Systematic placement of the recently discovered beetle family Meruidae (Coleoptera: Dytiscoidea) based on molecular data

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The family Meruidae has been established recently for the newly discovered species *Meru phyllisae* Spangler & Steiner, 2005 from Southern Venezuela. These beetles are morphologically highly distinct and at a body length of 0.8 mm represent the perhaps smallest individuals of Adephaga. Here, we use DNA sequence data to place this enigmatic taxon relative to other aquatic groups in this suborder. Meruidae was most closely associated with Noteridae, supporting a previous analysis of morphological structures which had suggested this sister relationship, albeit with weak support. While different alignment strategies did not affect the topology, the precise placement of Meruidae was affected by the choice of tree reconstruction method. Bayesian inference suggests a sister relationship of Meruidae + Noteridae, while parsimony analyses retrieve Meruidae + *Notomicrus* (a basal noterid genus), which combined are the sister group of all remaining Noteridae. Considering morphological evidence, the former placement appears more plausible.

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Introduction

The suborder Adephaga consists of almost 50 000 species and contains the extremely diverse terrestrial ground and tiger beetles (Carabidae), the wood-associated Rhysodidae, and several distinctive aquatic or semiaquatic lineages. Among the latter, whirligig beetles (Gyrinidae) and diving beetles (Dytiscidae) are the largest families with *c.* 1000 and 4000 described species, respectively, whereas Paelobiidae (formerly known as Hygrobiidae) and Amphizoidae comprise only a few species each (e.g. Balke 2005; Dettner 2005). New species of two morphologically highly divergent taxa could not be assigned to any of the known adephagan groups so that two new genera in two new families were described, both were placed in the superfamily Dytiscoidea.

The Aspidytidae (Ribera *et al.* 2002a) contains *Aspidytes niobe* from South Africa and *A. wrasei* from China (Beutel *et al.* 2008a).Meruidae (Spangler & Steiner 2005) was established for *Meru phyllisae* from Southern Venezuela, which is perhaps the smallest adephagan species attaining a total length of only about 0.8 mm (Beutel *et al.* 2008b).The phylogenetic relationships of the aquatic adephagan families, especially Dytiscoidea, were analysed in recent studies based on morphology and DNA sequence data (Ribera *et al.* 2002a,b; Balke *et al.* 2005). These studies did not include

M. phyllisae which was described only in 2005 and placed in a separate family due to its highly peculiar morphology (Spangler & Steiner 2005). Based on an informal analysis of morphological features, it was thought to be closely related either to the burrowing water beetles (Noteridae) or the crawling water beetles (Haliplidae) (Spangler & Steiner 2005). A cladistic analysis of Adephaga using comprehensive morphological data confirmed the former, that is, a sister group relationship with Noteridae (Beutel *et al.* 2006), although support levels were low (Jackknife 75%, Bremer support of 1). The purpose of the present study is to test this hypothesis with an independent data set composed of mitochondrial and nuclear genes. These markers have been used widely in phylogenetic analyses of aquatic adephagan beetles.

