A molecular phylogeny of the tribe Ochthebiini (Coleoptera, Hydraenidae, Ochthebiinae)

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**Abstract**

Ochthebiinae, with ca. 650 species distributed worldwide, are the second most speciose subfamily of the aquatic beetle family Hydraenidae. They are the ecologically most diverse subfamily, with terrestrial species as well as species in almost all types of aquatic habitats, including hypersaline waters. Ochthebiinae include the tribes Ochtheosini (four species in three genera) and Ochthebiini. We provide here the first comprehensive phylogeny of the tribe Ochthebiini, based on 188 species and four subspecies from most genera, subgenera and species groups. We obtained sequence data for a combination of mitochondrial and nuclear gene fragments including the 5’ and 3’ ends of the cytochrome c oxidase subunit 1, the 5’ end of 16S RNA plus the Leucine tRNA transfer plus 5’ end of NADH dehydrogenase subunit I, and internal fragments of the large and small ribosomal units. The analyses with maximum likelihood and Bayesian probabilities consistently recovered a generally well supported phylogeny, with most currently accepted taxa and species groups as monophyletic. We provide a new classification of the tribe based on our phylogenetic results, with three genera: *Meropathus* Enderlein, *Ochthebius* Leach and *Protochthebius* Perkins. *Meropathus* in its new sense includes five subgenera in addition to *Meropathus* s.str.: (1) *M.* (Hygrotymanogaster) Perkins comb.n.; (2) *M.* (Plesiotymanogaster) Perkins comb.n.; (3) *M.* (Topotymanogaster) Perkins comb.n. (the last three transferred from *Tymanogaster*); (4) *M.* (Tympalopatrum) Perkins comb.n.; and (5) *M.* (Tymanogaster) Janssens comb.n. (both currently considered as genera). The genus *Ochthebius* is here divided into nine subgenera in addition to *Ochthebius* s.str.: (1) *O.* (Angiochthebius) Jäch & Ribera; (2) *O.* (Asiobates) Thomson; (3) *O.* (Aulacochthebius) Kuwert; (4) *O.* (Cobalius) Rey; (5) *O.* (Enicocerus) Stephens; (6) *O.* (Gymnanthelius) Perkins comb.n.; (7) *O.* (Gymnochthebius) Orchymont comb.n.; (8) *O.* (Hughleechia) Perkins comb.n.; and (9) *O.* (Micragasma) Sahlberg. Within *Ochthebius* s.str., 17 species groups are proposed, five of them newly established (3, 9, 11, 13 and 16): (1) andraei; (2) atriceps; (3) corrugatus; (4) foveolatus; (5) kosiensis; (6) lobicollis; (7) marinus; (8) metallescens; (9) nitidipennis; (10) notabilis; (11) peisonis; (12) punctatus; (13) quadricollis; (14) rivalis; (15) strigosus; (16) sumatrensis; and (17) vandykei. We could not obtain sequences of any species of *Protochthebius*, which is therefore maintained as a distinct genus of Ochthebiini. We elevated to species rank two subspecies of *Ochthebius: O. fallaciosus* Ganglbauer stat.n. (former subspecies of *O.*
viridis Peyron) and O. deletus Rey stat.rest. (former subspecies of O. subpictus Wollaston).

Introduction

Ochthebiinae, with ca. 650 species and 11 subspecies described, are the second most diverse subfamily of the water beetle family Hydraenidae (Hansen, 1998; Jäch & Balke, 2008; Tables 1, S1). They occur on all biogeographic regions, including the Antarctic islands of Kerguelen and Heard, where they are the only Hydraenidae present (Hansen, 1998). Ochthebiinae are the ecologically most diverse hydraenid subfamily, with terrestrial species, species living in the interface between land and water, as well as in most types of aquatic environments (Jäch et al., 2016). A large number of species are tolerant to hypersaline waters, living either in coastal rockpools (e.g. Cobalius Rey or Calobius Wollaston, Antonini et al., 2010; Sabatelli et al., 2016), coastal or inland salt pans or in inland hypersaline streams (especially the O. notabilis group, but also many other species in different groups, Abellán et al., 2009; Millán et al., 2011).

The external morphology of the species of Ochthebiinae is more heterogeneous than in other Holarctic lineages of Hydraenidae (e.g. Hydraena Kugelann, Trizzino et al., 2013, or Limnebius Leach, Rudoy et al., 2016). This has resulted in a more complex taxonomy, with mostly all of the described genera or subgenera with uncertain relationships. Thus, the genus Ochthebius Leach was divided in 16 subgenera by Kuwert (1887), but all of them with the exception of Aulacochthebius Kuwert were later synonymised (see Tables 1, S2 for a synopsis of the classification and Table S1 for a complete checklist of Ochthebiini).

Ochthebiinae were divided by Perkins (1980) in two tribes, Ochthebiini and Ochtheosini, the latter for the single terrestrial genus Ochtheosus Perkins, with two species. The monotypic genera Edaphobates Jäch & Díaz and Ginkgoscia Jäch & Díaz, for which we could not obtain fresh material for DNA extraction, were tentatively hypothesized to be related to Ochtheosus by Jäch & Díaz (2003, 2004) and thus we tentatively consider them within Ochtheosini. Perkins (1997) divided Ochthebiini in five newly defined subtribes: Enicocerina (for the single genus Enicocerus Stephens), Meropathina (Meropathus Enderlein, Tympanopatrum Perkins and Tymanogaster Perkins), Neo-echthiini (Neo-echthiini Orchymont), Ochthebiina (Ochthebius, Gymnochthebius Orchymont, Hughleechia Perkins, Gymnanthelius Perkins,
Aulacochthebius and Micragasma Sahlberg) and Protochthebiina (Protochthebius Perkins). Enicocerus was treated as a subgenus by several subsequent authors (e.g. Jäch, 1998; Ribera et al., 2010; Jäch & Skale, 2015), and Neochthebius was treated as a synonym of Ochthebius s.str. by Jäch & Delgado (2014b), leaving ten genera in Ochthebiini, most of them described in the 20th century (Table 1). Ochthebius is the oldest available generic name (Leach, 1815), with 540 species (Hansen, 1998; Table 1), grouped into four recognised subgenera with mostly Palaearctic distribution: Asiobates, Calobius, Enicocerus and Ochthebius (Jäch & Skale, 2015; Tables 1, S1; see the detailed taxonomic history of subgenera and species groups in the Discussion below). Within Ochthebius s.str., the most diverse subgenus, several informal species groups have been defined, which have undergone important modifications through their taxonomic history (Tables S1, S2).

