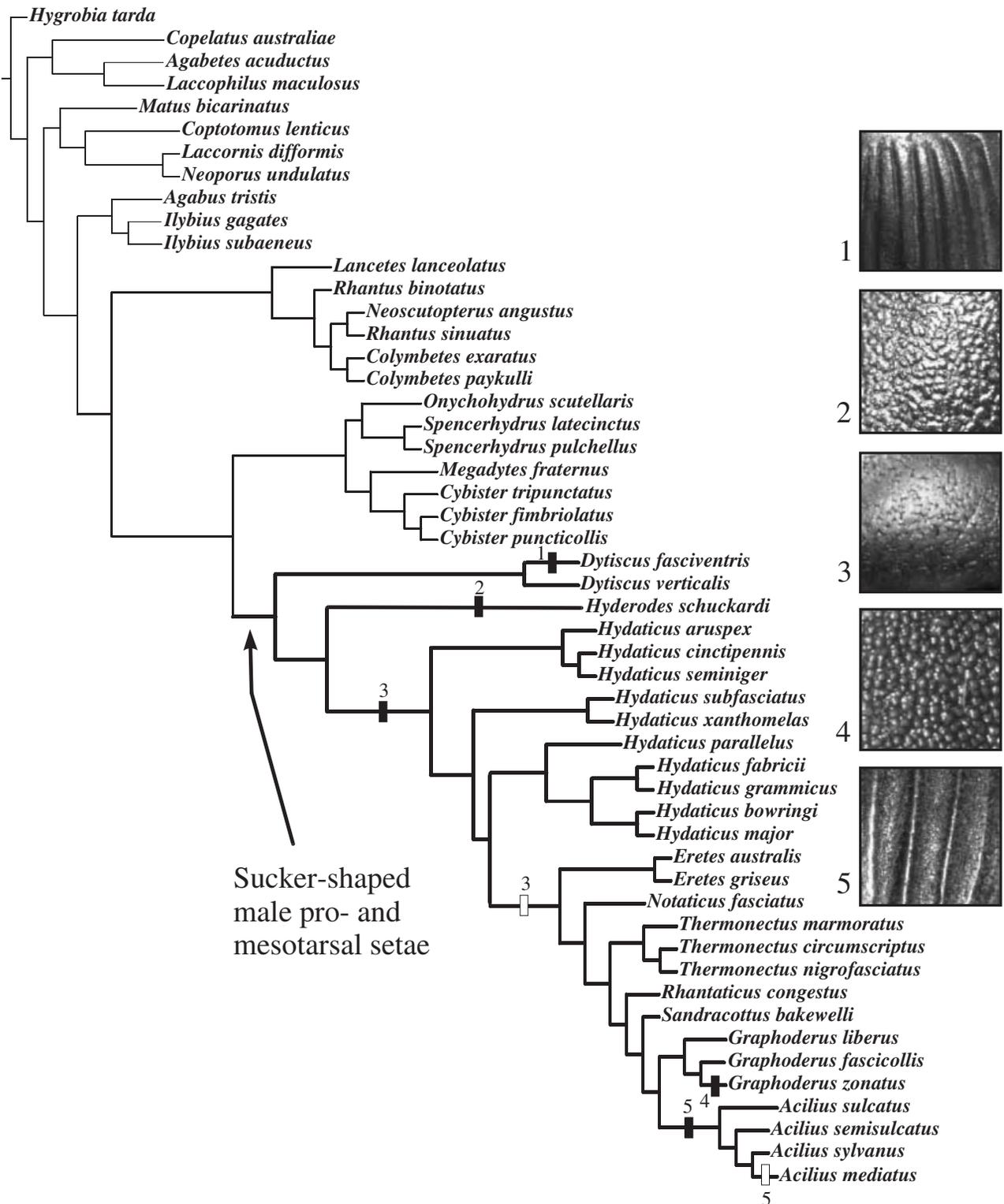
Figure 28. Phylogeny of the Dytiscinae and other dytiscid taxa with characters of interest for the sexual selection scenario mapped. Hashmarks and numbers indicate female modifications; filled hashmarks indicate transformation to the modified state, unfilled hashmarks indicate reversals.

until a male exhibits a particular behavioural or morphological signal. Many grasping devices may have more to do with this than with forcefully overcoming female resistance (Eberhard, 1985). For example, in sepsid flies, male grasping devices which seem to function as mechanisms for coercing a resistant female were found instead to stimulate her to mate (Eberhard, 2001). The taxa most clearly influenced by sexual conflict should possess a male grasping device combined with evidence of female morphological responses to the structure. If a female morphological response is absent the conclusion that an arms-race scenario is operating in a taxon may be unwarranted, or, at the least, selection pressure on the female to avoid mating may not be especially strong (but see Alexander *et al*., 1997 for an opposing perspective).

