

Molecular Phylogeny of Pheretimid Earthworms (Haplotaxina: Megascolecidae) Based on Mitochondrial DNA in Hainan Island, China

Qi Zhao^{1,2}, Daniel Cluzeau², Jibao Jiang¹, Eric J Petit³, Charlene Briard², Jing Sun¹, Andreas Prinzing² and Jiangping Qiu^{1*}

¹Ecology Lab, School of Agriculture and Biology, Shanghai Jiaotong University, 200240, Shanghai, China

²Université de Rennes 1, UMR CNRS 6553 EcoBio, Station Biologique, 35380 Paimpont, Rennes, France

³INRA, UMR985 ESE, F-35042 Rennes cedex, France

Abstract

In the Chinese Hainan Island, *Amyntas* and *Metaphire* are two dominated pheretimid earthworm species belonging to the family Megascolecidae. They differ from earthworms in the adjacent regions in their morphology and at the molecular level, which could have occurred due to the unique geography of Hainan Island. Hainan Island is made of two tectonic parts linked to South China and Indochina blocks, respectively. In addition, there is an apparent complicated link between Hainan Island and mainland China because of the different plate movements and sea level changes. In order to understand the evolution of pheretimid earthworms in Hainan Island, we first investigated the earthworm biodiversity here. Bayesian phylogenetic tree was then constructed from 3511 base pairs from five mitochondrial genes: COI, COII, 16S, 12S, and ND1. Their divergent time was finally traced based on the molecular clock of 2.0-2.4% substitutions/Ma. The results showed three species differentiations between Hainan and Guangdong earthworm species. According to the paleogeography of Asiatic regions (including Hainan Island itself), we hypothesized that the Hainan pheretimid earthworms might originate from Southeast Asia, and then they dispersed to the south and finally arrived at South China and Hainan Island. Meanwhile, due to the geology movement of the adjacent plates, there was also the migration of earthworms among adjacent regions. Furthermore, we also considered that the relationship between genera *Metaphire* and *Amyntas* might be re-considered as one genus.

Keywords: Mitochondrial phylogeny; Geographical history; *Amyntas*; *Metaphire*; Hainan Island

Introduction

Hainan Island is the second largest island in China. Currently, it is separated from mainland China by the Qiongzhou Strait from Leizhou Peninsula, Guangdong Province. It has a unique geographical history that has made it resource-rich tropical region and one of the biodiversity hotspots in China [1,2]. It is hypothesized that Hainan Island is made of two tectonic parts: North Hainan and South Hainan, which are linked to South China and Indochina blocks, respectively but likely merged during the middle Permian. During the late Mesozoic and early Cenozoic, Hainan Island separated from South China as the result of Yanshan and Himalaya Movements [3-8]. However, during the Pleistocene, Hainan Island connected with mainland China and separated from it several times because of different plate movements and sea level changes [9-12]. Finally, Hainan Island and mainland China separated completely in the late Quaternary.

Metcalf [10,11] once pointed out that the organisms in Hainan Island belonged to the typical Cathaysian flora. Our previous research also showed a similar composition of earthworm community in Hainan Island as in Asiatic mainland and adjacent islands. Two pheretimid genera in the family Megascolecidae, *Amyntas* and *Metaphire*, dominate the Hainan Island representing 91% of the islands' earthworm species [13]. However, both genera differ in their morphological characteristics regardless of whether they are from the Asiatic mainland or adjacent islands [13,14]. How did the differences occur? Is it affected by the geographical events or could it be useful to trace the paleogeography of Hainan Island? The study on the phylogenetic relationship of Hainan earthworms may give us the answer.

Mitochondrial DNA is a valuable tool to construct the phylogeny of organisms because of the ease in isolation, high copy number, assumed lack of recombination, conservation of sequence and structure across metazoa, and range of mutational rates in different regions of the molecule [15-19]. Patterns of variation in the mtDNA have allowed inferences of past biogeographic events on every geographic scale from continental to

local [20,21]. In addition, the rapid evolution of mtDNA provides robust data to interpret the evolutionary relationships within genera [22].

The aim of this study was to i) Construct the detailed phylogeny of pheretimid earthworm species in Hainan Island with five combined mitochondrial genes: COI, COII, 12S, 16S, and ND1; ii) Combine the molecular data and the evolutionary time to estimate the evolution or dispersal of Hainan pheretimid earthworms with respect to the paleogeography of Hainan Island; iii) Address the controversial relationship between *Amyntas* and *Metaphire*.

