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Phylogenetic evaluation of *Amynthas* earthworms from South China reveals the initial ancestral state of spermathecae

Jing Sun[a], Samuel W. James[b], Jibao Jiang[c], Bo Yao[a], Liangliang Zhang[c], Manqiang Liu[a], Jiangping Qiu[c,*], Feng Hu[a,*]

[a] College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210007, China
[b] Sustainable Living Department, Maharishi University of Management, Iowa City 52557, USA
[c] School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai, 200240, China

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ABSTRACT

Our knowledge of the phylogeny of the earthworm genus *Amynthas* under the family Megascolecidae, which is comprised of a huge number of species, is very limited compared to the better-known and much smaller family Lumbricidae. In order to investigate the phylogenetic relationships among the species within the genus *Amynthas*, which is the largest genus of the Megascolecidae family, nuclear and mitochondrial DNA sequences of 77 species, including 76 in-group *Amynthas* species collected from South China and 1 out-group species, were analyzed. A 5402 bp segments composed of whole nuclear 18 S rDNA and the mitochondrial genes COI, COII, ND1, 12 S, and 16 S was assembled from 77 species. Maximum Likelihood and Bayesian analyses of the concatenated sequences were performed. The results revealed evolution of two geographically independent lineages, both showing the ancestral state of two pairs of spermatheca (Sp.p 7/8/9). We found the species groups described by Sims and Easton (1972) to be non-monophyletic, and the origin of the parthenogenetic species group to likely be a quadthecal ancestor. These results provide modest evidence in support of an Indochinese peninsula origin of the Chinese *Amynthas* species and divergence of the genus once it had spread to mainland China. The findings of this study are consistent with a divergence scenario that resulted in at least one branch spreading to the Southeast of China and another branch spreading to the Southwest of China, but further research is required to confirm this interpretation of the *Amynthas* phylogeny.

1. Introduction

The genus *Amynthas* is though to have diversified upon colonizing China (Chen, 1956). Since Michaelsen' s investigation of 16 Chinese *Pheretima* species published in 1929 (Michaelsen, 1929), 302 *Amynthas* species have been reported in China (Sun, 2013). This genus was included in the *Pheretima* auct. before 1972. In an attempt to establish conveniently manageable units within the genus *Pheretima* auct., Sims & Easton used numerical phenetic methods to investigate the affinities of more than 700 *Pheretima* species (Sims and Easton, 1972). The species groups making up the *Amynthas* genera, which are mainly distinguished by counts of spermathecal pores, were proposed by Sims and Easton in 1972 and have since been used widely by earthworm taxonomists. Although Sims and Easton believed these grouping to be only out of convenience, rather than representations of monophyletic units, sometimes the groupings are though ro reflect evolutionary processes (Omodeo, 2000). Therefore, we employed molecular techniques to investigate whether Sims and Easton's species groups are monophyletic. Fortunately, we were able to obtain samples from a large number of species belonging to 8 *Amynthas* species groups, enabling us to test for mono- or nonmono-phyly of many groups.

Most publications on *Amynthas* have focused on the taxonomy of new and previously known species (Blakemore, 2008; Chang et al., 2009a; Sun et al., 2009; Jiang et al., 2014). Until now, phylogenetic analyses of the *Amynthas* have been restricted to small data sets with limited sampling of the *Amynthas* taxa. The first phylogeny for *Amynthas* distinguished *Amynthas formosae* (Michaelsen, 1922) and *A. yuhsii* (Tsai, 1964) as separate species, and transferred both species to the genus *Metaphire* (Chang and Chen, 2005). The relative phylogenetic position of *A. gracilis* (Kinberg, 1867) was then successfully identified (Chang et al., 2005). Afterwards, based on analyses of 8 *Amynthas* and 13 *Metaphire* species, many species groups under these two genera were identified as monophyletic, paraphyletic, or polyphyletic (James, 2005), but these findings were based on analyses of a limited number of

* Corresponding authors.
E-mail addresses: jpq@sjtu.edu.cn (J. Qiu), fenghu@njau.edu.cn (F. Hu).

