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Molecular phylogeny of the highly disjunct cliff water beetles (Coleoptera, Aspidytidae) and description of *Chinaspidytes* gen.nov. for the Chinese lineage

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Running title: Molecular Systematics of Dytiscoidea

The superfamily Dytiscoidea contains six families with an aquatic lifestyle, with most of its extant diversity in two families: the burrowing water beetles (Noteridae) and the diving beetles (Dytiscidae). The other families have few species (up to 6) and generally highly disjunct extant distributions. Aspidytidae currently contains one genus with two species, one in China and one in South Africa. Here we provide the first molecular data for the Chinese species, allowing us to explore the phylogenetic relationships and position of both species of this small family for the first time. Based on a matrix of 11 genes we inferred a phylogenetic hypothesis for Dytiscoidea including all extant families. Unexpectedly, Aspidytidae were consistently recovered as paraphyletic relative to Amphizoidae, despite being well characterized by apparently synapomorphic adult features. A re-examination of larval characters in the two aspidytid species revealed that the larva of the Chinese species is strikingly similar to that of Amphizoidae. Both share a series of plesiomorphic features but also some potential synapomorphies, including a dense vestiture of short hairs on the head capsule, anteriorly shifted posterior tentorial grooves, and widely separated labial palps. Arguably these features may belong to the groundplan of the clade Aspidytidae + Amphizoidae, with far-reaching secondary modifications (including reversals) in the South African *Aspidytes niobe*. At present we retain the family Aspidytidae, however, due to the strong adult morphological synapomorphies of the two extant species, and the fact that the molecular paraphyly of the family may result from the highly divergent nature of the two extant species. This long evolutionary separation and strong divergence, in terms of gene sequences and larval features is undeniable, substantial levels of saturation in third codon positions of protein-coding genes being present between the two taxa. We address this issue taxonomically by introducing the new genus *Chinaspidytes* for the Chinese *Aspidytes wrasei*. The continued contentious relationships amongst Dytiscidae, Hygrobiidae, Aspidytidae

and Amphizoidae highlight the need for more data to address dytiscoid phylogenetics, possibly involving a genomic approach.

ADDITIONAL KEYWORDS: *Aspidytes*, Coleoptera, Dytiscoidea, Hydradephaga, new genus

Chinaspidytes

INTRODUCTION

About 13,000 of nearly 400,000 described species of beetles (Zhang, 2011) are aquatic (Jäch & Balke, 2008, total estimate ca. 18,000). Seven extant aquatic families and more than 5,000 aquatic species belong to the second largest beetle suborder Adephaga, which comprises more than 40,000 species in total. In recent years two new adephagan families were discovered in hygropetric habitats, Meruidae (*Meru phyllisae*) from Venezuela, and Aspidytidae with the two species *Aspidytes niobe* from South Africa and *A. wrasei* from China (Spangler & Steiner, 2005; Ribera *et al.*, 2002; Balke *et al.*, 2003; Beutel *et al.*, 2010). Both families are placed in Dytiscoidea, along with Dytiscidae, Noteridae, Hygrobiidae and Amphizoidae. Several studies have addressed the phylogeny of Dytiscoidea (or Adephaga) as a whole, or parts thereof, using morphology and/or DNA sequence data (e.g. Balke *et al.*, 2005, 2008; Beutel *et al.*, 2006; Hawlitschek *et al.*, 2012; Miller & Bergsten, 2014). Larval instars of Meruidae and Aspidytidae were described recently, with analyses of phylogenetically relevant structures (Alarie *et al.*, 2005, 2011; Dressler *et al.*, 2011). Finally, new fossil evidence was presented by Prokin *et al.* (2013), and a morphology based phylogeny of extant and fossil Adephaga by Beutel *et al.* (2013). Even though these efforts place adephagan aquatic families among the best studied groups of beetles, our understanding of their phylogenetic relationships remains incomplete.