Materials and Method

Sampling of earthworms

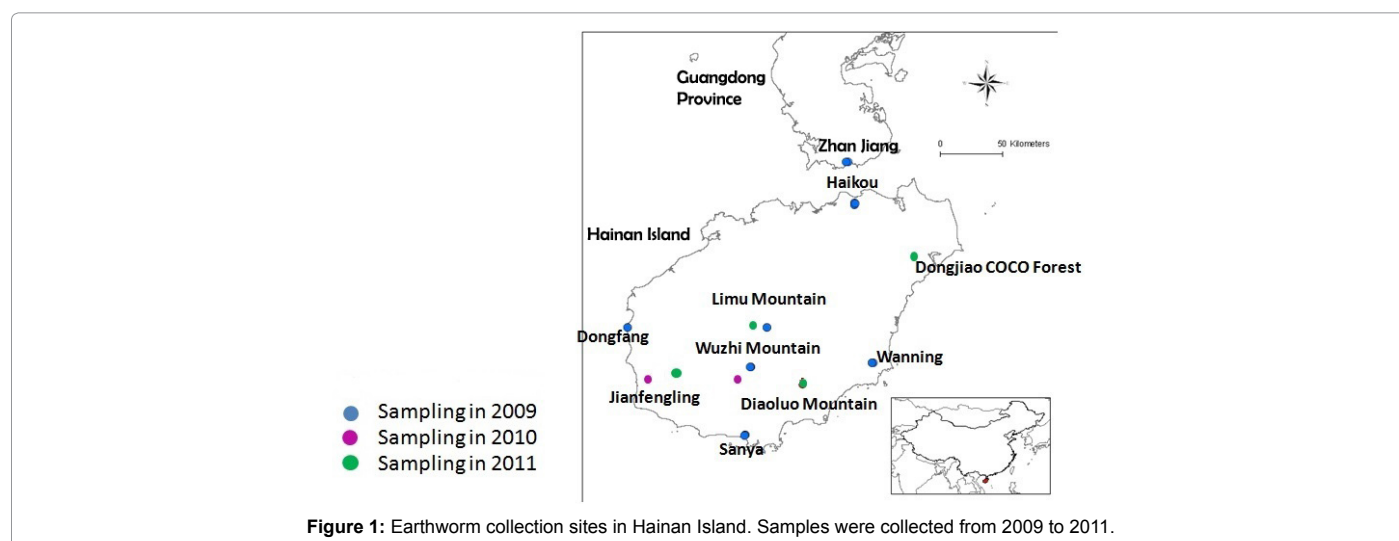
To understand the historical population structure of the Hainan earthworm species, we collected earthworms from various habitats in this island including natural rain forests (e.g., Jianfengling, Diaoluo Mountain, Wuzhi Mountain and Limu Mountain), agriculture fields, orchards (e.g., Dongjiao COCO forest) and roadsides (Figure 1) from 2009 to 2011. All specimens were preserved in 95% ethanol solution. All collected species were observed with a microscope (10 × 1 ~ 6.5, Nikon SMZ800). They were described based on features such as size, pigmentation, first dorsal pore, male pore and spermathecal pore regions, spermathecae, prostate and intestinal caeca. All samples were identified by the same specialist.

***Corresponding author:** Jiangping Qiu, Ecology Lab, School of Agriculture and Biology, Shanghai Jiaotong University, 200240, Shanghai, China, Tel: +86 (0)21342061 42; Fax: +86 (0)2134206142; E-mail: jqq@sjtu.edu.cn

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DNA extraction, amplification, and sequencing

