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Molecular phylogeny and biogeographic distribution of pheretimoid earthworms (clitellata: Megascolecidae) of the Philippine archipelago

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ABSTRACT

Philippine earthworms are highly diversified both locally and among sites collected on many islands and isolated mountain ranges within large islands. We conducted a molecular phylogenetic and biogeographic analysis of the earthworms from the Philippine islands to provide insight on the species diversification and distribution of these animals in relation to the geological history of the archipelago. The phylogeography of the earthworms was then viewed in light of the geological history of Southeast Asia. The resulting tree shows that the taxonomy of Philippine species of *Amynthas*, *Pithemera*, *Polypheretima* and *Pheretima* requires revision due to widespread non-monophyly. There appears to have been rapid diversification of *Pheretima* lineages. Phylogeographical patterns are not clear at the scale of taxon and gene sampling. Each of the four major islands that represented the archipelago during several Pleistocene periods of low sea level shows evidence of multiple colonizations. The lack of clear resolution in our results indicates that the dispersal of earthworms across the islands may have occurred intermittently and from different entry points in the neighboring archipelagoes. The likelihood of dispersal must have dramatically reduced during the times when the sea level rose dividing each of the major islands into several smaller ones. Climate change, the fluctuations in sea level, volcanism, and other ecological factors may have contributed to the rapid diversification of species. Further investigation of the evolutionary history of earthworms in Southeast Asian archipelagoes will require broader geographical sampling, including Indonesia, Malaysia and Australasia.

1. Introduction

Species diversification in SE Asia cannot clearly be understood without considering the region's geological history. For the Philippine islands, there have been various hypotheses as to how the archipelago formed, which resulted to the rich biodiversity it possesses today. The Zamboanga peninsula and Sulu archipelago are parts of the Sunda Shelf of the Eurasian Plate [e.g. 1,2], while Palawan, southern Mindoro, the Romblon Island Group, and western Panay make up a microcontinental block that collided with the Philippine Mobile Belt (PMB; composed basically of the rest of the Philippines) [3]. Lee and Lawver [4] proposed that the PMB may have moved as a single entity from the western margin of the Philippine Sea Plate since 60 MYA. On the other hand, a more robust tectonic reconstruction by Hall [1] based on the combination of geological and paleomagnetic data, proposed that the PMB may have been composed of islands coming from different directions at different times. Recent biogeographic studies on SE Asia such as that of de Bruyn et al. [5] and Lohman et al. [6] support the latter hypothesis.

It is during the Pleistocene when the Philippine islands began to have the topography close to the present-day topography of the archipelago. Repeated sea-level fluctuations have occurred which is recognized to be an important factor that influenced the distribution and organization of biodiversity in the Philippines [e.g. 7,8]. During the times when the sea level was 120–160 m below the current sea level, the Philippines formed the four major areas recognized as the Philippine biogeographic faunal regions: Greater Luzon, Greater Palawan, Negros-Panay and Greater Mindanao [7]. Esselstyn and Brown [9] conducted a study on the role of the fluctuations of the sea level in the generation of shrew diversity in the Philippine archipelago. They proposed that shrews must have entered the Philippines from Borneo via a land bridge in Palawan and consequently spread and colonized other parts of the archipelago. On the other hand, meta-analyses of geological, climatic, and biological data sets conducted by de Bruyn et al. [5] to understand the biological diversity in SE Asia showed that some floral and faunal species likely dispersed from Borneo to the Philippines (other than Palawan) through the Sulu Archipelago [10], while others

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likely dispersed from Indochina, probably through Taiwan [11] between the Miocene and the Pleistocene. Emigration and immigration of some floral and faunal species across water must have been achieved by rafting [12,13].

De Bruyn et al.'s [5] meta-analyses included studies of various plant and animal groups in SE Asia with fossil or molecular sequence data. Unfortunately, no data on soil-dwelling animals such as the earthworms was included due to lack of information. A large portion of the earthworm species, estimated to be ~1160 species occupying the eastern Asia and the western margin of the Pacific Basin, are pheretimoid species (members of the *Pheretima* complex) belonging to family Megascolecidae [14]. However, due to lack of molecular data and conflicting morphological character data, the evolutionary relationships of the members of this group and the pattern of their distribution remains unclear. Only recently have phylogenetic analyses of some subsets of the earthworms in this region been conducted [e.g. 15–19] but a collective study that covers the entire region has not been conducted so far. Here, a molecular phylogenetic and biogeographic analysis of the native earthworms of the Philippine islands was conducted to determine the pattern of species diversification and distribution in relation to the geological processes in the formation of the archipelago. Previous phylogenetic studies of Philippine earthworms have all had limited geographic or taxonomic coverage [15,19]. This is the first study to include a geographic sampling which more or less represents the Philippine archipelago. The data in this study will pave the way to a bigger study on molecular phylogeny and biogeography of the species in eastern Asia and the western Pacific archipelagos.