Dytiscine sucker-setae are followed within the clade by no less than five independent derivations of female morphology that appear to be responses to the male grasping device. In this respect, dytiscines may represent one of the best examples of a taxon influenced by sexual conflict presented to date. Nevertheless, even in dytiscines, sexual conflict may explain only a portion of the mating system. Males of at least some species exhibit mating behaviours such as shaking or other movements (Aiken, 1992; K.B. Miller, unpubl. observ.) suggesting that they must undergo some signalling or stimulating in order to mate. Therefore, it is probable that other types of female choice are operating in combination with sexual conflict in the evolution of dytiscine mating.

Alexander *et al*. (1997) argued that in sexual arms races an 'inevitable development' is the acceleration in the evolution of complexity in structure and function of characters related to mating. This is certainly the case in other groups putatively influenced by an arms race such as sepsid flies (e.g. Duda, 1925; Duda, 1926; Silva, 1993) and water striders (e.g. Matsuda, 1960; Andersen, 1982) which have very complex, species-specific male grasping devices and/or genitalic structures. However, dytiscines do not appear to exhibit the complex variation in protarsal structure and function that is expected from the model. In dytiscines, male protarsal setae do exhibit some phylogenetic variability (Chatanay, 1911). For example, in *Dytiscus* and *Acilius*, male protarsal setae occur in two distinct sizes, two or three very large setae and a lot of smaller ones. Within the species *Graphoderus zonatus*, disparity in setal size is correlated with the proportion of modified females in a population (the greater the proportion of modified females the greater the disparity in size between the larger and smaller setae, and the more numerous the smaller setae) (Bergsten *et al*., 2001). Thus, it is possible that setal size variation represents an additional innovation by males to gain further control over mating in some way, perhaps by

allowing males to adhere to smaller smooth surfaces among the roughened surfaces on female pronota or elytra. However, great setal size disparity occurs not only in species of *Dytiscus* and *Acilius* with modified females, but also in unmodified species and in the genus *Eretes*, the females of which are unmodified. Even given these examples of variation, dytiscine male protarsal features generally vary only slightly between species, and even at higher taxonomic levels they are often exceptionally uniform in their structure (Chatanay, 1911). Thus, at least in this group and for these characters, the rapid divergence prediction of the model of sexual conflict is not met or is much less dramatic than might be expected (Alexander *et al*., 1997).

Knowledge of mating evolution in the Dytiscidae is just beginning. There are several important lines of evidence that are lacking which could more strongly establish whether sexual conflict is operating. Experimental evidence of the effect of female cuticular modifications on male mating success is clearly needed. Presumably, under a sexual conflict model, male mating attempts would be successful less frequently with modified females. Also useful would be direct evidence that multiple or prolonged mating has a negative effect on overall female fitness. Fitness and associated costs of mating, though important in understanding sexual selection models, are difficult to measure and few studies have examined them empirically (Watson *et al*., 1998). Some potential costs are evident in dytiscids. Mating pairs often rest at the surface and appear to swim less effectively which may make them easier targets for predators as has been shown for some gerrids (Fairbairn, 1993; Rowe, 1994). Also, since these beetles breathe atmospheric oxygen they must extend the apex of the abdomen through the surface of the water to replenish the oxygen in an air bubble carried under the elytra. Mating occurs under water with the male dorsal to the female, and mating pairs often rest at the surface of the water with the male accessing the air and the female below him. In *Dytiscus alaskanus* (Aiken, 1992) and several other species (K.B. Miller, unpubl. observ.) females are commonly denied access to atmospheric oxygen for very long periods of time during mating or postcopulatory guarding. However, in at least some species the male periodically inclines the apex of the female abdomen to the surface so she can replenish her subelytral oxygen.

Dytiscids exhibit several features related to this phenomenon that provide additional fodder for research. For example, although all females of some species are modified, such as many *Acilius* and some *Dytiscus*, in most species females are dimorphic with some modified and others entirely smooth. In *Hydaticus*, the female modifications are often continuously variable from strongly modified to nearly smooth in

the same species or population. The evolutionary maintenance of these intraspecific variations is a potentially interesting avenue of study. In some insects, the relative cost of mating and female mating behaviours changes depending on the availability of mates (Moore, 1989; Rowe *et al.*, 1994). It is possible that the proportion of modified females in a population is related to the density of individuals or the sex ratio (Bergsten *et al.*, 2001). If potential mates are rare, unmodified females may be favoured since stress from repeated, long matings is more limited. If numbers of potential mates increase, constant harassment by males may tend to reduce the numbers of smooth females since repeated matings could reduce their overall fitness, and numbers of modified females may increase.

