1 **A molecular phylogenetic study of pheretimoid species**
2 **(Megascolecidae) in Mindanao and associated islands, Philippines**

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14 **ABSTRACT**

15 The knowledge on earthworm taxonomic studies in the Philippines over the past decade
16 dramatically increased and the results indicate an astonishingly high diversity and high local
17 endemicity of earthworms in the country. The earthworm fauna of the Philippines is composed
18 of pheretimoid species of the family Megascolecidae. A molecular phylogenetic study was done
19 in attempt to infer phylogenetic relationships among the pheretimoid species in Mindanao and
20 associated islands. Gene markers used include the mitochondrial cytochrome *c* oxidase subunit I
21 (COI) and 16S rRNA, and the nuclear 28S rRNA and protein-coding histone H3 genes. Despite
22 having limited taxa and limited genes included in the analyses, the combined data set generated a

phylogeny more or less consistent with morphology-based expectation. Sims and Easton's classification scheme published in 1972 does not reflect the phylogeny with respect to the genus *Amyntas* and the subgenus *Parapheretima*; the characters they used to define these taxa are homoplasious. The species groupings in *Pheretima* based on the location of spermathecae are partially reflected the pheretimoid phylogeny. Particularly, members of the *P. sangirensis* group formed a clade supported with 0.99 posterior probability with the inclusion of *P. (Parapheretima) pandanensis* (also with spermathecal pores at 7/8), the monothecate *Pheretima vergrandis* with spermathecal pore at 7/8, and an athecate conspecific of *P. apoensis*. The members of the *P. urceolata* group also form a weakly supported clade with the inclusion of the monothecate *P. (Parapheretima) boaensis* (with spermathecal pores at 5/6) and the athecate *Amyntas dinagatensis*. Moreover, loss of spermatheca or fusion of two spermathecae into one can occur in pheretimoid evolution, such as in the case of the monothecate *P. vergrandis* and the athecate conspecific of *P. apoensis*. Several of the branch nodes of the tree based on combined data set have support values that are very weak and formed polytomies, which is most likely due to insufficient data. The results could have improved if more data were available. The addition of genes with divergence rates slower than that of COI and 16S but faster than that of 28S and H3, such as 12S and ITS2, may also improve the resolution. Further molecular work including more taxa is needed to be able to establish a more robust system of classification of the pheretimoid species and come up with a better-resolved phylogeny. A consolidated phylogenetic study in the East Asian archipelagos will provide insights into the evolutionary, biogeographic, and ecological processes involved in island radiations of soil-dwelling animals.

Keywords: Molecular phylogeny; pheretimoid; *Pheretima*; *Parapheretima*; *Amyntas*;
Mindanao, Philippines

1. Introduction

Over the past decade the knowledge on earthworm taxonomic studies in the Philippines dramatically increased and the results indicate an astonishingly high diversity and high local endemism of earthworms in the country. The Philippines now has around 200 native species that are all pheretimoids representing eight genera in Megascolecidae [1], a large earthworm family widely distributed in Australia and the Pacific region, in North America, in Madagascar, and especially in eastern Asia [2]. Previous taxonomic works on Philippine earthworms done were concentrated on Luzon (e.g. [3,4,5]), the largest island located on the northern part of the country and there was only one study on Mindanao [6], the second largest island located on the southern part of the country. The discovery of new species does not appear to stop anytime soon as more species continue to be described. Recently, more species from Mindanao have been added to the growing list of Philippine species (e.g. [1,7,8]), which shows that *Pheretima* Kinberg, 1867 is the most speciose genus among the native earthworms in the country.