The classification and proposed relationships within Ochthebiini have also experienced many modifications during the last four decades. Perkins (1980) revised the by then known American species, and proposed a phylogeny derived from the examination of some morphological characters. Gymnochthebius was placed as sister to the remaining taxa, which were divided in two lineages: (1) Meropathus plus Neochthebius (currently a synonym of Ochthebius, Table S2) and (2) Ochthebius plus Asiobates. Subsequently, Perkins (1997) synonymized four subgenera with Ochthebius (Calobius, Cobalius, Liochthebius Sahlberg and Notochthebius Orchymont), and described three additional genera (Tables 1, S2). Based mostly on the exocrine secretion delivery system (ESDS), he divided the subfamily in two tribes, Ochtheosini for the newly described Ochtheosus and Ochthebiini, divided in turn in subtribes, with unresolved relationship among them. Ochtheosus was considered to have some plesiomorphic characters similar to some southern African genera (e.g. antennae with 11 antennomers, as in many Prosthetopinae, Perkins, 1997; see also Beutel et al., 2003), and did not share several of the most characteristic synapomorphies with the remaining Ochthebiinae, in particular the structure of the tentorial arms, galea and lacinia.

The first formal cladistic analysis of the family Hydraenidae was published by Beutel et al. (2003), but sampling was too incomplete to resolve internal relationships within Ochthebiinae other than the sister relationship of Meropathus with Ochthebius + Gymnochthebius. There is no published global molecular phylogeny of the entire family Hydraenidae or subfamily Ochthebiinae, but in recent years some detailed molecular
phylogenies for some lineages have been published, such as the *Ochthebius notabilis* group (Abellán et al., 2009) and *Enicocerus* (Ribera et al., 2010). In Abellán et al. (2009) an extensive phylogeny of *Ochthebius* and some related genera using only mitochondrial markers was used to estimate the phylogenetic diversity of the Iberian fauna. The sampling of some geographical areas was, however, very incomplete, as the intention was not to produce a phylogenetic study. Still, most Palaearctic lineages were represented, which allowed to establish the monophyly of most of the included genera/subgenera and of the recognised species groups, although internal groups had poor relationships between them. Sabatelli et al. (2016) used these data to study the origin of species typical of rockpools, recovering basically the same relationships and establishing a new species group for the South African *O. capicola* Péringuey. In the same paper the subgenus *Cobalius* was found to be outside *Ochthebius* s.str., but *Calobius* nested within it, referring to it as the “*Calobius*” lineage.

In this study we provide a comprehensive phylogeny of Ochthebiini, based on mitochondrial and nuclear sequence data including representatives of most lineages. We introduce several changes in the taxonomic classification to accommodate our phylogenetic results, and provide a complete checklist based on our new classification (Table S1).

**Material and Methods**

**Taxon sampling**

We studied 188 species and four subspecies of the 641 described species and 11 described subspecies of Ochthebiini, plus 27 specimens corresponding to undetermined or still undescribed species (Table S3). For two species with an isolated or unsupported placement (*O. guerreroi* Jäch & Ribera and *O. peisonis* Ganglbauer, see below) we sequenced two specimens to test for possible sequencing mistakes. We included examples of eight of the ten genera currently recognised in the tribe, all subgenera but two (within genus *Tympanogaster*), and all recognised species groups within the genus *Ochthebius* but one (*O. kosiensis* group, Tables 1, S1, S3). The two missing genera, *Tympalopatrum* (Australia) and *Protochthebius* (Asia), have four and seven species respectively (Table S1).
We used as outgroups 31 species of other Hydraenidae genera (*Hydraena*, *Laeliaena* Sahlberg and *Limnebius*) and of Ptiliidae. Trees were rooted in the split between Hydraenidae and Ptiliidae, considered to be sister groups both based on molecular (e.g. Hunt et al., 2007; McKenna et al., 2015) and morphological evidence (Hansen, 1997; Lawrence et al., 2011).

**DNA extraction and sequencing**

Specimens were killed and preserved in absolute ethanol. DNA was extracted with a standard phenol-chloroform extraction or by commercial extraction kits (mostly Quiagen DNeasy Tissue Kit, Hildesheim, Germany) following the instructions of the manufacturers. DNA samples and voucher specimens are kept in the collections of the Institute of Evolutionary Biology (IBE, Barcelona), Museo Nacional de Ciencias Naturales (MNCN, Madrid) and Naturhistorisches Museum Wien (NMW, Vienna). We sequenced fragments of six genes in five sequencing reactions, three mitochondrial: (1) 5’ end of the cytochrome c oxidase subunit 1 (the standard barcode, Hebert et al., 2003) (COI-5’), (2) 3’ end of cytochrome c oxidase subunit 1 (COI-3’), (3) 5’ end of 16S RNA (16S) plus the Leucine tRNA transfer (tRNA-Leu) plus 5’ end of NADH dehydrogenase subunit I (NAD1); and two nuclear: (4) an internal fragment of the large ribosomal unit, 28S RNA (28S) and (5) an internal fragment of the small ribosomal unit, 18S RNA (18S) (see Table S4 for details on primers used and typical polymerase chain reaction (PCR) conditions). Sequences were assembled and edited with GENEIOUS v10.1 (Kearse et al., 2012); new sequences (a total of 897) were deposited in the ENA database with accession numbers XXX–XXX.

**Phylogenetic analyses**

Edited sequences were aligned using the online version of MAFFT v.7 with the G-INS-I algorithm (Katoh et al., 2009). We used PartitionFinder v1.1.1 (Lanfear et al., 2012) to estimate the evolutionary model that best fitted the data, using one partition for each gene fragment (six partitions in total), and using Akaike Information Criterion (AIC) scores as selection criteria. Phylogenetic analyses were made using Bayesian probabilities in BEAST 1.8 (Drummond & Rambaut, 2007), using the partition and evolutionary models selected by PartitionFinder, with a Yule speciation process as tree...
prior. To obtain a general calibration of the tree we dated the root (i.e. the split between
Hydraenidae and Ptiliidae) with a normal distribution with an average of 170 Ma and a
standard deviation of 1 Ma following recent estimates based on a range of fossils (Hunt
et al., 2007; McKenna et al., 2015), and used an uncorrelated lognormal relaxed clock.
We ran 100 million generations login results each 5,000, and checked convergence to
estimate the burn-in fraction with Tracer v1.6 (Drummond & Rambaut, 2007). We ran
an additional ML phylogenetic reconstruction with RAxML-HPC2 (Stamatakis, 2006)
in the CIPRES portal (Miller et al., 2010), using the same partition scheme as in
BEAST with a GTR+G model estimated independently for each partition. Node
supports values were estimated with 100 pseudoreplicas using a rapid bootstrapping
algorithm (Stamatakis et al., 2008). The same ML analysis was repeated only with the
nuclear sequence (18S and 28S).