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genes and sequences (i.e., a ~ 480 bp 16S rRNA fragment). Consequently, confirmation of these monophyletic, paraphyletic, and polyphyletic groupings of *Amynthas* and *Metaphire* species with a larger data set is required. In this study, we investigate patterns of morphological evolution and the phylogenetic relationships within the *Amynthas* genus, especially for species found within the main landmass of China, where the species diversity of this genus is very high.

The spermathecal pores of other genera closely related to *Amynthas* have also been well studied. Chang et al. examined relatively long sequences (i.e., a ~ 2440 bp concatenated fragment from CO1, ND1, and 16S rRNA) to show that 13 taxa of the *Metaphire formosae* species group form a monophyletic clade (Chang et al., 2008). Aspe et al., based on sequences less than 1429 bp long and concatenated from CO1, 16S rRNA, and protein-coding histone H3 genes, reported that the *Pheretima sangirensis* group and the *Pheretim aurceolata* group formed a clade separately but weakly supported (Aspe et al., 2016). The evolutionary history of the quantitative and spatial changes to spermathecae in *Amynthas* remains unknown.

The potential for multiple rates of sequence divergence is an important consideration during phylogenetic reconstruction (Chang and James, 2011). As several researchers have shown, the three mitochondrial protein-coding genes (COI, COII, and ND1) evolved faster than others and are suitable for analyzing inter-species, inter-species-group, and inter-genera relationships (Heethoff et al., 2004; Chang and Chen, 2005; Chang et al., 2007; Huang et al., 2007; King et al., 2008; Chang et al., 2009 b; Novo et al., 2009; Rougerie et al., 2009; Voua Otomo et al., 2009; James et al., 2010; Knott and Haimi, 2010; Richard et al., 2010). On the other hand, mitochondrial 16S and 12S rRNA genes are suitable for analyzing inter-family or deeper level relationships (Jamieson et al., 2002; Pérez-Losada et al., 2009). The 18S rRNA gene is suitable for analyzing inter-family or deeper level relationships (Erseus, 2005; Halanych and Janosik, 2006; Chang and James, 2011). Nevertheless, when some of the above genes were concatenated, better-resolved earthworm phylogenetic trees were obtained (Jamieson et al., 2002; Pérez-Losada et al., 2009; Novo et al., 2010; Novo et al., 2011; James and Davidson, 2012). Therefore, our knowledge of the *Amynthas* systematics and phylogeny could benefit from an analysis of 5402 base positions from six genes even though historical heterogeneity of different genes (nuclear, mitochondrial, coding, and non-coding) and/or long branch repulsion effects may still influence (Jamieson et al., 2002; Chang and James, 2011).

In this study, we address the evolution of species and species-groups within the *Amynthas* genus. In total 76 *Amynthas* species and 1 *Perionyx* species were sampled for mitochondrial and nuclear genes, including the cytochrome c oxidase subunit I mitochondrial gene (COI), cytochrome oxidase subunit II mitochondrial gene (COII), NADH dehydrogenase I mitochondrial gene (NDI), mitochondrial small subunit (12S) rRNA gene, mitochondrial large subunit (16S) rRNA gene, and nuclear small subunit (18S) rRNA gene. We used the data to explore the following hypotheses: (1) The species groups designated by Sims and Easton (1972) are monophyletic; (2) The evolution of spermathecae differs among clades; (3) The phylogenetic placements for the parthenogenetic species are developed from non-parthenogenetic ones. We should also point out that the discovery of new earthworm species in China is ongoing (most of the taxa used in the analysis for this study have not yet been described), and that large areas of the country have not yet been explored. The findings of this study will partially resolve the evolution of *Amynthas* in Southern China.

