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

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ABSTRACT

The knowledge on earthworm taxonomic studies in the Philippines over the past decade dramatically increased and the results indicate an astonishingly high diversity and high local endemicity of earthworms in the country. The earthworm fauna of the Philippines is composed of pheretimoid species of the family Megascolecidae. A molecular phylogenetic study was done in attempt to infer phylogenetic relationships among the pheretimoid species in Mindanao and associated islands. Gene markers used include the mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rRNA, and the nuclear 28S rRNA and protein-coding histone H3 genes. 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 classification scheme published in 1972 does not reflect the phylogeny with respect to the genus *Amynthas* 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 *Pheretima 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 *Pheretima apoensis*. The members of the *Pheretima 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 *Amynthas 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.

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 endemicity 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. Refs. [3–5]), 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

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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. Refs. [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 *Amynthas* 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 *Pheretima urceolata* Horst 1893 group; those with a pair of spermathecal pores at intersegments 7/8 belong to the *Pheretima sangirensis* Michaelsen 1891 group; those with four pairs of spermathecal pores at intersegments 5/6/7/8/9 belong to the *Pheretima 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 *Amynthas* and *Metaphire* in China and Taiwan (eg. Refs. [11–13]) 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 to 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 [11,14–17].

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 *Pheretima malindangensis* Aspe and James, 2014, *Pheretima boniaoi* Aspe and James, 2014, *Pheretima apoensis*, Aspe and James, 2016, *Pheretima timpoongensis* Aspe and James, 2016, *Pheretima camiguinensis* Aspe and James, 2016, and a *Pheretima* morphospecies from Mt. Busa (hereafter, *Pheretima* Busa1); and five morphospecies are of the

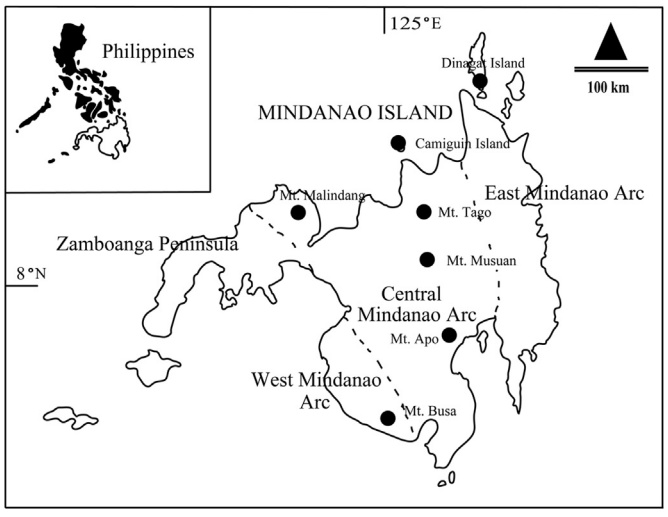

Fig. 1. Map of Mindanao and associated islands showing the seven collecting sites included in the study.