Results

The final matrix included 252 terminals with 3,656 aligned characters. Protein
coding regions had no indels except for the 3' end of COI-3, where some species had an
additional codon. The best partitioning scheme obtained with PartitionFinder had six
partitions corresponding to (1) COI-5, (2) COI-3, (3) 16S+tRNA-Leu, (4) NAD1, (5)
18S and (6) 28S. The optimal evolutionary model was GTR+I+G for all partitions
except for NAD1 (best model TMV) and 28S (best model SYM). The BEAST run
implementing the best models did, however, not converge properly, mostly due to the
parameters related to the estimation of the branch lengths, especially for the genes
NAD1, 18S and 28S. We thus did a second run with simpler models for these genes
(HKY+G+I), which converged adequately. The topologies of the two Bayesian analyses
were, however, almost identical (Figs 1, S1), and unless specified we report only the
results of the analyses with the better parameter convergence (i.e. with the simpler
evolutionary models).

Molecular phylogeny

The topologies obtained in the ML and the two Bayesian analyses were very
similar, differing only in some poorly supported nodes (Figs 1, S1, S2), most notably in
the position of Hughleechia (see below). The ML tree with the nuclear sequence only
had a topology very similar to that obtained with the combined data, although with a
generally lower resolution and support. Main difference was the recovery of
Ochthebiini as paraphyletic, with the genus *Hydraena* as sister to *Tympanogaster* plus
*Meropatus*, although with very low support (bootstrap support, BS= 53%; Fig. S3).
Genera, subgenera and most species groups were, however, recovered as monophyletic
with strong support, with internal topologies very similar to that of the combined ML
tree (Figs S2, S3).

In the ML and Bayesian trees with the combined nuclear and mitochondrial data
the monophyly of Ochthebiini was strongly supported, as well as their separation in two
clades, (1) *Meropatus* plus *Tympanogaster* and (2) *Ochthebius* sensu lato (s.l. herein).
*Meropatus* was nested within a paraphyletic *Tympanogaster* in the ML tree (combined
and nuclear only) and in the Bayesian tree with the best models, and sister to
*Tympanogaster* with low support in the Bayesian tree with simpler models (posterior
probability, pp= 0.63; Figs 1a, S1, S2).

Within *Ochthebius* s.l. *Asiobates* and *Aulacochthebius* were sister groups in the
Bayesian tree with low support (pp= 0.85), and both sisters to the rest of Ochthebiini. In
the ML analysis *Asiobates* and *Aulacochthebius* were paraphyletic with respect to the
rest of Ochthebiini, also with low support (BS< 50%) (Figs 1a, S2). In both analyses
Ochthebiini minus *Asiobates* and *Aulacochthebius* were monophyletic with strong
support (BS= 80%; pp= 1; Fig. 1a).

The remaining Ochthebiini were divided in a series of well supported clades
corresponding to traditionally recognised genera or subgenera, but with poorly resolved
relationships among them. (1) *Enicocerus*, strongly supported and with well resolved
internal relationships, sister to the Australian *Hughlechia* in ML and the Bayesian
analysis with the simpler models (BS = 81%, pp= 0.76; Figs 1b, S2). In the Bayesian
analysis with the best models *Hughlechia* was sister to the clade formed by
*Micragasma* and *Cobalius*, with low support (pp= 0.88; Fig. S1). (2) A clade including
*Gymnochthebius* and *Gymnanthelius*, the latter as sister to *Angiochthebius* Jäch &
Ribera (*Gymnochthebius plesiotypus* group of Perkins, 1980; see Jäch & Ribera, 2018)
(BS= 94%, pp= 0.96). Within *Gymnochthebius*, the Australian and American species
were respectively monophyletic and sisters, with very strong support both in the ML
and Bayesian trees (Figs 1b, S2). (3) *Cobalius*, with a strongly supported monophyly
(BS= 98%, pp= 1) and sister to the only sequenced species of *Micragasma*, also with
strong support (BS= 100%, pp= 1) (Figs 1b, S2). (4) *Ochthebius* sensu stricto (s.str.
herein), including *Calobius*, strongly supported both in the ML (BS= 94%) and Bayesian (pp= 1) trees (Figs 1b, S2).

Within *Ochthebius* s.str. most established Palaeartic species groups were recovered as monophyletic (see Discussion, Figs 1b, 1c, S2). Their monophyly was strongly supported in the ML and Bayesian trees, with the only exception of the group of species related to the *O. atriceps* and *O. notabilis* groups in the ML analyses (see below). The main difference with established groups was the expansion of the *O. marinus* group to include the south African *O. capicola* and the American *O. biincisus, bisinuatus* and *interruptus* groups of Perkins (1980). The *O. foveolatus* group of Jäck (1991) was split in three clades: (1) *O. foveolatus* group, sister to the *O. metallescens* group with strong support in both ML and Bayesian trees (BS= 71%, pp= 0.99); (2) *O. atriceps* group and (3) *O. corrugatus* group. The latter two formed a clade with the species of the *O. notabilis* and *O. andraei* groups, strongly supported in the Bayesian tree (pp= 1) but not in the ML tree (BS< 50%), in which the group also included one of the two sampled species of the *O. rivalis* group (Figs 1c, S2). Two coastal lineages, *Calobius* and the *O. vandykei* group (formerly genus *Neochthebius*), were nested within *Ochthebius* s.str., the former as sister to the *O. lobicollis* + *O. strigosus* groups (BS< 50%, pp= 0.92) and the latter as sister to the *O. marinus* group (BS< 50%, pp= 0.97) (Figs 1b, 1c, S2).