Pheretima was the largest genus of earthworms in Megascolecidae sensu Gates, 1959 until Sims and Easton [9], based on phenetic treatment of morphological data, reallocated members of this group into 'convenient' species groups comprising 10 genera (*Pheretima* complex or *Pheretima* group of genera.), which present earthworm taxonomists follow and collectively refer to as pheretimoid species. The general characteristics of pheretimoid species include having perichaetine setal arrangement, metanephridial excretory system, single gizzard

in viii, a pair of racemose prostates opening through male pores in xviii, and testes contained within testis sacs. Sims and Easton [9] assigned pheretimoid species with nephridia on the spermathecal ducts, with copulatory bursae, and with caeca originating in xxvii to *Pheretima* s. str. On the other hand, they assigned pheretimoid species with no nephridia on the spermathecal ducts, with no copulatory bursae, and with caeca originating in xxvii to *Amyntas* Kinberg, 1867. They also assigned pheretimoid species with no nephridia on the spermathecal ducts but with copulatory bursae, and with caeca originating in xxvii to *Metaphire* Sims and Easton, 1972. For members of *Pheretima* s. str. with no secretory diverticula projecting from the copulatory bursae, they assigned them to the subgenus *Pheretima* while those that have, they assigned them to the subgenus *Parapheretima* Cognetti, 1912. As pheretimoid species are morphologically widely varied, Sims and Easton [9] also assigned species groupings for different genera primarily basing on the number and position of spermathecal pores: e.g. members of the subgenus *Pheretima* with a pair of spermathecal pores at intersegments 5/6 belong to the *P. urceolata* Horst 1893 group; those with a pair of spermathecal pores at intersegments 7/8 belong to the *P. sangirensis* Michaelsen 1891 group; those with four pairs of spermathecal pores at intersegments 5/6/7/8/9 belong to the *P. darnleiensis* Fletcher 1887 group.

James [10] conducted a preliminary molecular phylogenetic study to verify the intrageneric relationships among pheretimoids and as well the species-groups in *Pheretima* s. str. as assigned by Sims and Easton [9]. Previous and recent molecular phylogenetic studies on pheretimoids are on *Amyntas* and *Metaphire* in China and Taiwan (eg. [11,12,13]) and James' [10] work was the first molecular phylogenetic study on Philippine earthworms that includes species in *Pheretima* s. str. The specimens he worked on were collected from Luzon group of islands using the data of mitochondrial 16S rDNA and nuclear 28S rDNA. The trees generated

from 16S and 28S respectively poorly resolved the phylogeny and although the tree generated from the combined gene markers have better resolution, most of the internal nodes were weakly supported (< 0.8 posterior probability) and many of the branches formed polytomy. To produce a well-resolved phylogeny at different taxonomic levels, it is recommended that a combination of three or more genes with different mutation rates and having a total length of more than 2000 bp should be used [11,14,15,16,17].

This paper is a molecular phylogenetic study of the pheretimoid specimens collected from Mindanao and associated islands with the goal to verify Sims and Easton's [9] generic and species-group assignments that were based on morphological data. This work also attempts to resolve the intergeneric and interspecific phylogeny of the pheretimoid taxa using four gene markers with different mutation rates having a total length of more than 2000 bp.

2. Materials and methods

2.1. Collection sites and sampling

Collection of specimens was conducted intermittently from May 2003 to April 2004. The collection sites were chosen based primarily on the Key Conservation Sites of the Philippines identified by the Haribon Foundation [18]. These include Mt. Timpoong in Camiguin Island, approximately 10 km north of the Misamis Peninsula; Cagdianao Municipality in Dinagat Island northeast of Mindanao; Mt. Tago and Mt. Musuan in Bukidnon Province, both in central Mindanao; Mt. Apo in Davao del Sur Province; Mt. Busa in Sarangani Province; and Mt. Malindang Range in Misamis Occidental Province at the base of the Zamboanga Peninsula (Fig. 1). As most of the sites are protected areas under the Protected Areas and Wildlife Bureau (PAWB) of the Department of Environment and Natural Resources (DENR), Prior Informed

Consent certificates were obtained from the Protected Area Management Board for the respective sites prior to collection. Sampling was done from soil, ferns, mosses, and the insides of rotten logs in primary and secondary forests at high elevations away from human settlements or trails. Worms were sorted in the field to putative species, using body size, coloration, and number and location of spermathecal pores as identifying characters. The earthworms were rinsed in tap water, killed in 10% ethanol and then preserved in 95% ethanol.