2. Materials and methods

2.1. Collection sites and sampling

Collection of specimens was conducted from 2001 to 2006 in the in different localities of the archipelago (in black circles in Fig. 1). The collection sites were chosen based primarily on the Key Conservation Sites of the Philippines [20]. 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), Gratuitous Permits to collect were obtained. As part of the procedures prior to the collection of specimens, Prior Informed Consent certificates were obtained from the Protected Area Management Board for the respective sites. Sampling was done from soil, ferns, mosses, and the insides of rotten logs in primary and secondary forests as much as possible at high elevations away from human settlements or trails to ensure that the collected specimens are native. Using body size, coloration, and number and location of spermathecal pores as identifying characters, the collected worms were sorted to putative species. 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 using a stereomicroscope. The generic diagnoses and taxonomic assignments follow Sims and Easton [21]. The native species of earthworms in the Philippines are pheretimoids which belong to family Megascolecidae, a large group dominating the Asia-Pacific region with 55 genera. The Megascolecidae includes members with generally racemose prostate glands, whose ducts generally are joined by the sperm ducts in combined male and prostatic pore(s) on segment xviii or nearby and the spermathecal pores open into some or all of the intersegmental furrows from 4/5 to 9/10 (rarely intra-segmentally). The excretory system may be meronephric or holonephric and the setal arrangement may be perichaetine or lumbricine [21,22]. Pheretimoids, or members of the *Pheretima* Kinberg [23] complex which is composed of 10 genera, were reallocated to genera by Sims and Easton [21] based on phenetic treatment of morphological data. The general characteristics of the species in this group include having perichaetine setal arrangement, meronephridial excretory system, single gizzard in viii, a pair of racemose prostates opening through male pores in xviii, and testes contained within testis sacs. Table 1 shows the comparison of morphological features among the genera included in the analysis. *Pheretima* is the most speciose genus among the Philippine earthworms [14].

For *Pheretima*, Sims and Easton [21] assigned species with no secretory diverticula projecting from the copulatory bursae, to the subgenus *Pheretima* while those that have are assigned to the subgenus *Parapheretima* Cognetti [25]. As pheretimoid species are morphologically widely varied, Sims and Easton [21] 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 (monothecate) at 5/6 belong to the *P. urceolata* group; those with a pair of spermathecal pores at 7/8 belong to the *P. sangirensis* group; those with four pairs of spermathecal pores (octothecate) at 5/6/7/8/9 belong to the *P. darnleiensis* group; while those that have three pairs of spermathecal pores (sexthecate) at 6/7/8/9 belong to the *P. dubia* group.

Sixty-two morphospecies collected from different parts of the Philippines were examined and identified (Table 2). Among these, there are 41 morphospecies of *Pheretima*, nine morphospecies of *Pithemera* Sims and Easton [21], five morphospecies of *Polypheretima* Michaelsen [24], and seven morphospecies of *Amynthas* Kinberg [23]. The morphospecies of *Pheretima* were categorized by the number and location of spermathecae: seven morphospecies have spermathecal pores at 5/6, in which six have paired spermathecal pores and one (*P.* (*Parapheretima*) *boaensis*) has a single spermathecal pore at the midventral area; 14 morphospecies have spermathecal pores at 7/8, in which 13 have paired spermathecal pores and one (*P. vergrandis*) has a single spermathecal pore at the midventral area; 12 morphospecies have four pairs of spermathecal pores at 5/6/7/8/9; seven morphospecies have three pairs of spermathecal pores at 6/7/8/9; one morphospecies has two pairs of spermathecal pores at 6/7/8. For the taxa that have not been identified to the species level, the names of the locality to which the earthworms were collected are indicated after the genus. Color-coded fonts are assigned to each genus except for the members of *Pheretima*, which have been assigned color-coded fonts based on the position (or number) of spermathecae.

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 five gene markers, which include the mitochondrial 16S rRNA (hereafter, 16S), cytochrome *c* oxidase subunit I (COI), 12S rRNA (hereafter, 12S), 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 dH_2O, 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). Alternative to BSA, 1 µl of DMSO was added to the samples that failed to amplify. The cycling profile was as follows: denaturation for 30 s at 95 °C, annealing for 30 s 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