According to our calibration scheme, with a separation between Hydraenidae and Ptiliidae at 170 Ma, the estimated age of crown Hydraenidae was 106 Ma (HPD 122.8–90.2 Ma), and that of the crown Ochthebiini 93 Ma (HPD 109.7–80.8 Ma). The basal diversification of Ochthebiini was reconstructed as having occurred in a relatively short temporal window, with genera, subgenera and most species groups with an origin between ca. 87–60 Ma (Fig. 1; see Table 2 for the estimated evolutionary rates of all partitions).

**Discussion**

Our results strongly support the monophyly of Ochthebiini, but our sampling did not allow to test for the monophyly of Ochthebiinae, or its position within Hydraenidae. Within Ochthebiini our results recover two well supported clades, which we consider here as genera: *Meropathus* and *Ochthebius* (Fig. 2; see below for a detailed discussion.
of the taxonomic classification of Ochthebiini). We did not find evidence to support the five subtribes proposed by Perkins (1997), which are therefore not considered here.

We did not find evidence for a clear separation between the studied species of *Tympanogaster* and *Meropathus*, in agreement with previous studies (Hansen, 1991). We could not obtain material of the genus *Tympallopatrum*, considered to be closely related to *Tympanogaster* by Perkins (1997), and two of the subgenera of *Tympanogaster* (*Plesiotympanogaster* Perkins and *Topotympanogaster* Perkins), so until more data become available we consider *Tympanogaster* s.str., the other subgenera of *Tympanogaster*, and *Tympallopatrum* as subgenera of *Meropathus* (Tables 1, S1; Fig. 2) (see Perkins, 2006 for a discussion on the subgeneric classification of *Tympanogaster*). The species of *Meropathus* are found in the Australian Region, on two Antarctic islands (Kerguelen and Heard) and on several Subantarctic islands, such as Campbell Island, Crozet Islands, Prince Edward Island and Falkland Islands.

The second lineage, genus *Ochthebius* s.l., included all the non-Australian Ochthebiini, as well as several Australian species. Our results agree remarkably well with the currently recognised subgenera and many of the established species groups, which were recovered as monophyletic with general strong support (Fig. 2). The relationships between these lineages, however, do not confirm some previous hypotheses on their relationships. Thus, *Aulacochthebius* was not found to be closely related to *Gymnochthebius*, as proposed in Hansen (1991), but to *Asiobates*; *Micragasma* and *Hughleechia* were not among the basal lineages and *Cobalius* and *Calobius* were not closely related, as hypothesised in Perkins (1997). Novel relationships found here are the possible sister relationship between *Hughleechia* and *Enicocerus*, and the close relationship between *Gymnochthebius* and *Gymnanthelius*. Interestingly, within the clade *Gymnochthebius + Gymnanthelius + Angiochthebius* there are two cladogenetic events separating American from Australian species: one within *Gymnochthebius*, dated at 73 Ma (95% HPD 87–60 Ma), and another separating the Australian *Gymnanthelius* and the Chilean *Angiochthebius*, dated at 60 Ma (95% HPD 78–51 Ma). Although a detailed biogeographic analysis is outside the scope of this paper, it is interesting to note that these estimations are too recent for a tectonic split between Australia and South America (i.e. west and east Gondwana), dated at ca. 130 Ma (McIntyre et al., 2017). Our calibration would thus require a different scenario, probably through the colonization of some southern islands or the Antarctica. An older age for these nodes is unlikely, given that our rate estimations are already slower than
most recent estimations for the same genes in other groups of Coleoptera (Table 2; see e.g. Papadopoulou et al., 2010; Andújar et al., 2012; Cieslak et al., 2014).

**Taxonomic classification of Ochthebiini** Thomson, 1859

1. Genus Meropatus Enderlein, 1910

   Type species: Meropatus chuni Enderlein, 1910, by monotypy.

   Meropatus was described as genus, considered as subgenus of Ochthebias by Orchymont (1938) and reinstated again as genus by Jeannel (1940). Bameul (1989) redescribed the genus and recognised 12 species (in two species groups), transferring *O. schizolabrus* Deane to Meropatus. Hansen (1991) noted the difficulty in establishing clear distinctions within the Meropatus-Tympanogaster complex. Perkins (1997) erected the subtribe Meropathina for the genera Meropatus, Tympanogaster and the newly described *Tympallopatrum*, the latter two including the Australian and Tasmanian species. Subsequently, Perkins (2004a, 2006) revised *Tympallopatrum* and *Tympanogaster* respectively, and described three new subgenera and 76 new species in the latter. As defined here, *Meropatus* includes 96 species in six subgenera (Fig. 2; Table S1).


   Type species: Tympanogaster maureenae Perkins, 2006, by original designation.

   Hygrotympanogaster Perkins was described by Perkins (2006) as a subgenus of *Tympanogaster*, to include mostly hygropetric species in south-western Australia. Currently it includes 36 species (Perkins, 2006) (Table S1).

   1.2. Subgenus Meropatus Enderlein, 1910

   Type species: Meropatus chuni Enderlein, 1910, by monotypy.

   *Meropatus* s.str. includes the species of the former genus *Meropatus* that were not transferred to *Tympanogaster* by Perkins (1997, 2006), i.e. all seven New Zealand, Antarctic and Subantarctic species plus *M. labratus* Deane from Queensland (Table S1).

   1.3. Subgenus Plesiotympanogaster Perkins, 2006 comb.n.

   Type species: Tympanogaster thayerae Perkins, 2006, by original designation.
Plesiotympanogaster was described by Perkins (2006) as a subgenus of Tympanogaster to include the type species plus Ochthebius costatus Deane (Table S1). Both species were considered to have plesiomorphic characters within the genus.

1.4. Subgenus Topotympanogaster Perkins, 2006 comb.n.
Type species: Tympanogaster crista Perkins, 2006, by original designation. Topotympanogaster was described by Perkins (2006) as a subgenus of Tympanogaster to include eight Australian species, all described in Perkins (2006) (Table S1).

1.5. Subgenus Tympallopatrum Perkins, 1997 comb.n.
Type species: Tympallopatrum longitudum Perkins, 1997, by original designation. Tympallopatrum was described by Perkins (1997) as a monotypic genus within Meropathina. Subsequently, Perkins (2004a) revised the genus and described three additional species, all of them from western Australia (Table S1). We could not obtain any representative of this and the two previous subgenera for our study, and thus their phylogenetic placement within Meropathus remains untested.