2.2. Morphological examination

External and internal characters were examined in the laboratory using a stereomicroscope. The generic diagnoses and assignment to genus and species groups follow Sims and Easton [9]. Eighteen morphospecies from Mindanao and associated islands were examined and identified (Table 2). Among these, 12 are identified as *Pheretima* (*Pheretima*): six are of the *P. sangirensis* species group of Sims and Easton [9], namely *P. malindangensis* Aspe and James, 2014, *P. boniaoi* Aspe and James, 2014, *P. apoensis*, Aspe and James, 2016, *P. timpoongensis* Aspe and James, 2016, *P. camiguinensis* Aspe and James, 2016, and a *Pheretima* morpospecies from Mt. Busa (hereafter, *Pheretima* Busa1); and five morphospecies are of the *P. urceolata* species group of Sims and Easton [9], which include *Pheretima* Apo1, *Pheretima* Apo3, and *Pheretima* Apo4 from Mt. Apo, *Pheretima* Busa2 from Mt. Busa, and *Pheretima* Tago1 from Mt. Tago. One morphospecies is identified as the monothecate *P. vergrandis* Aspe and James, 2014. One *Pheretima* morphospecies collected from Mt. Musuan is assigned to *P. apoensis* due to morphological similarity despite being athecate and despite having relatively larger body size. There are also two species of the *P. (Parapheretima)*, namely *P. (Parapheretima) pandanensis* Aspe and James, 2016 from Camiguin Island and *P. (Parapheretima) boaensis* Aspe and James, 2016 from Dinagat Island. Members of the subgenus *Parapheretima* are distributed in Borneo

and western New Guinea and these are the first records of *Parapheretima* in the Philippines. There are also three species of *Amyntas*, which include *A. dinagatensis* Aspe and James, 2016 from Dinagat Island, one *Amyntas* morphospecies from Mt. Apo and a morphospecies from Mt. Tago, hereafter, *Amyntas* Apo1 and *Amyntas* Tago1, respectively. Table 3 shows the summary of the comparison of morphological features among the pheretimoid taxa included in the analyses.

2.3. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from muscle tissues of the specimens using the DNeasy Blood & Tissue Kit (Qiagen, USA). Regions of the mitochondrial 16S rRNA (hereafter, 16S), cytochrome *c* oxidase subunit I (COI), nuclear 28S rRNA (28S) and histone H3 (H3) genes were amplified using the polymerase chain reaction (PCR). The mixture (total volume 10 µl) contained 1 µl DNA and 9 µl PCR-mix (3.76 µl sterile dH₂O, 2.68 µl of 2 µg/µl bovine serum albumin (BSA), 0.45 µl of each primer [forward and reverse primers, 10 pmol/µl], 0.9 µl of 10× buffer, 0.71 µl of dNTP, 0.05 µl Ex *Taq*-polymerase). The cycling profile was as follows: denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C, and extension for 1 min at 72°C for 35 cycles with an initial denaturation step for 1 min at 95°C and a final extension step for 7 min at 72°C. An alternative cycling profile was followed to amplify genes that failed to be amplified using the above-mentioned cycling profile: denaturation for 1 min at 94°C, annealing for 1 min at 48°C, and extension for 1 min at 72°C for 35 cycles with an initial denaturation step for 4 min at 95°C. PCR amplifications were confirmed by electrophoresis in 2% agarose gel, visualized by SYBR Green. Sequencing reactions were performed with BigDye Terminator Cycle Sequencing Kit ver 3.1 (Applied Biosystems, USA) using 0.8 pmol/µl of the same primers as for amplification. Sequencing was done by an ABI Prism 3730 Genetic Analyzer (Applied

Biosystems, USA). Table 1 shows the summary of the primers used and the lengths of the genes acquired.