For DNA extraction, fragments of the muscular body wall from the tail of each earthworm were dissected out. All DNA extractions were performed using OMEGA E.Z.N.A.TM Mollusc DNA Kit or Chelex following the manufacturer's protocol for invertebrate tissues. The isolated DNA was stored at -20°C. Five mitochondrial genes (COI, COII, 16S, 12S, and ND1) were sequenced using previously reported primer pairs and sequences: COI-LCO1490, COI-HCO2198, COI-HCO2198 advanced [23,24], COII-H, COII-L, tRNA-Leu-ND1-LumbF2, tRNA-Leu-ND1-LumbR1b, 12S-tRNA-Val-16S-LumbF1, 12S-tRNA-Val-16S-LumbR1 [25], and 16S-Sar, 16S-Sbr [26]. PCR amplifications were performed in a total volume of 50 µL containing 1 µL DNA, 9.6 µL enzyme mix, 2 µL of each primer and 35.4 µL ddH₂O. PCR cycling profile was as follows: 30 s 94°C, 1 min at 50°C, and 1 min at 72°C for 32 cycles with an initial denaturation step at 94°C for 5 min, and a final extension step at 72°C for 10 min. After amplification, all amplified PCR products were directly sent to and sequenced by Beijing Genomics Institute (Shanghai Branch, China) and Roscoff Geopôle ouest (France). Sequences of each gene from various earthworm samples were aligned individually using Muscle [27]. A previous study reported that well-resolved earthworm phylogenetic trees can be reconstructed using DNA sequences containing three or more genes and >2000 bp [28]. Therefore, in this study we used the combined sequences of COI, COII, 16S, 12S, and ND1 to construct a phylogenetic tree of earthworm species. Sequences of the outgroup *Lumbricus terrestris* were obtained from GeneBank (JN869946.1) and sequences of four earthworm species of Guangdong province were supplied by Jibao Jiang (unpublished data). The most appropriate model of DNA substitution was chosen using hierarchical likelihood ratio tests with Modeltest 0.1.1. Maximum likelihood (ML) analysis was performed by PhyML in www.phylogeny.fr [29,30]. Bayesian (BI) analysis was applied to generate a posterior probability distribution using the Metropolis-coupled Markov Chain Monte Carlo (MCMC) with MrBayes 3.1.2. The search was run for 1 × 10⁸ generations, sampling every 100th generation. Posterior probabilities for each branch in each tree were calculated from the sampled trees.

BEAST v.1.7.2 was used to estimate the divergence time based on the uncorrelated log-normal relaxed clock and Bayesian statistics. The search was run for 1 × 10⁸ generations, and sampling every 100th generation. The results were presented in Tracer v.1.5 [31]. A Yule speciation prior was then used for the tree prior. Each partition was analyzed again under the best-fit model of sequence evolution. For

the combined mitochondrial genes, we used a calibration of 2.0-2.4% substitutions/Ma based on previous studies [28,32-34].

Results

Identification of earthworms

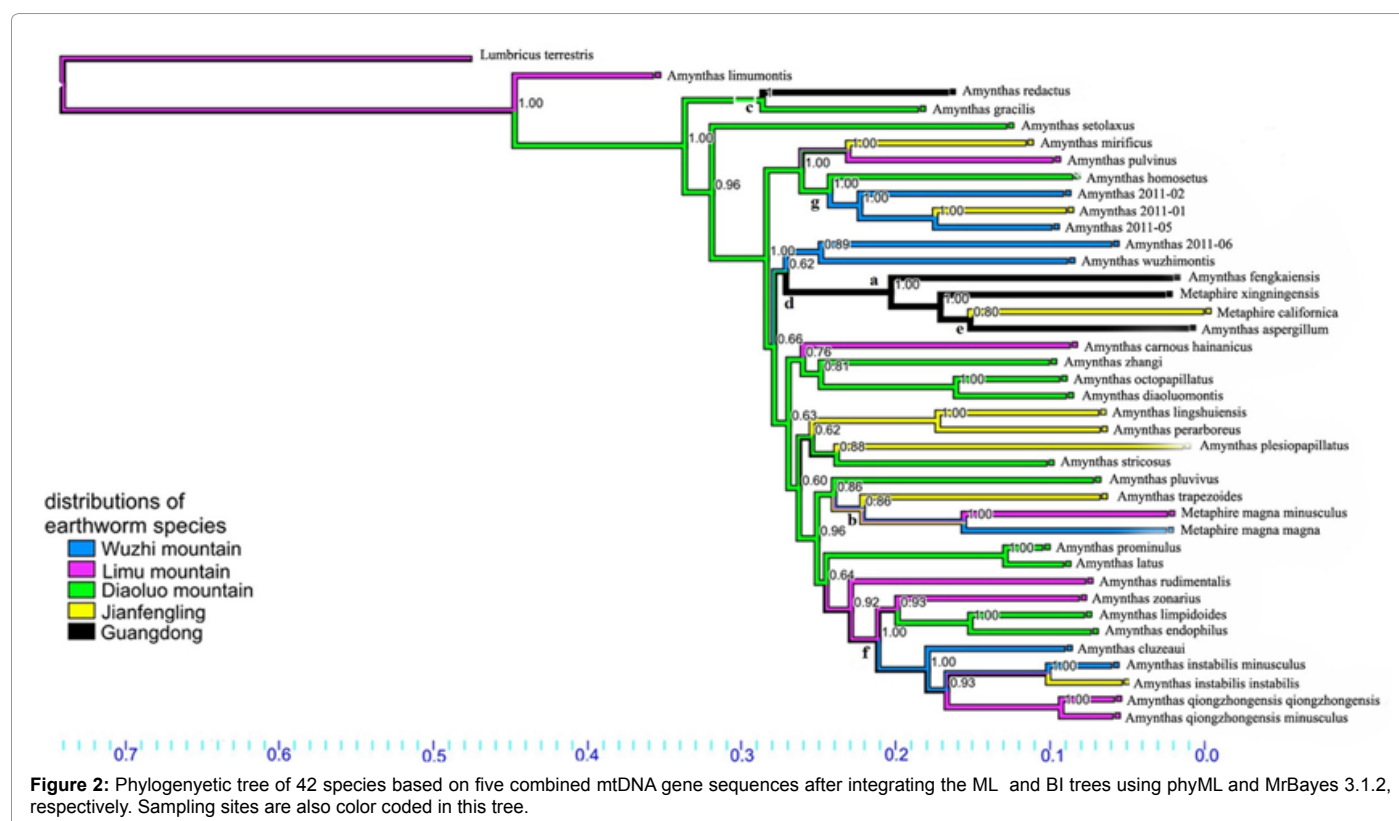
In Hainan Island, 38 pheretimid earthworm species were identified based on their morphological characteristics including body length, pigment, first dorsal pore, genital markings, genital papillae, form of male pore, spermathecae and prostate gland. These species were identified using the COI gene sequence homology. And the description of them was previously published or in press [2,13,35-42].