Materials and methods

DNA was extracted non-destructively from a single specimen using the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany). We amplified and sequenced the following loci: the 3′ region of Cytochrome Oxidase I (*cox1*); a central fragment of Cytochrome b (*cob*); the 3′ of the large subunit rRNA (*rrnL*), $tRNA^{Leu}$ and 5′ end of NAD Dehydrogenase 1 (*nad1*); the 3′ region of the small subunit rRNA (*rrnS*); and the entire nuclear SSU (18S rRNA) gene. We failed to sequence histone 3 for Meruidae, although a fragment of this gene available for all other taxa was used in the current analysis. Primer sequences and laboratory procedures were as described by Balke *et al.* (2005). Taxon sampling was drawn widely from the superfamily Dytiscoidea (families Noteridae, Aspidytidae, Amphizoidae, Paelobiidae, Dytiscidae), with Haliplidae used as outgroup. Alignment of length-variable ribosomal genes was conducted with ClustalW (with default settings: gap opening/extension 15/6.66) (Thompson *et al.* 1994), Muscle (Edgar 2004) and Prank (Loytynoja & Goldman 2005), implementing various progressive alignment procedures. The hypervariable SSU gene regions V4 and V6 (Tautz *et al.* 1988) were excluded from all analyses. We also removed all remaining gapped characters in one of the analyses of each aligned data set, to evaluate the stability of our results. Bayesian phylogenetic analysis (Rannala & Yang 1996) was implemented in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001) using a GTR model with γ-distributed among-site rate variation and estimating the proportion of invariable sites. Model parameters were estimated independently for each of the sequenced loci. Searches were conducted using the default priors starting with random trees, with three heated and one cold Markov chains for 1 000 000 generations, sampled at intervals of 100 generations. Stationarity of the Markov chains was determined by plotting the log-likelihood scores against generation time and visually assessing when values reached a stable equilibrium. Posterior probabilities were used to assess node support. Parsimony analyses were conducted in PAUP* 4.0b10 (Swofford 2002), using TBR heuristic searches with 1000 random addition sequences and gaps coded as a fifth character state. We reweighed the characters *a posteriori* according to the retention index. Non-parametric bootstrapping was conducted with 1000 pseudoreplicates of 100 random additions each (Felsenstein 1985). New sequence data were deposited at GenBank under accession numbers FM163588-FM163592.

Results

Bayesian and parsimony analysis combining all markers were conducted on alignments obtained with various procedures (Materials and Methods). Topologies were very similar among these analyses and resulted in generally high nodal support values. In agreement with earlier studies that did not include *Meru* (Balke *et al.* 2005), all dytiscoid families were monophyletic. Aspidytidae + Amphizoidae was the sister group to Dytiscidae, with Paelobiidae as the sister to all of them. All analyses strongly supported (bootstrap 100, posterior probability > 95) a clade comprising Meruidae and Noteridae. The Bayesian analyses suggested a sister group relation of Meruidae + Noteridae in all alignments (Fig. 1). However, in the parsimony analysis Meruidae was retrieved as sister group of *Notomicrus*, and both of them as sister taxon to the rest of the Noteridae, also with strong support (bootstrap 100). This topology was also retrieved with high support when all gapped characters were removed. The branch leading to *Meru phyllisae* is comparably long, indicating that this morphologically highly derived species is genetically highly divergent also. Clear differences are evident, for example, from the base composition of the SSU which is more AT rich than in related Noteridae (Table 1). The position of this divergent taxon near the base of the tree might suggest that Meruidae is not part of the ingroup altogether. However, we conducted exploratory analyses using a wider range of taxa, including species of the families Gyrinidae and Carabidae, and still recovered *Meru* within Dytsicoidea (data not shown). The results therefore confirm the placement of Meruidae within a well supported Dytiscoidea (Beutel *et al.* 2006).

Discussion

Morphological studies had already suggested a close association between Meruidae and Noteridae (Beutel *et al.* 2006), but the combination of six gene fragments used here now provides high levels of support for this relationship. Topologies and levels of node support in the molecular analyses were not affected by the method of alignment, but the precise relationships differed dependent on the procedure for tree reconstruction. Bayesian analyses supported *Meru* + Noteridae, whereas parsimony supported a clade comprising *Meru* + *Notomicrus*, which would challenge the family status of Meruidae. The sister group relationship between *Meru* and Noteridae is