Type species: Tympanogaster deanei Perkins, 1979 (replacement name for Ochthebius longipes Deane, 1931), by monotypy. Described by Janssens (1967) as a monotypic genus for O. longipes Deane (= T. deanei Perkins), Perkins (1997) redescribed Tympanogaster and transferred some species from Meropathus. Perkins (2006) revised the genus and described four subgenera (see Meropathus above) and raised the total number of the species in the here defined subgenus Tympanogaster to 38 (Table S1), all distributed in Australia and Tasmania.

2. Genus Ochthebius Leach, 1815
Type species: Helophorus marinus Paykull, 1798, fixed by Orchymont (1942). The second well supported lineage within Ochthebiini includes the remaining genera/subgenera with in some cases uncertain relationships among them. We consider Ochthebius a single genus with 540 species and nine subspecies in ten well supported subgenera, corresponding in most cases to currently recognised taxa (Fig. 2). One additional species, O. eremita Knisch from Fiji, cannot be confidently placed in any of
the described subgenera, and it is left as incertae sedis within the genus *Ochthebius* (Hansen, 1998; Table S1).

2.1. Subgenus *Angiochthebius* Jäch & Ribera, 2018

Type species: *Ochthebius guerreroi* Jäch & Ribera, 2018, by original designation.

The subgenus *Angiochthebius* was created for the *Gymnochthebius plesiotypus* species group (sensu Perkins, 1980), which now includes four South American species (Jäch & Ribera, 2018; Table S1). The species of the *G. plesiotypus* group were included within *Gymnochthebius* by Perkins (1980) as they share a bifid apex of the aedeagus, but external characters (e.g. the pubescent 5th abdominal ventrite) and some aedeagal characters (Jäch & Ribera, 2018) as well as molecular data (Figs 1b, S2) warrant their removal from *Gymnochthebius* and their status as a distinct subgenus of *Ochthebius*.

2.2. Subgenus *Asiobates* Thomson, 1859

Type species: *Ochthebius rufimarginatus* Stephens, 1829 (= *O. bicolon* Germar, 1824), by monotypy.

Originally described as a genus, but downgraded to subgenus by Seidlitz (1875), and treated as such by most authors (e.g. Jäch, 1990a; Hansen, 1991; Perkins, 1997). Jäch (1990a) divided the Palaearctic species in the *bicolon* and *minimus* groups, which were recovered as respectively monophyletic with strong support. The sampled American species were divided in the *puncticollis* group of Perkins (1980), with only one sampled species being sister to the rest of the subgenus with strong support (BS= 88%, pp= 1; Figs 1a, S2), plus the *discretus* group of Perkins (1980). The placement of the studied Nearctic species of the *A. discretus* group and two of the Afrotropical species (*O. andreii* Régimbart and *O. andronius* Orchymont) was uncertain both in the ML and the Bayesian trees (Figs 1a, S2). We provisionally consider them within the *A. minimus* group due to the similarities in their aedeagi and the morphology of the pronotum (Orchymont, 1948; Perkins, 1980; Jäch, 1990a). The subgenus *Asiobates* currently includes 105 described species and three subspecies occurring in all biogeographical regions, except the Oriental and Australian Realms. While the *puncticollis* group is restricted to the Nearctic Region, the *bicolon* and *minimus* groups are more widespread. The former occurs in the Palaearctic and (with several undescribed species) Afrotropical Regions, and the latter occurs in the Nearctic, Neotropical, Palaearctic, and Afrotropical
Regions. Many additional species of *Asiobates* await description, several of them included in our phylogeny (Table S3).

2.3. Subgenus *Aulacochthebius* Kuwert, 1887.

Type species: *Ochthebius exaratus* Mulsant, 1844, by monotypy.

Considered as a subgenus until Perkins (1997) raised it to genus level. There are no species groups defined within this subgenus, and our sampling is too incomplete to draw firm conclusions. Currently the subgenus includes 13 Palaearctic, Oriental and Afrotropical species (Table S1), although the taxonomy of the subgenus is in clear need of revision and it is expected that the number of species will increase considerably (Table S3).

2.4. Subgenus *Cobalius* Rey, 1886.

Type species: *Ochthebius lejolisii* Mulsant & Rey, 1861, fixed by Jäch (1989b).

Described as a subgenus of *Ochthebius* by Rey (1886), synonymised by Perkins (1997) with *Ochthebius* s.str. and considered again as subgenus by Sabatelli *et al.* (2016). We recovered it here as a strongly supported monophyletic lineage clearly outside *Ochthebius* s.str., confirming its status as subgenus. Its nine recognised species and two subspecies occur along the coasts of the Mediterranean Sea, the Black Sea and the eastern Atlantic Ocean from Cape Verde to Scotland (Jäch, 1989b; Jäch & Skale, 2015; Jäch & Delgado, 2017a). Its taxonomy is in need of revision (Sabatelli *et al*., 2016; Jäch & Delgado, 2017a; unpublished results).

2.5. Subgenus *Enicocerus* Stephens, 1829.

Type species: *Enicocerus viridiaenbius* Stephens, 1829 (= *Ochthebius exsculptus* Germar, 1824), by monotypy.

*Enicocerus* was originally described as a genus, downgraded to subgenus of *Ochthebius* by Chenu (1851), reinstated again as genus by Perkins (1997) (within its own subtribe, Enicocerina), but treated as a subgenus by subsequent authors (e.g. Jäch, 1998; Jäch & Skale, 2015; Ribera *et al*., 2010). Our results support the exclusion of the East Palaearctic and Oriental species, confirming Jäch (1998) and Skale & Jäch (2009), and are in agreement with the phylogeny of Ribera *et al.* (2010). *Enicocerus* in its current sense includes 16 species with a mostly Mediterranean distribution, with some species reaching the British Isles, Central Europe, the Middle East and the Caucasus. One
species from eastern North America (O. benefossus LeConte, Perkins, 1980), not
included in our phylogeny, possibly belongs to Enicocerus as well.

Type species: Ochthebius hieroglyphicus Deane, 1933, by original designation.
The genus Gymnanthelius was introduced by Perkins (1997) for O. hieroglyphicus.
Subsequently, Perkins (2004b) revised the genus and transferred to Gymnanthelius two
other Australian species described by Deane (1931, 1937) within Ochthebius (Table
S1). The eight described species are distributed mostly in south-eastern Australia, with
some reaching as far north as Queensland (Perkins, 2004b).