In addition to continuously variable female modifications, the genus *Hydaticus* differs from other genera with modified females in several ways. Females have only the pronotum or the pronotum and the humeral region of the elytra modified instead of the elytron or the entire dorsal surface as in other taxa. Presumably, in most dytiscines the female modifications interfere with male mating attempts during an initial struggle as the male attempts to grasp a female's elytral or pronotal surface with his sucker-setae. In *Hydaticus*, the modified pronotum may instead interfere with the adhesive setae during or at the end of the mating event. During mating, many dytiscid species grasp the female by the edge of the pronotum with the protarsal claws and the adhesive setae attached to the lateral surface of the pronotum (Régimbart, 1877; K.B. Miller, unpubl. observ.). If this is the case in *Hydaticus* (mating has not been documented in this taxon), the protarsi would be orientated directly over the sides of the pronotum where the greatest modification is generally exhibited. Thus, the modifications may aid a female in determining the endpoint of mating by making it easier to dislodge the male. In addition, males of this group possess an apparent stridulatory device on the front legs (Larson & Pritchard, 1974). That the device is present only in males and is apparently not used defensively (such as when handled) suggests that it is used in courtship. Presumably, intersexual conflicts should be reduced when a female uses courtship information to assess males (Alexander *et al.*, 1997). However, Alexander *et al.* (1997) have argued that even in situations where males lure females, costs resulting from male adaptations that increase their control over fertilization could favour females who can regain this control, a possible scenario for *Hydaticus*.

Based on the historical pattern of characters related to mating in the Dytiscinae and closely related taxa (Fig. 28), it appears that these beetles may be a particularly good example of a group influenced by an intersexual 'arms race'. The situation in these beetles is unique in that one prediction of the model that appears to be borne out, evolution of female morphological counter-adaptations to a male grasping device, is rarely evident in other taxa. However, another prediction of the model, the extreme diversification of features related to mating (Alexander *et al.*, 1997), is not. The lack of extreme variability in male grasping devices suggests that this component of the model may require reassessment, at least as applied to dytiscines.

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APPENDIX

Morphological characters

Adult morphological characters included here are derived mainly from previous analyses (Miller, 2000; Miller, 2001) though some are new or homology assessments are revised. Larval characters included here have been used extensively in the historical classification of the Dytiscidae, but have never before been included in a cladistic analysis.