Sequencing

All newly obtained DNA sequences were submitted to GenBank and the Accession numbers are given in Supplementary 1. The lengths of the aligned sequences were 658 bp (COI), 534 bp (COII), 996 bp (12S), 467 bp (16S), and 856 bp (ND1). The combined length of all fragments was 3511 bp with a GC% of 34.4%. The best fit substitution model was GTR+I+G with a proportion of 0.4960 invariable sites and a gamma shape parameter of 0.6310 (Base frequencies: A=0.3941; C=0.1511; G=0.1407; T=0.3141).

Phylogenetic relationships among Hainan earthworms

In this study, we analyzed 42 earthworm species, which had all the five gene sequences. ML and BI trees (Supplementary 2) showed high congruence and the differences between the two methods stemmed from the relative positions of *Amyntas gracilis* and *Amyntas redactus*. In the ML tree, their positions were retained due to their pp values (pp value=1). The position of *Amyntas mediocus* also differed between two trees. Both the pp values were too low (0.75 and 0.76) to support the branches. Although *Amyntas zhangii*, *Amyntas pluvius*, and *Amyntas trapezoide* were monophyletic in the ML tree, their pp values were too low (0.71 and 0.52) to support the branches when compared to their relationships in the BI tree. Furthermore, although the position of *Amyntas* 2011-03 was stable in both trees, it was discarded because it was supported neither by the ML tree nor by the BI tree (no pp value). (Figure 2) showed the final phylogenetic tree after the above mentioned modifications. The integrated phylogenetic tree also indicated that the *Metaphire* and *Amyntas* species did not only constitute monophyletic group (Figure 2, branch a and b).



Distribution of earthworm species and estimation of their divergent times in Hainan Island

It was evident from Figure 2 that species separation occurred three times between the Guangdong province and Hainan Island (Figure 2, branch c-e). Based on the evolutionary rate of mtDNA, the first separation event between Guangdong province (*Amyntas redactus*) and Hainan Island (*Amyntas gracilis*) likely took place about 9.5-11.4 Ma ago (Figure 2, branch c); the second one (*Amyntas wuzhimontis* and *Amyntas 2011-06* from Hainan Island, and *Amyntas fengkaiensis* and *Metaphire xingningensis* from Guangdong province) might have occurred about 9-10.8 Ma ago (Figure 2, branch d) and the last one might have appeared about 5.6-6.7 Ma ago with the separation of *Metaphire californica* (Hainan Island) and *Amyntas aspergillum* (Guangdong province) (Figure 2, branch e). All these events likely occurred in the mid-Cenozoic.