2.7. Subgenus Gymnochthebius Orchymont, 1943
Type species: Ochthebius nitidus LeConte, 1850 by original designation.
Gymnochthebius was originally described as a subgenus of Ochthebius (Orchymont,
1943) to place several American species described under Ochthebius that could not be
placed in any of the described subgenera, which had been established mostly for
Palaearctic species. Orchymont (1943) also included three Australian species for which
he could examine the aedeagus and confirmed that they had the same general structure
as the American species. Perkins (1980) revised the American species and Perkins
(2005) the Australian and Papuan species, recognising another four species in addition
to the three previously noted by Orchymont (1943) and raising the total number of
species in the subgenus to 57 (Table S1). The Australian and the American species of
the subgenus form two well supported clades, the O. australis and O. fossatus groups,
with 36 and 21 species respectively (Table S1).

Type species: Hughleechia giulianii Perkins, 1981, by original designation.
Originally described as a monotypic genus (Perkins, 1981), a second species was
described by Perkins (2007a). Both species inhabit coastal rockpools in southern
Australia and Tasmania, in the intertidal zone and (most frequently) above the tide
(Perkins, 2007a).

2.9. Subgenus Micragasma Sahlberg, 1900 comb.n.
Type species: Micragasma paradoxum Sahlberg, 1900, by monotypy.
Described as a monotypic genus for *M. paradoxum* (Sahlberg, 1900). Jäch (1997a) redescribed the genus and transferred *O. substrigosus* Reitter to *Micragasma*. A third species was recently described from Crete (Hernando *et al.*, 2018), and there are two additional undescribed species from Central Asia (unpublished data). Our results clearly show that *Micragasma* is nested within *Ochthebius* s.l., and thus we consider it a subgenus of *Ochthebius*.

2.10. Subgenus *Ochthebius* Leach, 1815

Type species: *Helophorus marinus* Paykull, 1798, fixed by Orchymont (1942).

Within *Ochthebius* s.str. we recovered, with strong support, most of the currently recognised species groups as monophyletic. Most species groups are entirely Palaearctic, or with mostly Palaearctic species, and thus the basis for the taxonomy of the subgenus is the revisionary work of Jäch (e.g. 1989a, 1990a, 1991, 1992a), who distinguished 13 groups and subgroups. With only one exception (*O. jengi* group), they were all, with some modifications, recovered as monophyletic. According to our results, the 322 described species and four subspecies of *Ochthebius* s.str. are separated in 17 species groups, five of them newly established herein (Fig. 2). A few species still have an uncertain phylogenetic placement. This is particularly the case for *O. belucistanicus* Ferro, *O. fissicollis* Janssens and *O. pierottii* Ferro, which presently cannot be confidently included in any of the recognised species groups, mainly because their original descriptions lack information about relevant characters (Table S1).

(1) *O. andraei* group: Defined and revised in Jäch (1992a), with additional species described in Jäch (2002) and Jäch & Delgado (2010). Currently this group includes six species and one subspecies of Palaearctic distribution (Table S1), typical of saline or hypersaline habitats. We could study a single species (*O. patergazellae* Jäch & Delgado, Table S3), included in a clade together with the species of the *O. notabilis, corrugatus* and *atriceps* groups (Fig. 1). The close relationship between the species of the *O. andraei, corrugatus, notabilis* and *atriceps* groups were already suggested in Jäch (1991, 1992a).

(2) *O. atriceps* group: In Jäch (1991) the species of the *O. foveolatus* group were divided in two subgroups, (A) *foveolatus* subgroup, sharing some characters with the species of the *O. metallescens* group, and (B) *atriceps* subgroup, sharing some
characters with the species of the *O. notabilis* group. We recovered both subgroups as respectively monophyletic, and confirm the suspected relationships proposed by Jäch (1991) (see below). *Ochthebius burjkhalifa* Jäch & Delgado and *O. despoliatus* Jäch & Delgado, both from the UAE and of uncertain affinities although hypothesized to be related to the *O. atriceps* group (Jäch & Delgado, 2014a), were found to be sister to the rest of the species of the group, with strong support in the Bayesian analysis (pp= 0.95) but weaker in the ML (BS= 55%) (Figs 1, S2). With the inclusion of these two species the *O. atriceps* group includes 21 species and one subspecies (Table S1). They have a mostly Palaearctic distribution but extending to East Africa (Djibouti) (Jäch & Delgado, 2017b).

(3) *O. corrugatus* group: Jäch (1992a) suggested that *O. corrugatus* Rosenhauer, despite being related to the species of the *O. andraei* and *notabilis* groups, could not be included in either of them. Our results confirm this hypothesis, but extend the *O. corrugatus* group to include two additional Mediterranean species previously included in the *O. atriceps* subgroup (Jäch, 1991; Table S1).

(4) *O. foveolatus* group: The *O. foveolatus* group as here defined corresponds to the *O. foveolatus* subgroup of Jäch (1991), recovered as sister of the *O. metallescens* group with strong support (Fig. 1). After the additions and corrections of Delgado & Jäch (2009) and Jäch & Delgado (2010, 2014b) it currently includes 27 species, all Palaearctic (Fig. 1; Table S1).

(5) *O. kosiensis* group: Jäch (1997b) established this group for *O. kosiensis* Champion, described within *Ochthebius* but originally not placed in any subgenus (Champion, 1920). Knisch (1924) placed it in *Asiobates* due to the resemblance of the general habitus, although the male genitalia do not correspond to those of the species of *Asiobates* (Jäch, 1997b). Jäch (2003) recognized the similarity between *O. kosiensis*, *O. strigosus* and related species, and included both species in the *strigosus* subgroup of the *metallescens* group. The study of some undescribed species deposited in the NMW (M.A. Jäch, manuscript in prep.) suggests that the *strigosus* subgroup as defined in Jäch (2003) should be divided in the *kosiensis* and *strigosus* groups, with two and 16 described species respectively (see below; Table S1). We could not obtain any species of the *kosiensis* group suitable for DNA extraction, and thus their phylogenetic
relationships (and composition) remain untested. Based on described and undescribed species the group is so far known from the Himalaya and Myanmar.

(6) *O. lobicollis* group: Jäch (1990b) revised the *lobicollis* group, with subsequent additions by Jäch (1994) and Jäch et al. (1998). It currently includes 11 species with a West Palaearctic distribution (Table S1; Fig. 1b).