Adult characters

1. Anterior margin of eyes: (0) not emarginate; (1) emarginate.
2. Medial mandibular setae: (0) absent; (1) present.
3. Medial mandibular setae: (0) with mesal line of setae not extending along apicoventral surface and with an isolated patch of setae medially on ventral surface; (1) with continuous line of setae from mesal margin in curved line along apicoventral surface.
4. Fronto-clypeal suture: (0) obliterated medially; (1) complete.
5. Lateral pronotal margin: (0) not beaded; (1) beaded.
6. Scutellum: (0) concealed with elytra closed; (1) visible with elytra closed.
7. Female dorsal cuticular modification: (0) none of the following; (1) with closely set, deep sulci on elytron; (2) pronotum and elytron covered with granulations; (3) pronotum and base of elytron covered with irregular cavities; (4) elytron strongly granulate, pronotum strongly rugulose; (5) elytron with broad, shallow grooves bearing short, spinous setae.
8. Ventral setal patch at apex of elytron: (0) absent; (1) present.
9. Ventral setal patch at apex of elytron: (0) small, composed of coarse setae; (1) extensive, composed of fine setae; (2), a linear apical series of setae parallel to the margin.
10. Series of short spines along apicolateral margin of elytron: (0) absent; (1) present.
11. Characteristic punctures on dorsal surface of head, pronotum and elytron in both sexes: (0) absent; (1) present.
12. Prosternal process: (0) not declivitous; (1) declivitous.
13. Longitudinal groove along prosternal process: (0) absent; (1) present.
14. Anterolateral margin of metasternal wing: (0) curved; (1) straight.
15. Transverse carinae on dorsal surface of abdominal sternum 2: (0) absent; (1) present.
16. Male anteroapical protibial spur: (0) absent; (1) present.
17. Male posteroapical protibial spur: (0) absent; (1) present.
18. Apex of adhesive setae on ventral surfaces of male protarsomeres 1–3: (0) oval or elongate, apex bilaterally symmetrical (Figs 1, 3), (1) round, sucker-shaped disc, radially symmetrical (Figs 2, 4). The nature of male pro- and mesotarsal setae are coded as two separate characters (see characters 29–30) since the form of the setae is not perfectly correlated. Some taxa lack adhesive setae on the mesotarsomeres and in others these setae are simple rather than apically expanded.
19. Protarsal stridulatory device formed by reticulate ridges on dorsal surface of protarsus and pegs on dorsal margin of protibia of male: (0) absent; (1) present.
20. Accessory, medial spinous setae on dorsal surface of protarsomere one of male: (0) absent; (1) present.
21. Spinous setae along ventral margin of mesofemur: (0) shorter than or subequal to width of mesofemur; (1) very long, much longer than width of mesofemur.
22. Posteroapical marginal setae on mesotibia: (0) absent medially; (1) present in continuous marginal series.
23. Apices of posteroapical series of setae on mesotibia: (0) simple; (1) bifid.
24. Posterodorsal series of setae on mesotibia: (0) absent; (1) present.
25. Apices of posterodorsal series of setae on mesotibia: (0) simple; (1) bifid.
26. Apices of posteroventral series of setae on mesotibia: (0) simple; (1) bifid.
27. Basal brush of setae ventrally on male mesotarsomere 1: (0) absent; (1) present.
28. Basal brush of setae ventrally on male mesotarsomere 1: (0) small, linear series; (1) larger field of setae.
29. Ventral adhesive setae on male mesotarsomeres: (0) absent; (1) present.
30. Apices of ventral adhesive setae on male mesotarsomeres: (1) oval or elongate, apex bilaterally symmetrical; (0) simple; (2) round, sucker-shaped disc, radially symmetrical.
31. Posterodorsal setae near apical margin of mesotarsomeres of males and pro- and mesotarsomeres of females: (0) absent; (1) present.
32. Metacoxal processes: (0) not concave laterally; (1) concave laterally.
33. Oblique groove across posterior surface of metatrochanter: (0) absent; (1) present.
34. Natatory setae on dorsal margin of metafemur: (0) absent; (1) present.
35. Femoral linear setal patch: (0) absent; (1) present.
36. Posterior surface of metafemur: (0) without punctures or setae; (1) with punctures, generally bearing setae.
37. Posterior surface of metafemur: (0) with irregular, curved line of punctures; (1) with distinct, dense line of setose punctures.
38. Anterior surface of metafemur: (0) without characteristic punctures; (1) with many, very fine punctures.
39. Metatibial spurs: (0) simple; (1) bifid.
40. Natatory setae on ventral margin of metatibia: (0) absent; (1) present.
41. Anterior metatibial spur: (0) similar in size and

shape to posterior spur; (1) aciculate, broader than posterior spur.

42. Anterior surface of metatibia: (0) with only large, setae-bearing punctures or none; (1) with characteristic, fine punctures between larger, setae-bearing punctures.

43. Posterodorsal series of setae on metatibia: (0) apically simple; (1) apically bifid.

44. Posterodorsal series of setae on metatibia: (0) in linear series; (1) linear series distinctly curved ventrally at proximal end; (2) with a dense posterodorsal apical 'brush' of setae and other scattered setae on posterior surface; (3) with strongly oblique linear series and bases of setae contiguous.

45. Posteroapical setae on metatibia: (0) absent; (1) present.

46. Posteroapical setae on metatibia: (0) simple; (1) apically bifid.

47. Posteroventral series of setae on metatarsomere 1: (0) absent; (1) present.

48. Posterodorsal series of setae on metatarsomere 1: (0) absent; (1) present.

49. Short, aciculate setae on surfaces of metatarsomeres: (0) absent; (1) present.

50. Fine, adpressed series of setae present along antero- and posteroapical margins of basal four meso- and metatarsomeres: (0) absent; (1) present.

51. Natatory setae on posteroventral margins of metatibia and metatarsomeres of female: (0) absent; (1) present.

52. Metatarsal claws: (0) different lengths, anterior claw shorter than posterior claw in both sexes; (1) same length in both sexes; (2) male claws equal in length, female posterior claw shorter than anterior; (3) both sexes with posterior claw shorter than anterior; (4) male with a single claw, female with posterior shorter than anterior; (5) both male and female with a single claw.