2.4. Data analysis

The sequences were aligned using MUSCLE [23]. Phylogenetic reconstructions for combined (concatenated) data set were performed with the maximum-likelihood (ML) method (raxmlGUI ver 1.2) [24] and with the Bayesian inference (MrBayes ver 3.2.1) [25]. Gene concatenation was done using FASconCAT [26]. For the ML analysis, GTR+G+I was selected as the best-fit model in jModelTest ver 2.1.4 [27]. For the Bayesian analysis, a best-fit model for each gene was selected in jModeltest ver 2.1.4: GTR+G+I for 16S and COI; F81 for 28S; and HKY+G for H3. Nodal supports were assessed by bootstrapping with 1000 pseudoreplicates for ML. Posterior probability values and tree topology for BI were calculated with 1×10^7 generations with trees sampled every 100 generations, and 25% of the tree samples were discarded as burn-in. The tree was rooted using *Dichogaster* sp. (Acanthodrilidae) as outgroup.

3. Results

There was difficulty in obtaining DNA from the specimens and in PCR amplification as the specimens were collected from the field around 11 years ago. Purification of the DNA and the addition of BSA to the PCR-mix somehow improved the results of amplification although amplification remained unsuccessful for many of the samples. Regardless of modifications made to the reaction mix and PCR profile, amplification was unsuccessful for many specimens, suggesting DNA degradation and/or primer mismatch: out of 50 specimens extracted for DNA, 16S was successfully amplified for only 18 specimens*; COI and H3 fragments were successfully amplified for 10 and 11 specimens, respectively; and despite addition of DMSO to

the PCR mix as being used in James [10], amplification of 28S was unsuccessful for all but one specimen (Table 2).

The intraspecific sequence divergence for the pheretimoid species of Mindanao calculated using K2P model based on 16S sequences ranged from 0.000–0.011 (0%–1.1%) (Table 4). On the other hand, the interspecific sequence divergence rate for the same group of species ranged from 0.022–0.142 (2.2–14.2%).

The resulting tree for the combined data of 16S, COI, 28S, and H3 (Fig. 2) showed the clade for the Mindanao pheretimoid species is strongly supported with posterior probability of 1.0 and is moderately supported with bootstrap value of 69%. There are three major clades that have formed: a clade composed of *Amyntas* Tago1 and *Amyntas* Apo1, strongly supported with posterior probability of 1.0 and bootstrap value of 100%; a weakly supported clade that is composed of members of the *P. urceolata* group, *Amyntas dinagatensis* and *P. (Parapheretima) boaensis*; and a clade that is supported with 0.99 posterior probability and 61% bootstrap value, composed of *P. (Parapheretima) pandanensis*, members of the *P. sangirensis* group, the monothecate *P. vergrandis*, and the morphospecies that is assigned to *P. apoensis*. The clade comprised of *Amyntas* Apo1 and *Amyntas* Tago1 separated from *Amyntas dinagatensis*, making the genus *Amyntas* non-monophyletic. The two *Parapheretima* species are separated: *P. (Parapheretima) boaensis* is a sister taxon to *Amyntas dinagatensis* (with posterior probability of 0.99 and bootstrap value of 57%) within the weakly supported clade that also contains the members of the *P. urceolata* group, while *P. (Parapheretima) pandanensis* formed a clade with members of the *P. sangirensis* group supported with 0.99 posterior probability and 61% bootstrap value. This makes the subgenus *Parapheretima* non-monophyletic. A sub-clade composed of members of the *P. urceolata* group (*Pheretima* Busa2, *Pheretima* Apo4 and

Pheretima Tago1) within the weakly supported clade is well-supported with 0.98 posterior probability and 81% bootstrap value. The position of the monothecate *P. vergrandis* among members of the *P. sangirensis* group is supported with 0.90 posterior probability and 59% bootstrap value. Also, the position of the athecate morphospecies from Mt. Musuan that is assigned to *P. apoensis* together with the thecate *P. apoensis* from Mt. Apo is strongly supported with 1.0 posterior probability and 100% bootstrap value.