A clade Noteridae (including Phreatodytinae) + Meruidae is generally retrieved as sister to the remaining dytiscoid families. In contrast, relationships between Dytiscidae, Aspidytidae, Hygrobiidae and Amphizoidae remain contentious (see Alarie *et al.*, 2005 p. 429). Morphology based analyses are ambivalent, suggesting either a clade Hygrobiidae + Dytiscidae, with Amphizoidae as their sister and Aspidytidae basal (Beutel *et al.*, 2006, 2013; Alarie *et al.*, 2005 in part), or alternatively, Hygrobiidae + Dytiscidae are found sister to Amphizoidae + Aspidytidae (Balke *et al.*, 2005; Alarie *et al.*, 2005 in part, 2011). Analyses based on DNA sequence data place Amphizoidae + Aspidytidae as sister to Dytiscidae, with Hygrobiidae as sister to these three families (Balke *et al.*, 2005, 2008). In the only combined analysis

to date Hygrobiidae + Dytiscidae were recovered as sister to Aspitytidae, and Amphizoidae sister to these three (Ribera *et al.*, 2002). Clearly the inter-relationships between these dytiscoid families remain very ambiguous, and the absence of a robust phylogenetic hypothesis impedes our understanding of the evolutionary history of this large group of beetles, including the evolution of swimming behavior and other features related to life in different aquatic or semiaquatic habitats (Ribera *et al.*, 2002).

In this study, DNA sequence data of the Chinese species *Aspidytes wrasei* were included in a phylogenetic analysis for the first time. Previous datasets (Ribera *et al.*, 2002a; Balke *et al.*, 2005; Hawlitschek *et al.*, 2012) were supplemented with five protein-coding genes and taxon sampling across basal lineages of the superfamily was extended. Our goals were to: (1) present a molecular phylogeny of Dytiscoidea focused on Aspitytidae, (2) clarify family level relationships within the Dytiscoidea using new data, and (3) summarize morphological (from adults and larvae) and molecular evidence to reassess the taxonomic status of *Aspidytes wrasei* for which we here suggest *Chinaspidytes* gen.n.

MATERIAL AND METHODS

TAXON SAMPLING AND MOLECULAR BIOLOGY

We included 30 taxa representing all extant families of Hydradephaga. For Gyrinidae only one species was included to root the phylogenetic tree (Table 1). We extracted total genomic DNA from legs or thoracic tissues of freshly collected specimens kept in 96% ethanol using the DNeasy kit (Qiagen, Hilden, Germany). Using standard PCR protocols (Hawlitschek *et al.*, 2012; http://zsm-entomology.de/wiki/The_Beetle_D_N_A_Lab) we amplified and then sequenced the following genes: mtDNA - Cytochrome c oxidase subunit 1 (CO1 - 756 bp), Cytochrome b (Cytb - 306 bp), ribosomal 12S (373 aligned bp), ribosomal 16S (492 aligned bp); nDNA - ribosomal 18S (566 aligned bp), Arginine kinase (ARK - 672 bp), Carbamoylphosphate synthetase (CAD - 672 bp), Enolase (ENO - 663 bp), Histone 3 (H3 - 303 bp), Histone 4 (H4 - 159 bp) and Wingless (WGL - 486 bp). Sequences from previous

studies (Ribera *et al.*, 2002; Balke *et al.*, 2005; Hawlitschek *et al.*, 2012) were used to complete the dataset. Both sequence strands were assembled and errors/ambiguities corrected in Geneious R6 (Biomatters, <http://www.geneious.com/>), aligned using Muscle (Edgar, 2004) and the reading frames checked with Mesquite 3.01 (<http://mesquiteproject.org>). New sequences were deposited in GenBank under accession numbers XXXX-XXXX.