Furthermore, differentiation also happened in Hainan Island itself. Our study revealed that *Amyntas limumontis* was the deepest node in the tree which implied that it owned the most ancestral characteristics. And next, earthworm species in lineage Diaoluo Mountain was populated by the descendants of *Amyntas limumontis*. The most recent lineages appeared in Wuzhi Mountain and Jianfengling species, but sometimes also in Limu Mountain species (Figure 2). The first migration of earthworm species from Diaoluo Mountain to Wuzhi Mountain occurred about 9-10.8 Ma ago, which was earlier than Jianfengling and Limu Mountain about 8.7 Ma ago.

Discussion

Mitochondrial phylogeny of pheretimid earthworm species in Hainan Island

The high congruence between the ML and BI trees indicated

the reliability of the estimated phylogenetic relationship among the pheretimid earthworms in Hainan Island. However, minor differences were also observed between the two trees likely because our phylogenetic tree was made only of one locus, the mtDNA.

Although the rapid mutation rate of mtDNA made it ideal to study the evolutionary relationships between organisms, it is a strictly female-inherited gene. Thus, genes outside of the mitochondrial region like nuclear genes (Globin chain C or ITS) should be considered in the future to complement this phylogenetic analysis. In addition, molecular analyses should be combined with morphological features and geographical characteristics to obtain a comprehensive picture of the evolutionary history of an organism. This approach is not only important for species identification [43] but is also helpful to understand the structure-function relationships and species evolutionary processes [44]. Thus, evolution of earthworm can be better understood by combining morphological and molecular analysis.

Questions about the relationship of genera *Metaphire* and *Amyntas*

Since 1972, *Metaphire* and *Amyntas* have been treated as two genera [45]. However, our phylogenetic analysis indicated that these two genera are always grouped together (Figure 2). Indeed, distinguishing *Metaphire* from *Amyntas* has always been controversial with the only taxonomical difference between them being the shape of the male pore. In *Metaphire*, the male pore is in a copulatory pouch while in *Amyntas* it is superficial.

However, the evagination of the copulatory pouch often leads to misidentification of the two earthworm species.

For example, James et al [46] named *Metaphire hengchunensis* as *Amyntas hengchunensis* based on its taxonomical characteristics.

However, Chang et al [47] re-identified this species with molecular techniques and re-named it as *Metaphire*. Furthermore, Sims and Easton [45] renamed *Pheretima lubricates* (Chen 1936) as *Amyntas* species, but Blakemore [48] considered it as *Metaphire* species again. Meanwhile, these phylogenetic analyses also showed the noncommittal relationship among *Amyntas*, *Metaphire* and *Pheretima* species [37, James, personal communication 2012]. Taken together, these results suggest that *Metaphire* and *Amyntas* could not be considered as monophyletic groups, respectively. It is likely that they evolved together at the beginning as one monophyletic group and then diverged. Based on this, they might be reconsidered as one genus.

Paleogeographical interpretation of Hainan pheretimid earthworms

Hainan Island tectonically lies at the southern margin of the South China block, adjacent to the northern Indochina block [9] (Figure 3). It is once part of a northern extension of Gondwana in the Cambrian but then drifts from Gondwana about 200 Ma later in the Carboniferous. During the period when North Hainan block was affected by South China block, the southern part was still linked to the Indochina block. In early Jurassic, the eastern extension of South China block was displaced to what is now the position of north Hainan Island [49]. North Hainan then connected with South Hainan due to the effects of Yanshan Movement during the late Mesozoic and early Cenozoic [3-8]. During the Pleistocene, Hainan Island connected and separated from mainland China several times because of the rising and falling of sea levels. Finally, they separated completely in the late Quaternary [9-11,50,51]. Furthermore, the west-north-west trending structural patterns (such as Wangwu-Wenjiao fault, Changjiang-Qionghai fault, Jianfeng-Diaoluo fault, and Jiusuo-Lingshui fault) in Hainan Island are tectonically temporally compatible with and spatially linked to those in Indochina and South China Blocks, respectively [8]. In general, the species diffusion and the population differentiation are strongly affected by the geographical events, which also directly lead to the fauna's migration among different regions [12,52]. Our previous investigation demonstrated that the 81% of the