(7) *O. marinus* group: The Palaearctic species of the *O. marinus* group, the most speciose within *Ochthebius* s.str., were revised by Jäch (1992b). According to our results it includes the species of the *O. jengi* group sensu Jäch (1998) and also species from the Nearctic and Neotropical Regions (*O. biincisus*, *bisinuatus* and *interruptus* groups of Perkins, 1980); the Afrotropical Region (*O. extremus* and *salinarius* groups of Perkins & Balfour-Browne, 1994; Perkins, 2011; *O. capicola* group of Sabatelli et al., 2016), including Madagascar (*O. alluaudi* Régimbart; Perkins, 2017); the Oriental Region (*O. masatakasatoi* Jäch; Jäch, 1992b; Jäch & Delgado, 2017a); and the Australian Region (*O. queenslandicus* Hansen; Jäch, 2001a; Perkins, 2007b).

Two of the studied subspecies were not recovered as sisters to the nominal subspecies in any of the analyses: *O. viridis fallaciosus* Ganglbauer (sister to *O. arefniae* Jäch & Delgado and another specimen likely representing an undescribed species), and *O. subpictus deletus* Rey (sister to *O. marinus* plus *O. auropallens Fairmaire*), in both cases with strong support (Figs 1, S1, S2; Table S1). We thus upgrade the two subspecies to species, *O. fallaciosus* Ganglbauer, 1901 stat.n. and *O. deletus* Rey, 1885 stat.rest. (see Jäch, 1992b and Jäch & Delgado, 2008 for a detailed description of the species). The *O. marinus* group as here defined includes 78 species, plus two species of uncertain adscription (Table S1). Most of these species seem to be associated to lentic habitats, frequently saline and, especially those outside the Palaearctic, coastal.

(8) *O. metallescens* group: The *O. metallescens* group was revised by Jäch (1989a). It is well defined morphologically, but many species have variable isolated populations, difficulting species recognition and diagnosis. This difficulty is reflected in the complex taxonomic history of the group, with multiple changes in the status of some species (e.g. Jäch, 1989a, 1999, 2001b). A number of species are typical of hygropetetic habitats covered by a thin film of water, such as seepages or the marginal areas of stony surfaces.
in streams. The species group has currently 56 Palaearctic species and one subspecies (Table S1). Due to the somewhat cryptic habits and restricted geographic ranges of many species it is expected that this number will increase considerably.

(9) *O. nitidipennis* group: We include in the *O. nitidipennis* group the Asian species formerly included in the subgenus *Enicocerus* (see above). As suggested by previous authors (Jäch, 1989b; Skale & Jäch, 2009; Yoshitomi & Satô, 2011), morphological similarities between these species and those of *Enicocerus* are the result of evolutionary convergence, likely due to occupying similar microhabitats on the surface of rocks and stones partially submerged in streams. The group currently includes 12 species in the Himalaya Region and East Asia (Table S1).

(10) *O. notabilis* group: Jäch (1992a) recognised the *O. notabilis* group for species formerly included in the subgenus *Doryochthebius* Kuwert, establishing its synonymy with *Ochthebius* s.str. and differentiating the members of this group from the species of *Calobius* (see below). The group includes 13 Palaearctic species, all typical of saline or hypersaline habitats.

(11) *O. peisonis* group: *Ochthebius peisonis* was included in the *O. marinus* group by Jäch (1992b). Our results, however, place the species in a very isolated and uncertain position within *Ochthebius* s.str. We provisionally consider it in its own group, until additional evidence clarifies its phylogenetic relationships.

(12) *O. punctatus* group: The *punctatus* group was defined by Jäch (1992c) to include the species formerly considered under subgenus *Bothochius* Rey, with irregular elytral punctation (Jäch, 1989c), and a series of species with similar morphological characters but with regular elytral striae. The *Ochthebius punctatus* group includes 53 species and one subspecies, mostly Palaearctic (reaching the Oriental region) but with some Afrotropical species, among them the *namibiensis* group of Perkins & Balfour-Browne (1994) (Jäch, 1992c; Hansen, 1998; Perkins, 2011; Jäch & Delgado, 2017b; Table S1).

(13) *O. quadricollis* group: The *O. quadricollis* group corresponds to the genus *Calobius*, described for *C. heeri* Wollaston from Madeira. The concept of *Calobius* was expanded by subsequent authors to include species now in different species groups (e.g.
Ritter (1886) included among them *O. notabilis* Rosenhauer and *O. quadrifoveolatus* Wollaston, and was usually treated as a subgenus. It was revised by Jäch (1993), who still considered it a subgenus, but was synonymised with *Ochthebius* s.str. by Perkins (1997), who considered it to be closely related to *Cobalius*. Its status remained, however, uncertain, with some authors treating it as a genus (e.g. Audisio et al., 2010) or subgenus (e.g. Jäch & Skale, 2015). Finally, Sabatelli et al. (2016) provided evidence of the phylogenetic position of *Calobius*, demonstrating its derived status within *Ochthebius* s.str. and considering it as the “*Calobius*” lineage, named here the *O. quadricollis* group for consistency with other species groups within *Ochthebius* s.str. Sabatelli et al. (2016) also found that the group includes more than the five species currently recognised (Table S1), in agreement with previous results from the Italian species (e.g. Urbanelli et al., 2008; Audisio et al., 2010). Our results support this impression, as happens with the subgenus *Cobalius*, which is also in need of taxonomic revision. All species of the *O. quadricollis* group are found in coastal rockpools in the Mediterranean basin and the islands of Madeira and the Canaries.

(14) *O. rivalis* group: *Ochthebius rivalis* Champion and two similar species were originally considered to be a subgroup of the *O. metallescens* group (Jäch, 2003). Our results, however, do not support a close relationship with the species of the *O. metallescens* group, but with *O. peisonis* and the *O. notabilis, corrugatus* and *andraei* groups (Figs 1, S2), with low support. In the Bayesian analysis the two sampled species of the group were sister with strong support (pp= 1), but in the ML analysis they were not placed together, although with low support (BS< 50%) (Fig. S2). Currently the group includes four Asian species (including *O. himalayae* Jách, originally described within the *O. metallescens* group, Jách, 1989a), distributed from the Himalaya to Hainan Island (Table S1).