PHYLOGENETIC RELATIONSHIPS

We tested for possible saturation in the sequence data using DAMBE 5.5 (Xia, 2013). We calculated the index of substitution saturation (ISS) of each non-coding gene and each position of the protein coding genes, and compared it with a critical index of substitution saturation (ISSc) representing a threshold for significant saturation in the data (Xia, 2013). Saturation is assumed when the ISS value either exceeds the ISSc value, or is not significantly different (Xia, 2013).

All phylogenetic inference analyses were performed including or excluding saturated positions. Phylogenetic relationships were investigated in Maximum Likelihood (ML) and Bayesian Inference (BI). Optimal partitioning schemes were estimated with PartitionFinder v1.1.1 (Lanfear *et al.*, 2012) using the ‘greedy’ algorithm, either the ‘*mr bayes*’ or ‘*raxml*’ set of models and the corrected Akaike Information Criterion (AICc) to compare the fit of different models. The PartitionFinder input file was configured to include the non-coding gene fragments as separate entities and decompose the coding genes by codon positions, yielding a total of 27 partitions to test. We also used the reversible-jump MCMC method developed by Huelsenbeck *et al.* (2004) to explore the entire space of substitution models instead of using the ones selected in PartitionFinder. The BI analyses were conducted under MrBayes 3.2.2 (Ronquist *et al.*, 2012) using two runs of eight Markov Chain Monte Carlo (MCMC, one cold and seven incrementally heated) running for 50 million generations and sampling every 5,000 cycles. After checking the convergence of runs under Tracer 1.5 (<http://BEAST.bio.ed.ac.uk/Tracer>) and applying a conservative

burn-in of 25%, we used the command *sump* in MrBayes to calculate the posterior probabilities (PP) and produce a 50% majority rule consensus tree. The ML analyses were carried out under RAxML (Stamatakis, 2006) and 1,000 thorough *Bootstrap* replicates (BS) were computed to investigate the level of support at each node. A $PP \geq 0.95$ and a $BS \geq 70$ were recognized as indicating strong support for a given node (Hillis & Bull, 1993; Erixon *et al.*, 2003).

Finally, we conducted analyses on reduced datasets, using the full dataset but without 18s rRNA, as well as mtDNA only and nDNA only, to evaluate the possible influence of deviating gene sequences on topology.

RESULTS AND DISCUSSION

PHYLOGENETICS

The molecular matrix comprised a total of 5,448 aligned basepairs. All third positions of protein coding genes had substantial to high levels of saturation as estimated by the ISS index (Figure 1). We therefore built a matrix excluding all saturated positions, which comprised 4,109 bp. All analyses recovered broadly similar phylogenetic patterns (Figure 2). Few differences were revealed when we used different datasets (e.g. all data but without 18S rRNA) and different models of substitution. These alternative topologies were weakly supported.

Dytiscoidea was divided into three major clades:

- 1) Meruidae + Noteridae were robustly combined in a clade with *Meru phyllisae* as sister to a monophyletic Noteridae. Within Noteridae, the subfamily Noterinae was recovered as monophyletic with strong support, with Notomicrinae + Phreatodytinae as sister clades.
- 2) A clade containing Amphizoidae, Aspidytidae and Hygrobiidae was recovered in all analyses except the one including saturated positions, which placed Hygrobiidae as sister to a unit comprising ((Aspidytidae + Amphizoidae) + Dytiscidae), albeit with weak support. Amphizoidae was always recovered as

monophyletic, whereas Aspidytidae was recovered as paraphyletic in all analyses with moderate to strong support. *Aspidytes wrasei* was placed as sister taxon to a clade comprising Amphizoidae + *Aspidytes niobe*. Except in the analysis including saturated positions, Hygrobiidae was always sister to Amphizoidae + Aspidytidae, with the European *Hygrobia hermanni* well separated from the Australian *Hygrobia* species.

3) Dytiscidae was monophyletic with strong support, and the monophyly of the included subfamilies was strongly supported except for Copelatinae, which were rendered paraphyletic by the inclusion of *Sandracottus* (Dytiscinae) in three of the analyses. The main internal branching pattern did not agree with previous reconstructions focused on Dytiscidae (e.g. Miller, 2001; Ribera *et al.*, 2008; Miller & Bergsten, 2014), but node support along our Dytiscidae tree backbone was low (as was also the case in most previous studies).