2. Material and methods

2.1. Earthworm collection and identification

The earthworm specimens were collected by hand throughout 31 locations in 12 provinces in South China, within a total area 2,285,640 km^2 in 2010 and 2011 (Fig. 1). The specimens were preserved in anhydrous alcohol at 4 °C. For each specimen, the tail muscle tissue was removed for DNA extraction, and rest of the specimen was used for taxonomic identification. A total of 77 earthworm taxa were identified according to morphologic characteristics and DNA barcoding.

For each species, the morphological characteristic of spermathecal pores, collection location, and GenBank sequence accession numbers are reported in Table A (Supplementary). Species ID codes were used to represent species without published names.

2.2. DNA extraction, PCR, and sequencing

The extraction of total genomic DNA was performedusing the E.Z.N.A. ™Mollusc DNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The quality of DNA samples were checked with electrophoresis through a 1% agarose gel and stored at −20 °C.

Polymerase chain reaction (PCR) amplification of mitochondrial genes was performed using 50-μl of reaction mixture, with TransTaq™ DNA Polymerase High Fidelity MixTaq containing 0.6 μl of Trans TaqTM HIFI DNA Polymerase, 4 μl of 2.5 mM dNTPs, and 5 μl of 10 × TransTaq ™HiFi Buffer I; 2 μl of each primer; 35.4 μl of ddH_2O; and 1 μl of template DNA. For nuclear 18S gene amplification, we used a 50-μl of reaction mixture with TransTaq™ DNA Polymerase High Fidelity MixTaq containing 0.6 μl of TransTaq ™HIFI DNA Polymerase, 4 μl of 2.5 mM dNTPs, and 5 μl of 10 × TransTaq ™HiFi Buffer I; 1.75 μl of DMSO; 2 μl of each primer; 33.65 μl of ddH_2O; and 1 μl of template DNA. Mitochondrial genes were amplified through 32 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min, with an initial 5 min denaturation at 94 °Cand a final 10 min extension at 72 °C. The nuclear 18S gene was amplified through 32 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 1 min, with an initial 5 min denaturation at 94 °Cand a final 10 min extension at 72 °C.

The primers we used in amplification of the mitochondrial COI, COII, ND1, 12S, and 16S genes and nuclear 18S subunit ribosomal RNA genes are listed in Table 1. For the COI gene, when primer HCO2198 did not work well for some taxa, primer COI-E was used as a replacement. For the 18S gene, primers TimAF and TimBR were used for amplification, and the other three primers were used only in sequencing.

The PCR products were checked by electrophoresis through a 1% agarose gel and subsequently purified and sequenced by the Beijing Genomics Institute using an ABI 3730 DNA analyzer. Sequencing of mitochondrial genes was conducted in both directions using the same primers as for PCR. Sequencing of the nuclear 18S gene used the primers 600F, SJ-32122G-1F, and SJ-32122G-1R, which dffered from the PCR primers (Table 1).

2.3. Phylogenetic analysis

The phylogenetic analysis was conducted on a total of 76 in-group species, and 1 *Perionyx* species was successfully constrained as an outgroup.

SeqVerter 2.0.4.3 was used for sequence merging, splitting, and format changing, and Primer Premier 5 (Lalitha, 2000) was used to perform sequence order adjustments. Multiple alignments were performed for the concatenated sequences of 5 mitochondrial genes and the 18S nuclear gene. Given the large taxon sample and the set of 6 genes, we used clustalW 1.82 with default parameters (Thompson et al., 1994) in Cyberinfrastructure for Phylogenetic Research (CIPRES) to conduct the multiple alignments. Alignments were checked manually using BioEdit7.0.9.0 (Hall, 1999), and regions with ambiguous alignment were excluded. The best-fit models for each gene were selected using Akaike Information Criterion (AIC) (Akaike, 1973) in JModeltest 0.1 package.

After multiple alignments and manual sequence editing, the final