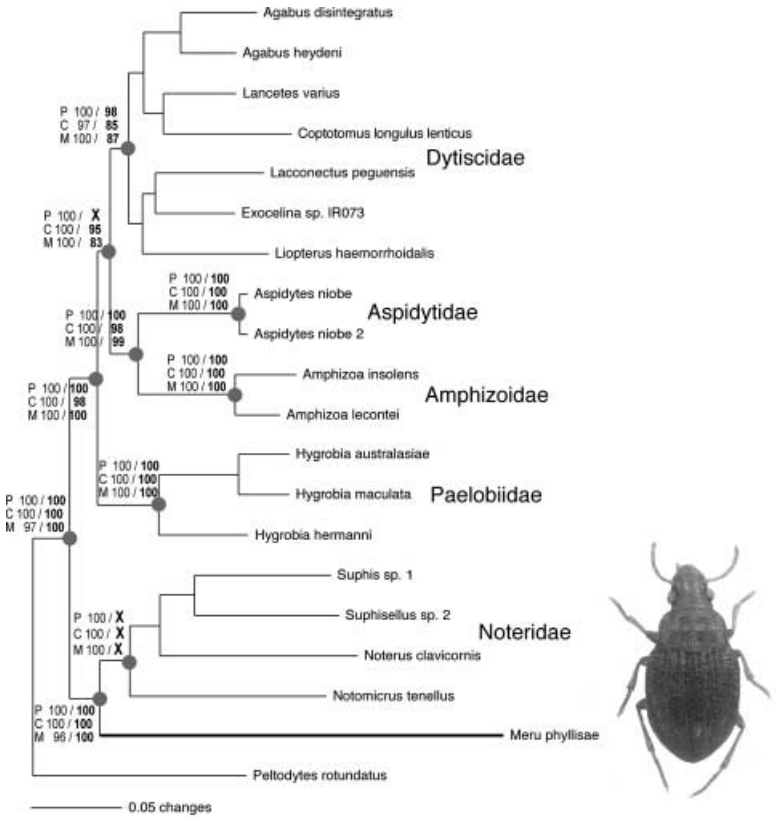

Fig. 1 Phylogram obtained in the Bayesian analyses of the Muscle alignment. Numbers at nodes are support according to different alignment strategies (P, Prank; C, Clustal; M, Muscle), left column are Bayesian posterior probabilities (×100), right column in bold are parsimony boostrap values, X indicates that this particular node was not retrieved under the parsimony criterium. Beetle habitus shows Meru phyllisae (photo by Harald Schillhammer).

Table 1 Base frequencies of 18S rRNA in *Meru* and selected Noteridae.

Taxon	A	C	G	T
Notomicrus	0.24431	0.23709	0.27429	0.24431
Noterus	0.24138	0.23971	0.27809	0.24082
Meru	0.26162	0.21405	0.26051	0.26383
Mean	0.24912	0.23026	0.27095	0.24968

congruent with the morphological data (Beutel *et al*. 2006). *Meru* does not show any of the diagnostic characters of Noteridae, and the apomorphic characters shared by *Notomicrus* and the remaining family (excluding *Phreatodytes*) are also absent (Beutel & Roughley 1987; Belkaceme 1991). In *Notomicrus*, like in other adult noterids, the surface of all exposed sclerites including the elytra is smooth. The body is streamlined and the head is shortened and rounded. Burrowing spurs are present on the fore tibiae. The hind legs are modified as swimming legs, with sparse fringes of long swimming hairs (Belkaceme 1991). None of these features is found in *Meru* (Spangler & Steiner 2005), and it appears very unlikely that all of them should have been secondarily modified. A secondarily elongated head, for instance, is not found in any of the known semiaquatic dytiscid beetles, and not even in the secondarily terrestrial species (e.g. Brancucci 1979). The typical noterid features of the hind coxa such as the presence of a distinct duplicature (metacoxal plates) or the anteromedian angle (Beutel & Roughley 1987; Belkaceme 1991) are also absent in *Meru*. However, metacoxal features may be modified in correlation with size reduction. The fusion of the anterior margin with the metaventrite found in *Notomicrus* (partial) and *Meru* (complete) is almost certainly a result of miniaturization. Fusion of thoracic sclerites is a common feature in extremely small beetle species (e.g. Ptiliidae, Jacobsoniidae, pers. obs. Beutel). The inclusion of molecular data of the subterranean species of the Japanese genus *Phreatodytes*, which were placed as the sister taxon of the remaining Noteridae by Belkaceme (1991), could be crucial to resolve these ambiguities, but it is unlikely that suitable material of these extremely rare and endangered beetles (Uéno 1957, 1996) could be available in the near future.

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