The analysis of the full dataset with the exclusion of the 18S rRNA gene resulted in a strongly supported topology, except for the position of *Sandracottus*, which was placed as sister to Copelatinae (*Exocelina*, *Copelatus*, *Lacconectus*) instead of being nested within Copelatinae as in the analyses of the full dataset (Supplementary Fig. S1).

In the analysis of the mitochondrial sequence, *Aspidytes wrasei* was sister to a clade containing *Aspidytes niobe* + Amphizoidae, rendering Aspidytidae paraphyletic, as in the analyses of the full dataset. Here the clade containing the *Aspidytes* species and Amphizoidae was placed as sister to Dytiscidae, Hygrobiidae as sister to Noteridae, and Haliplidae were paraphyletic (Supplementary Fig. S2).

Analyses of the nuclear sequences placed *Aspidytes wrasei* sister to Amphizoidae, and both as sister to *Aspidytes niobe* + Hygrobiidae. All these groups combined were sister to Dytiscidae (Supplementary Fig. S3).

Due to the morphological differences between both adults and larvae of the two species of extant Aspidytidae, coupled with the remarkably large genetic differences between them and the uncertainty in their relative phylogenetic position, we erect a new genus for *A. wrasei* below.

***CHINASPIDYTES* BALKE, BEUTEL & RIBERA, GEN.N.** (Figs 2—6)

Etymology

Derived from Qin (秦), a Chinese kingdom during the Zhou dynasty *c.* 900–246 BC.

Type species

Aspidytes wrasei Balke, Ribera & Beutel, 2003

Description including differential diagnosis versus Aspidytes niobe

Adult characters

Head: Anterior margin in dorsal view evenly rounded between eyes (*A. niobe*: anteriorly elongated with margin concave in front of each eye) (Fig. 3). Ligula large, T-shaped and without dense setation (*A. niobe*: short and roughly triangular with long, thick setae). Penultimate labial palpomere smaller than ultimate one and with four unusual sensilla: short hair on cupula surrounded by cuticular bulge (*A. niobe*: no such sensillae present and penultimate palpomere much thicker than ultimate one) (Fig. 4). Mentum slightly concave medially (*A. niobe*: with a V-shaped emargination).

Thorax: Prosternal process without bead, metacoxal process with a broad bead (vice-versa in *A. niobe*) (Fig. 5).

Male: Median lobe formed by a simple, curved lobe (*A. niobe*: with multiple sclerites and membranous structures) (Fig. 6).

Female: Gonocoxa and gonocoxosternum both with conspicuous long setae (*A. niobe*: without, or only few short setae. Vagina with a dorsal gland (*A. niobe*: gland on ventral side).

Larval characters

General (Instar III). Head with greatest width in middle region (as in *Amphizoa*; *A. niobe*: posterior third).

Head capsule with dense vestiture of short hairs (as in *Amphizoa*; *A. niobe*: absent). Middle part of anterior clypeolabral margin broad (as in *Amphizoa*; *A. niobe*: narrow). Anterior margin of nasale with 16 short sensorial setae (= lamellae clypeales) (2 + 24 in *Amphizoa*; 6 in *A. niobe*). Coronal suture not shortened, ca. 40% of length of head capsule (as in *Amphizoa*; *A. niobe*: less than 20% of length of head capsule).

Posterior tentorial grooves shifted to anterior third of head (as in *Amphizoa*; *A. niobe*: posterior half).