(15) *Ochthebius strigosus* group: *Ochthebius strigosus* Champion, described as *Ochthebius* s.str., was included in the subgenus *Asiobates* by Jách (1989b) based on the study of female specimens only. After the discovery of males of a related species (*O. strigoides* Jách) they were placed in their own subgroup within the *O. metallescens* group (Jách, 1998). We found the only sequenced species of the group to be sister of the *O. lobicollis* group with low support (BS= 56%, pp= 0.87), and we consider it here as a distinct species group within *Ochthebius* s.str. The *O. strigosus* group currently includes
16 described plus some undescribed species, one of them included here (voucher IBE-RA617). The group is distributed in the eastern Palaearctic, including Taiwan (Jäch, 2003; Table S1).

(16) *Ochthebius sumatrensis* group: In the original description, *O. sumatrensis* Jäch could not be placed in any of the by then described groups, although some similarities with *O. jengi* Jäch (currently in the *marinus* group) were noted (Jäch, 2001a). Several undescribed species similar to *O. sumatrensis* have been collected in recent years (Jäch *et al*. manuscript in prep.), among them the one from Hong Kong included here (specimen voucher MNCN-AC16; Table S1), recovered as sister to the rest of the species of *Ochthebius* s.str. with low support in the ML analysis (BS< 50%; Fig. S2) and as sister to the *punctatus* group in the Bayesian analysis, also with low support (pp< 0.5; Fig. 1). They live in hygropetric surfaces, which makes them prone to evolutionary convergence with non-related species sharing the same habitat, thus obscuring their relationships. The group is distributed from the Himalaya to eastern China and southward to Sumatra, where it is the only known species of *Ochthebius* s.str.

(17) *O. vandykei* group: The species of the *O. vandykei* group correspond to the former *Neochthebius*, originally described as subgenus but raised to genus (within its own subtribe, Neochthebiina) by Perkins (1997) based on peculiarities of their antennae and the lack of ESDS. Jäch & Delgado (2014b), based on unpublished molecular data and on aedeagal characters of several newly described species, synonymised *Neochthebius* and considered it as a species group within *Ochthebius* s.str. The group currently includes eight species from the northern Pacific coast, seven in Asia and one in North America (Jäch & Delgado, 2014b; Table S1). They are all typical of rocky seashores or other coastal microhabitats.

3. **Genus Protochthebius** Perkins, 1997

Type species: *Protochthebius satoi* Perkins, 1997, by original designation.

The genus *Protochthebius* was described by Perkins (1997) for *P. satoi* and *O. jagthanae* Champion, who erected also the subtribe Protochthebiina based on peculiarities of the antennae and the ESDS. Subsequently, Jäch (1997b) and Perkins (1998) described another two and three species respectively. All known seven species of *Protochthebius* are found in the Himalaya Region, Meghalaya and Laos (Table S1).
Some of them have been found by sifting forest litter or moss (Jäch, 1997b; Perkins, 1998). We could not obtain molecular data of any of the species of Protochthebius, and thus the phylogenetic placement of the genus remains uncertain. Perkins (1997) noted some presumably plesiomorphic characters of the pronotum and post-ocular area of the head. The species of the genus have also a reduced ESDS system (Perkins, 1997), but this might be as a secondary loss due to their microhabitat preferences. Their male genitalia are, however, typical of Ochthebius s.str., and when molecular data become available the taxonomic status of Protochthebius may have to be changed to a subgenus of Ochthebius or a species group within Ochthebius s.str., in which case P. satoi would become a junior homonym.

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**Figure legends**

Fig. 1: Majority rule consensus tree obtained with BEAST for the phylogeny of Ochthebiini, with the simple evolutionary models (see text). Numbers in nodes, posterior probabilities / bootstrap support values in RAxML. Names in nodes refer to the new classification. Habitus photographs correspond to species used in the analyses, with the addition of *Limnebius papposus* Mulsant, *Hydraena riparia* Kugelann (Fig. 1a), *Ochthebius* (s.str.) *bernhardi* Jäch & Delgado and *O. (Micrasagma) minoicus* Hernando, Villastrigo & Ribera (Fig. 1b).

Fig. 2: Summary tree of the phylogenetic relationships of the Ochthebiini main lineages. The width of the triangles reflects the number of species of the respective clade in the tree. Symbols in nodes: Circles, congruent topology in the maximum likelihood and Bayesian analyses; triangles: incongruent topologies; in black: nodes with good support (Bayesian posterior probability > 0.95 and maximum likelihood bootstrap support > 70%) in both analyses; in grey: in one analyses only; in white: not supported nodes. Pie charts reflect the geographical distribution of the species of the respective clades.
Supporting Information

Table S1: Table S1: Checklist of the species of Ochthebiini, with the current (following Jäch & Skale, 2015 and Jäch et al., 2016) and new classifications. In bold, type species. Phyl, species included in the phylogeny (in brackets, species for which the sequenced specimen was a female). Distribution: PAL, Palaearctic; AFR, Afrotropical; AUR, Australian; NAR, Nearctic; NTR, Neotropical; ORR, Oriental; ANR, Antarctic. In brackets, specimens considered to have a Palaearctic distribution in Jäch & Skale (2015), but including the Oriental or Afrotropical Regions in Hansen (1998).

Table S2: Current classification of Ochthebiini, with synonymies and type species (following Jäch & Skale, 2015 and Jäch et al., 2016). In bold, taxa included in the phylogeny.

Table S3: List of material used in the molecular phylogeny, including voucher numbers, accession numbers of the sequences and locality data.

Table S4: (A) primers used for DNA amplification and sequencing reactions; (B) Typical conditions for the polymerase chain reaction.

Fig. S1: Majority rule consensus tree obtained with BEAST for the phylogeny of Ochthebiini with the best partition models. Numbers in nodes, posterior probabilities.

Fig. S2: Phylogeny obtained with RAxML, including current Ochthebiini classification. Numbers in nodes, bootstrap support values.

Fig. S3: Phylogeny obtained with RAxML with the nuclear genes only. Numbers in nodes, bootstrap support values.
Asiobates montanus AN207
Limnebius millani AI920
Laeliaena sahlbergi HI19
Asiobates crenulatus AH159
Aulacochthebius narentinus AF161
Asiobates jaimei RA1081
Asiobates flavipes RA437
Hydraena croatica RA52
Rioneta uluguruensis RA1081
Acratichia sp. AI413
Ptiliolum sp. AI649
Ptenidium pusillum AI515