4. Discussion

The analyses of the combined data produced a tree more or less consistent with morphology-based expectation (Fig. 2). Despite having limited taxa and limited genes included in the analysis, some interesting points are raised that need to be pondered by the community of earthworm systematists. These can serve as hypotheses to be tested with expanded data. *Amyntas* differs from *Pheretima* in having no nephridia on the spermathecal ducts and in having no copulatory bursae whereas the latter possess both organs. Sims and Easton [9] acknowledged that there are *Amyntas* and *Pheretima* that are athecate and in such case, the athecate species may be assigned to either of the genera based on having copulatory bursae or not. Even at preclitellate stage, the spermathecae and the copulatory bursae in thecate *Pheretima* species should already be evident (e.g. [6]). An additional problem is that athecate individuals with copulatory bursae could also belong to *Metaphire*, a genus differing from *Pheretima* by lacking nephridia on the spermathecal ducts. Nonetheless, the issue between athecate *Pheretima* and *Metaphire* has been addressed in Aspe and James [1]. The non-monophyly of *Amyntas* as shown in the tree here shows that the character used by Sims and Easton [9], particularly the absence of copulatory bursae, to define *Amyntas* appear homoplasious. It is suggested in James [10] and Chang et al. [11] that

Amyntas may either be paraphyletic or polyphyletic implying that the development or loss of either the nephridia on spermathecal ducts and/or the secondary male pores happened more than once in the pheretimoid taxa. In another study on molecular phylogeny of pheretimoids in Hainan Island, China, it is proposed that *Amyntas* and *Metaphire* be reconsidered as one genus [13]. Our results reinforce these older concerns about the monophyly of pheretimoid genera.

The phylogenetic relationship between *Amyntas dinagatensis* and *P. (Parapheretima) boaensis* is supported with 0.99 posterior probability and 57% bootstrap value, despite having very little morphological similarity: *Amyntas dinagatensis* is athecate (vs. with single spermatheca at intersegment 5/6 in *P. (Parapheretima) boaensis*), has larger body size (240 mm $\times$ 7–9 mm vs. 94–133 mm $\times$ 3.5–4.5 mm in *P. (Parapheretima) boaensis*), the segmental equators are not pigmented (vs. pigmented equators in *P. (Parapheretima) boaensis*), the distance between male pores is wider (0.27 circumference apart ventrally vs. 0.16 circumference apart ventrally in *P. (Parapheretima) boaensis*), more setae between male pores (20 vs. 6–9), and the copulatory bursae are absent (vs. present in *P. (Parapheretima) boaensis*) [8]. What is common though between these two species is that both were collected from Dinagat Island while the rest of the species within the clade are from mainland Mindanao. Thus, the clade for these two species reflects their geographic affinity.

The separate positions of the two members of the subgenus *Parapheretima* as shown in the tree of the combined data (Fig. 2) suggests that the presence of secretory diverticula that Sims and Easton [9] used to define *Parapheretima* is homoplasious. In other words, the respective secretory diverticula on the copulatory bursae of *P. (Parapheretima) boaensis* and *P. (Parapheretima) pandanensis* evolved independently. The position of *P. (Parapheretima) pandanensis*, which has a pair of spermathecae at intersegment 7/8, among the members of the *P.*

sangirensis group, which is also defined by the same character, is rather well-supported with 0.99 posterior probability and 61% bootstrap value. On the other hand, *P. (Parapheretima) boaensis*, which has a single spermatheca midventrally in intersegment 5/6, is nested among the weakly supported clade that contains members of the *P. urceolata* group, defined by having a pair of spermathecal pores at intersegment 5/6. Therefore, it appears in this case (we do not intend this as a general rule) that the location of spermathecae is a better indicator of phylogeny than the presence of secretory diverticula on the copulatory bursae. If this is proven true, the subgenus *Parapheretima* should be discarded unless further morphological and molecular investigation supports the existence of a clade containing the type species of the subgenus and other members with secretory diverticula. However, at this point, we cannot draw any conclusion because the position of *P. (Parapheretima) boaensis* among the members of *P. urceolata* group is weakly supported. Further phylogenetic studies should be done involving other species of the (*P.*) *Parapheretima* in Borneo and western New Guinea to confirm the results and determine the placement of (*P.*) *Parapheretima* among the pheretimoids.