Ventral ecdysial line distinct (as in *Amphizoa*; *A. niobe*: not recognizable on SEM images). Stemmata with convex lenses (as in *Amphizoa*; *A. niobe*: completely flat). Labial palps widely separated, 2x the width of the basal palpomere (as in *Amphizoa*; *A. niobe*: less widely separated). Abdominal segments III–VII sclerotized dorsally and membranous ventrally (as in *Amphizoa*; *A. niobe*: completely sclerotized, ring-like). Siphon short but distinct (absent in *A. niobe*). Additional anterodistal tibial pore absent (present in *A. niobe* - Michat *et al.*, 2014).

Instar II. Egg bursters absent (present in *A. niobe*).

Distribution

China, Shaanxi Province, Hua Shan.

KEY TO GENERA OF ADULT ASPIDYTIDAE

Prosternal process without bead, metacoxal process with a broad bead; China ... *Chinaspidytes*

Prosternal process with a broad bead, metacoxal process lacking a bead; South Africa ... *Aspidytes*

FAMILY LEVEL RELATIONSHIPS

A subdivision of Dytiscoidea into clades comprising Noteridae + Meruidae and the remaining groups is in agreement with previous studies (e.g., Balke *et al.*, 2008; Beutel *et al.*, 2013). In contrast, a sister group relationship between Dytiscidae and a clade containing the three small families Hygrobiidae, Aspidytidae and Amphizoidae, as recovered here, is a novel and unorthodox result. The apparent instability in Dytiscoidea excl. Noteridae + Meruidae (e.g., Balke *et al.*, 2008) suggests that adding a limited number of gene fragments and terminal taxa is insufficient for reliable phylogenetic reconstruction. A possible reason for these difficulties is the large phylogenetic distance between the extant species of most of the families; their relict status is highlighted by exceptionally disjunct distribution patterns - e.g. Hygrobiidae (6 spp.) with one species in Europe, one in western China and four in Australia (Hawlitschek *et al.*, 2012), Amphizoidae (5 spp.) with two species in China and three in western North America, and Aspidytidae (2 spp.) with one species in China and one in South Africa. This issue is probably best reflected by the paraphyly of Aspidytidae throughout the different analyses presented here. The genus *Aspidytes* was originally erected for a single species, *A. niobe* from the Cape region of South Africa (Ribera *et al.*, 2002). Subsequently a second species from China, *A. wrasei*, was described within the same genus, despite their disjunct distribution and some very distinct morphological differences, especially in the male genitalia. This concept appeared justified given their great similarity in habitus, similar hygropetric habitats, and also on the basis of specific shared derived adult features, in particular a unique modification of the antennal base - an 8-shaped scapus partly enclosing the shortened pedicellus (Balke *et al.*, 2005). An unusual larval apomorphy of the two species is the dorsal orientation of the spiracles on abdominal segment VIII of instars II and III, but a similar condition is present in larvae of *Amphizoa* (e.g., Dettner, 2005). Other features shared by the two species are the presence of well-developed “metacoxal plates”