53. Long, dense setae along dorsal margin of lateral lobe: (0) absent; (1) present.

54. Male median lobe of aedeagus: (0) asymmetrical; (1) symmetrical.

55. Ventral sclerite of male median lobe of aedeagus: (0) absent; (1) present.

56. Dorsal series of setae on male median lobe of aedeagus: (0) absent; (1) present.

57. Female genitalia: (0) hydroporine-type; (1) amphizoid-type; (2) dytiscine-type. The coding of this character differs from my previous paper (Miller, 2001). Based on closer examination, members of Colymbetinae (*s.s.*) have a hydroporine-type of female genitalia rather than an amphizoid-type. However, in these taxa the spermathecal duct is attached to the bursa extremely near its posterior end.

58. Bursal gland: (0) absent; (1) present.

59. Bursa: (0) not long and twisted; (1) very long, slightly twisted.

60. Spermatheca: (0) reduced; (1) distinct, well-developed.

61. Gonocoxal shape: (0) not flattened and rounded; (1) dorsoventrally flattened with medial margins rounded.

62. Gonocoxal shape: (0) not long with rounded apices; (1) extremely long, apices expanded and rounded, apicolateral margins concave.

63. Gonocoxae: (0) not knifelike; (1) knifelike, apparently modified for endophytic oviposition.

64. Gonocoxal fusion: (0) not fused, independently articulable; (1) weakly fused, with strong membrane connecting gonocoxae and not independently articulable; (2) strongly fused, no membrane separating gonocoxae (additive).

65. Apical setal pencil on gonocoxa: (0) absent; (1) present.

66. Rami of female genitalia: (0) not modified; (1) together fused with medial portion bearing ventral teeth.

67. Accessory glands on each side of base of common oviduct: (0) absent; (1) present.

68. Thick muscles surrounding vagina: (0) absent; (1) present.

Third instar larval characters

69. Body shape: (0) not flexed medially; (1) distinctly flexed medially.

70. Anterior margin of clypeus: (0) evenly curved (Fig 21), (1) strongly bisinuate into three distinct lobes or teeth.

71. Nasale (anteriorly produced clypeal margin): (0) absent; (1) present.

72. Occipital foramen: (0) not deeply excised; (1) deeply excised on dorsal and ventral margins.

73. Stemmata: (0) not distinctly disparate in size; (1) strongly disparate in size, anterodorsal two larger and directed dorsally, anteroventral stemma directed anteriorly.

74. Antennomeres: (0) not subdivided; (1) subdivided.

75. Number of antennal segments: (0) 6; (1) 7; (2) 8; (3) 9 (additive). Since this character and the previous are not independent, this character applies only to those taxa with subdivided antennomeres (>4 antennomeres total).

76. Cardo of maxilla: (0) narrow, without series of long setae; (1) moderately broad, with medial and lateral rows of long setae; (2) very broad, with submedial and lateral rows of long setae (additive).

77. Galea: (0) absent; (1) present.

78. Maxillary palpomeres; (0) not subdivided; (1) subdivided.

79. Number of maxillary palpomeres (not including

palpiger); (0) 4; (1) 5; (2) 6; (3) 7; (4) 8 (additive). As with character 75, this character is not independent of the previous, and it only applies to those taxa with divided maxillary palpomeres (>3 palpomeres total).

80. Apicomedial margin of labial prementum; (0) unmodified; (1) with rounded, lobe-like projection; (2) with two, spine-bearing projections; (3) with a single, elongate projection.

81. Single, apicomedial process on labial prementum; (0) short, with four long spines; (1) long, apically simple; (2) long, apically bifid (additive).

82. Labial palpomeres: (0) not subdivided; (1) subdivided.

83. Number of labial palpomeres: (0) 3; (1) 4. As with characters 75 and 79, this character applies is not independent of the previous character, and it only applies to those taxa with divided labial palpomeres (>2 palpomeres total).

84. Natatory setae on anteroventral margin of femur and tibia: (0) absent; (1) present.

85. Abdominal terga: (0) large, each covering dorsum of each segment; (1) reduced, comprised of small plates anteriorly on dorsum.

86. Venter of abdominal segment 7: (0) sclerotized; (1) not sclerotized.

87. Fringe of natatory setae along lateral margins of abdominal segments 7–8: (0) absent; (1) present. I do not regard the condition in *Coptotomus* as homologous with state 1.

88. Cerci: (0) absent or very small; (1) present, elongate.

89. Fringe of natatory setae on cerci: (0) absent; (1) present.

90. Serrations on ventral margin of mandible: (0) absent; (1) present. I do not regard the mandible condition in *Copelatus* as homologous with this condition.

Codon gap characters in wingless sequence

91. Gap 1: (0) absent; (1) present (Table 3).

92. Gap 2: (0) absent; (1) present (Table 3).