As mentioned above, the members of the *P. urceolata* group formed a weakly supported clade that includes *Amyntas dinagatensis* and *P. (Parapheretima) boaensis*. Within this clade is a well-supported clade (0.98 posterior probability and 81% bootstrap value) that contains *Pheretima* Busa2, *Pheretima* Apo4 and *Pheretima* Tago1, partially reflecting Sims and Easton's [9] species-group assignment based on the location of spermathecae. In James [10], members of the *P. urceolata* group did not form a clade and it is suggested that some members of the *P. urceolata* group may be derived from an octothecal ancestor (with spermathecae in intersegments 5/6/7/8/9), which also gave rise to the hexathecal species (with spermathecae in

intersegments 5/6/7/8 and in 6/7/8/9, respectively). Here, we do not support any conclusions about the monophyly of the *P. urceolata* group.

The *P. sangirensis* group formed a well-supported clade with 1.0 posterior probability in James [10]. Here, a clade that is composed of members of the *P. sangirensis* group is supported with posterior probability of 0.82, with the inclusion of the monothecate *P. vergrandis* and the athecate morphospecies assigned to *P. apoensis*. It is hypothesized in James [6] and Aspe and James [1] that monothecate species must have evolved from bithecate species with the fusion of two spermathecae into one because the single spermatheca in these species have two identical diverticula. Such is the case in *P. ambonensis* Cognetti, 1913, *P. monotheca* James, 2004 and *P. conceptionensis* Aspe and James, 2014. In *P. vergrandis* (with midventral spermatheca at intersegment 7/8), the single spermatheca has only one diverticula, which could have resulted from either a loss of one spermatheca in bithecate ancestor or fusion of two spermathecae into one, followed by a loss of a diverticulum. The more parsimonious option would be to reject the fusion hypothesis, but we suggest that much more detailed investigation is necessary to be certain of the evolutionary history. The clade that is formed for *P. vergrandis*, *P. boniaoi* and *P. malindangensis* also reflects geographic affinity having these species being collected from the same locality, in the Mt. Malindang Range.

The relationship between the athecate morphospecies from Mt. Musuan provisionally assigned to *P. apoensis* and the thecate *P. apoensis* from Mt. Apo is strongly supported (with posterior probability of 1.0 and bootstrap value of 100%). The genetic divergence of 0.011 (or 1.1%) using K2P model based on 16S (Table 4) indicates that the two taxa are conspecifics and the population recently diverged. On another note, a study on cryptic species of hormogastrid earthworms showed an upper limit of 4.27% intraspecific divergence in *Hormogaster elisae*

using K2P based on 16S [14]. It is most likely that geographic isolation (e.g. the distance between Mt. Apo and Mt. Musuan is approximately 110 km) may have allowed divergence in some morphological features such as the body size (188 mm × 9 mm in *P. apoensis* from Mt. Apo vs. 324 mm × 10 mm in *P. apoensis* from Mt. Musuan) and loss of spermatheca, in this case, the *P. apoensis* from Mt. Musuan. A study on the reproductive traits of *Aporrectodea trapezoides* showed that isolation of an earthworm in a controlled environment can trigger the earthworm to reproduce parthenogenetically [28].

In general, several of the nodes have support values that are very weak and have formed polytomies, which is most likely due to the lack of data, preventing obtaining a more credible estimate of the relationships among the pheretimoid taxa. The results of this study could have improved if more 28S data had been available. The addition of genes with divergence rates slower than that of COI and 16S but faster than that of 28S and H3, such as 12S and ITS2, may also improve the resolution. Moreover, working on newly collected specimens would have reduced the difficulty in obtaining DNA from the specimens and in PCR amplification. Further molecular and morphological investigation should be done to trace the pheretimoid phylogeny and be able to establish a more robust system of classification.