(medium lamina of metacoxa) and remnants of a transverse suture on the metaventrite (Ribera *et al.*, 2002; Balke *et al.*, 2005). However, these are likely to represent plesiomorphic character states (at least the transverse suture) retained from the groundplan of Dytiscoidea (e.g., Beutel *et al.*, 2013). Interestingly, a re-examination of larval features has revealed that the larva of the Chinese species is strikingly similar to those of Amphizoidae, even though *A. niobe* is recovered as closer to *Amphizoa* in the analyses of combined or the mitochondrial DNA data (not so in the analyses of nuclear genes, where *C. wrasei* is sister to Amphizoidae). Larvae of *Chinaspidytes* and *Amphizoa* share a series of plesiomorphic features (or features with uncertain polarity). These include the head with the greatest width in the middle region (posterior 3rd in *A. niobe*), a fairly long coronal suture (short in *A. niobe*), the presence of a long and distinct ventral ecdysial line (not recognizable on SEM images of *A. niobe*), a broad middle part of the anterior clypeolabral margin (narrow and with deep lateral incisions in *A. niobe*), anteriorly shifted posterior tentorial grooves (on posterior half in *A. niobe*), stemmata with convex lenses (completely flat in *A. niobe*), widely separated labial palps (closer to midline in *A. niobe*), and abdominal segments III–VII sclerotized dorsally and membranous ventrally (completely sclerotized and ring-like in *A. niobe*). Shared derived features of *Chinaspidytes* and *Amphizoa* are the presence of a dense vestiture of short hairs, a distinctly increased number of clypeolabral sensilla, and possibly the anterior shift of the posterior tentorial grooves. The phylogenetic branching pattern revealed from our molecular data suggests that the former features belong to the groundplan of a clade comprising Aspdytidae and Amphizoidae, and that some conditions found in the larvae of *A. niobe* may be due to reversals or secondary modifications. Our analyses revealed long branches for the two species of Aspdytidae, suggesting an old phylogenetic divergence, and also a very low net diversification rate, unless many more species are yet to be discovered (which seems unlikely). Even though analyses of the molecular data used here do not support the monophyly of Aspdytidae, we have opted to maintain the family at present. This decision is based on the highly unusual apparent morphological synapomorphies of adult *Aspidytes* and *Chinaspidytes*, such as the aberrant antennal base, as well as the possibility that the paraphyly of Aspdytidae results from analytical

artefacts, as a result of a long history of independent evolution.

The fossil record contains species similar to Aspidytidae in the extinct †Liadytidae. The two families differ primarily by the absence of an anteromedian metaventral process (between the mesocoxae) in the latter (Prokin *et al.*, 2013). Unfortunately the antennal scapus and pedicellus are not visible in the available †*Liadytes* fossils. However, the very similar shape of the medium laminae of the metacoxa, combined with the presence of a transverse suture on the metaventrite (which is a plesiomorphy however) suggest that the Aspidytidae and †Liadytidae might be closely related. †Liadytidae are well documented in the fossil record of E Asia, *c.* 120–160 million years old, with five described species (Fig. 7). A phylogenetic analysis of extant and fossil Adephaga (Beutel *et al.*, 2013) did not retrieve Aspidytidae and †Liadytidae as sisters, but these families were closely associated in a paraphyletic series Aspidytidae / Amphizoidae / †Liadytidae. The position of †Liadytidae obviously remains ambiguous as larval and several adult characters cannot be scored at present. Their precise affinities with the species of Aspidytidae (or other groups of Dytiscoidea) will only be revealed with the discovery of additional fossil specimens showing critical features such as the antennal base.

In summary, the monophyly of Aspidytidae appears unlikely considering the extensive molecular data presented here but remains ambiguous based on morphology. The discovery of new extant or extinct taxa may possibly help to solve the problem, reducing possible long branch issues in the dataset. However, it is likely that a reliable solution of the problem may require more extensive taxon sampling across the Dytiscoidea in general, with the inclusion of the Chinese species of *Amphizoa*, as well as additional sequence data. More detailed anatomical studies, especially of larvae, and analyses of transcriptomes or even genomic data may eventually lead to a more robust solution.

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Figure legends

Figure 1. Results of the saturation test for individual genetic markers.

Figure 2. Phylogenetic relationships of Dytiscoidea. Support values indicated through coloration of boxes at the respective nodes,

Figure 3. Head and pronotum of: A, *S. wrasei* and B, *A. niobe*.

Figure 4. Ventral aspect of head showing structure of submentum, ligula and labial palps mentioned in text, in A, *S. wrasei* and B, *A. niobe*.

Figure 5. Ventral aspect of beetle showing structure of prosternal and mesosternal processes as well as medium lamina of metacoxa (“metacoxal plates”) in A, *S. wrasei* and B, *A. niobe*.

Figure 6. Male genitalia, from left to right: median lobe lateral view, ventral view, dorsal view and lateral lobe (paramere) of A, *S. wrasei* and B, *A. niobe*.

Figure 7. Distribution of †*Liadytes* species (source: Palaeobiology database, paleobiodb.org/).