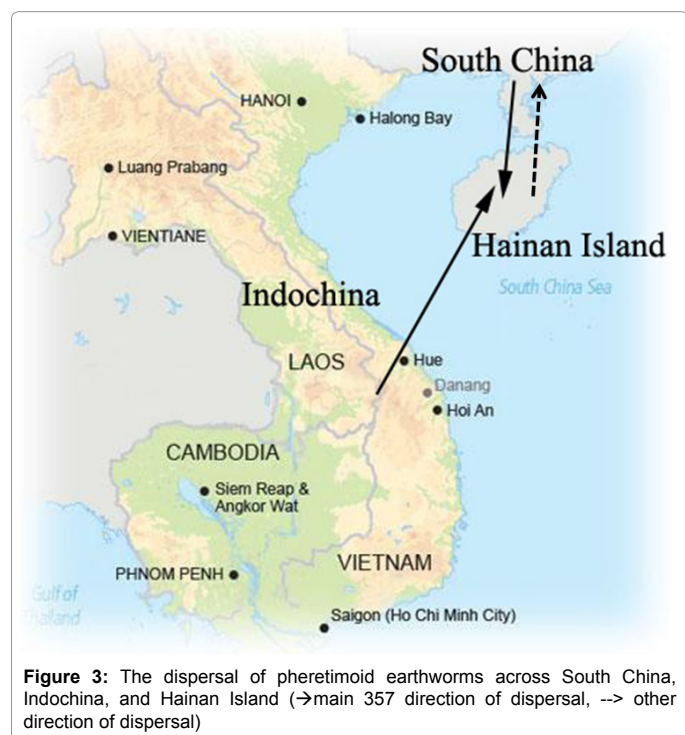
exotic earthworms in Hainan were also found in Vietnam, 75% in Laos, 56% in Cambodia, 50% in Malaysia, and 31% in China mainland and Taiwan Island [13,53]. Lack of molecular data of species in almost all of the above regions, our study only preliminarily showed three separations between species in Hainan Island and Guangdong province (Figure 2 and 3). Michaelsen [54] once pointed out that the pheretimid earthworm originated from Philipphines or Kalimantan. A part of them evolved, migrated to the west, and arrived at the Andaman Islands. And then they continually evolved to the south. Finally, pheretimid earthworms became a dominant population in South China due to their strong ability to adapt to various environmental factors. Geographically, South China plate resulted from the collision in the Indochina place in the late Permian to late Triassic. We partly agreed with Michaelsen and hypothesized that earthworms in Hainan Island had a Southeast Asian origin (Indochina plate). They likely migrated to the south and finally arrived at Hainan Island and South China. In Pleistocene, the frequent rising of sea level likely blocked the connection between South China and North Hainan Blocks, and also the migration of earthworms. The evolutionary process of these species may be more evident by including additional earthworm species from Southeast Asia. In Hainan Island, there is always controversy on its paleogeography. Hainan Island is constituted of two parts. The south part connects with Indo-China plate and the north part conjoins with South China plate. However, some researchers consider that the collision belt of these two plates occurs in the Changjiang-Qionghai belt, and some others point out that it is in the Jiusuo-Lingshui belt [8,55-58]. Our phylogeny showed that *Amyntas limumontis* owned the most ancestral characteristics, followed by species in Diaoluo lineage (Figure 2). Additional information on the paleogeography of Hainan Island and the phylogenetic analysis on the population level could provide a clear evolutionary map of Hainan earthworms.

Conclusion

In this study, we constructed a phylogenetic tree of 42 pheretimid earthworm species in the Hainan Island and Guangdong province based on 3511 bp long nucleotide sequence containing five mtDNA genes and estimated their divergence time based on a calibration with 2.0-2.4% substitutions/Ma. The phylogenetic tree presented three divergent events between the Hainan and Guangdong earthworm species. Our results also indicated that genera *Amyntas* and *Metaphire* should be combined into one genus. Finally, the evolutionary process of Hainan pheretimid earthworms appears to have been influenced by the paleogeographical history of the Hainan and South China plates. Future studies should consider using molecular markers outside of the mitochondrial region to enhance our knowledge of earthworm phylogeny. For example, molecular markers with a more precisely known mutation rate could be considered. Morpho-phylogenetic analysis could also complement the phylogenetic analyses of earthworms. Because of the unique spatial and temporal links between the Hainan Island and its adjacent regions, geographical information and phylogeny of earthworm species in Southeast Asia, East Asia, Guangdong province and/or Australia could be added for a comprehensive understanding of the evolutionary history of pheretimid earthworms in Asia.

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