5. Conclusions

Despite having limited taxa and limited genes included in the analyses, the combined data set generated a phylogeny more or less consistent with morphology-based expectation. Sims and Easton's [9] taxonomic assignment on the genus *Amyntas* and the subgenus *Parapheretima* do not reflect phylogeny and the characters they used to define these taxa are homoplasious. The species groupings in *Pheretima* based on the location of spermathecae, such as in the well-

supported clade of species that have spermatheca(e) at intersegments 7/8 and in the weakly supported clade of species that have spermatheca(e) at intersegments 5/6 are partially reflected in the pheretimoid phylogeny. Also, loss of spermatheca or fusion of two spermathecae into one can occur in pheretimoid evolution, such as in the case of the monothecate *P. vergrandis* and the athecate conspecific of *P. apoensis*. Several of the branch nodes have support values that are very weak and formed polytomies, which is most likely due to insufficient data. The results could have improved if more data were available. The addition of genes with divergence rates slower than that of COI and 16S but faster than that of 28S and H3, such as 12S and ITS2, may also improve the resolution. Further molecular work including more taxa is needed to be able to establish a more robust system of classification of the pheretimoid species and come up with a better-resolved phylogeny. A consolidated phylogenetic study in the East Asian archipelagos will provide insights into the evolutionary, biogeographic, and ecological processes involved in island radiations of soil-dwelling animals.

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Table 1. Summary of the primers used for the 16S rDNA, COI, 28S rDNA and Histone3 sequences

Amplified Region (size in bp; genetic position)	Primer name	Primer sequence (5'-3')	Reference
16S (455; 1-455)	16sAr	CCGGTCTGAACTCAGATCACGT	Palumbi [19]
	16sBr	CGCCTGTTTATCAAAAACAT	Palumbi [19]
16S (373; 1-455)	16SF-ME	GCAAAGGTAGCATAATCACTTGC	This study
	16SR-ME	AATTTTGCGCTATATAGATACCCTAAGC	This study
COI (641; 456-1097)	LCO1490	ACTTCAGGGTGACCAAAAAATCA	Folmer et al. [20]
	HCO2198	GGTCAACAAATCATAAAGATATTGG	Folmer et al. [20]
COI (591; 456-1097)	COI-F_N	TTTGAGCCGGAATAATTGG	This study
	COI-R_N	TCGAAGAATGATGTATTTAGGTTTCG	This study
28S (756; 1098-1854)	C1'	ACCCGCTGAATTTAAGCAT	Jamieson et al. [21]
	D2	TCCGTGTTTCAAGACGG	Jamieson et al. [21]
H3 (333; 1855-2188)	H3aF	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. [22]
	H3aR	ATATCCTTRGGCATRATRGTGAC	Colgan et al. [22]

Table 2. Taxon list and sequences obtained (O). Outgroup sequences were obtained from GenBank.

Taxon	Remark	16S	28S	COI	H3
<i>Pheretima</i> Apo1	<i>P. urceolata</i> group	O		O	O
<i>Pheretima</i> Apo3	<i>P. urceolata</i> group	O		O	O
<i>Pheretima</i> Apo4	<i>P. urceolata</i> group	O		O	
<i>Pheretima</i> Busa2	<i>P. urceolata</i> group	O			
<i>Pheretima</i> Tago1	<i>P. urceolata</i> group	O			
<i>Pheretima apoensis</i> (Mt. Apo)	<i>P. sangirensis</i> group	O			
<i>Pheretima apoensis</i> (Mt. Musuan)	athecate morphospecies	O		O	O
<i>Pheretima timpoongensis</i>	<i>P. sangirensis</i> group	O		O	O
<i>Pheretima camiguinensis</i>	<i>P. sangirensis</i> group	O		O	O
<i>Pheretima</i> Busa1	<i>P. sangirensis</i> group	O			
<i>Pheretima malindangensis</i>	<i>P. sangirensis</i> group	O		O	O
<i>Pheretima boniaoi</i>	<i>P. sangirensis</i> group	O		O	O
<i>Pheretima vergrandis</i>	monothecate morphospecies	O		O	O
<i>P. (Parapheretima) pandanensis</i>	Subgenus <i>Parapheretima</i>	O			O
<i>P. (Parapheretima) boaensis</i>	Subgenus <i>Parapheretima</i>	O		O	O
<i>Amyntas dinagatensis</i>	<i>Amyntas</i>	O			O
<i>Amyntas</i> Tago1	<i>Amyntas</i>	O	O		
<i>Amyntas</i> Apo1	<i>Amyntas</i>	O			
<i>Pontodrilus litoralis</i>	outgroup	AF406568	AY101576	LC018740.1	
<i>Dichogaster</i> sp.	outgroup	AF406571	AY101555		
Out of 50 specimens		18	1	10	11

*16S sequences of other representatives of some species here are not included in the ML and MrBayes phylogenetic reconstructions.

Table 3. Comparison of morphological features among pheretimoid taxa included in the analysis. “O” means present.

Diagnostic character	<i>Genus Amyntas</i>		<i>Genus Pheretima</i>	
		<i>Subgenus Parapheretima</i>	<i>Subgenus Pheretima</i>	
			<i>P. urceolata</i> group	<i>P. sangirensis</i> group
Nephridia on spermathecal duct		O	O	O
Spermathecae in 5/6	O	O	O	
Spermathecae in 7/8	O	O		O
Copulatory bursae		O	O	O
Secretory diverticula		O		

Table 4. Intraspecific and interspecific sequence divergences of some Mindanao pheretimoid species calculated using K2P model based on 16S sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 <i>Pheretima (Parapheretima) boaensis</i> 1														
2 <i>Pheretima (Parapheretima) boaensis</i> 2	0.011													
3 <i>Pheretima (Parapheretima) pandanensis</i> 1	0.112	0.105												
4 <i>Pheretima (Parapheretima) pandanensis</i> 2	0.116	0.101	0.008											
5 <i>Pheretima apoensis</i> 1	0.142	0.126	0.084	0.087										
6 <i>Pheretima apoensis</i> 2	0.142	0.126	0.084	0.087	0.000									
7 <i>Pheretima apoensis</i> (Musuan)	0.142	0.126	0.090	0.094	0.011	0.011								
8 <i>Pheretima camiguinensis</i> 1	0.102	0.087	0.061	0.064	0.067	0.067	0.080							
9 <i>Pheretima camiguinensis</i> 2	0.102	0.087	0.061	0.064	0.067	0.067	0.080	0.000						
10 <i>Pheretima camiguinensis</i> 3	0.102	0.087	0.061	0.064	0.067	0.067	0.080	0.000	0.000					
11 <i>Pheretima camiguinensis</i> 4	0.105	0.091	0.058	0.061	0.064	0.064	0.077	0.005	0.005	0.005				
12 <i>Pheretima timpoongensis</i> 1	0.105	0.090	0.055	0.058	0.074	0.074	0.084	0.022	0.022	0.022	0.022			
13 <i>Pheretima timpoongensis</i> 2	0.105	0.090	0.058	0.055	0.077	0.077	0.087	0.028	0.028	0.028	0.028	0.005		
14 <i>Pheretima timpoongensis</i> 3	0.105	0.090	0.055	0.058	0.074	0.074	0.084	0.022	0.022	0.022	0.022	0.000	0.005	
15 <i>Pheretima timpoongensis</i> 4	0.105	0.090	0.058	0.055	0.077	0.077	0.087	0.028	0.028	0.028	0.028	0.005	0.000	0.005

Figure Captions

Figure 1. Map of Mindanao and associated island showing the seven collecting sites included in the study.

Figure 2. Tree generated from the combined 16S, COI, 28S and H3 genes showing posterior probability values and bootstrap values. Posterior probability values lower than 0.8 and bootstrap values lower than 50% indicate weak support and are not shown.

Figure 1

Figure 2