



Molecular systematics and phylogeography of the gigantic earthworms of the *Metaphire formosae* species group (Clitellata, Megascolecidae)

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ARTICLE INFO

Article history:

Received 13 June 2008

Revised 29 August 2008

Accepted 31 August 2008

Available online 10 September 2008

Keywords:

Phylogeography

Systematics

Metaphire formosae species group

Megascolecidae

Pheretima complex

ABSTRACT

The earthworms of the *Metaphire formosae* species group distributed in Taiwan are members of the *Pheretima* complex within the Megascolecidae. In this study, the systematics and phylogeography of this species group were investigated using DNA sequences of mitochondrial cytochrome *c* oxidase subunit I (COI), 16S ribosomal (r)RNA, and NADH dehydrogenase subunit 1 (ND1). The results indicated that the 13 taxa of the *M. formosae* species group form a clade, including a cryptic species discovered in this study. In addition, *Metaphire hengchunensis* (James et al., 2005) should be regarded as a subspecies of *Metaphire paiwana* Tsai et al., 2000, and *Metaphire bununa glareosa* Tsai et al., 2000 should be elevated to specific status. Phylogeographical inferences showed that allopatric speciation occurred in this species group during the rapid uplift of the main island of Taiwan between 5.0 and 2.5 million years ago. Our analysis exposes non-monophyly within each of the genera *Amyntas* and *Metaphire*, and more generally within the *Pheretima* complex. Further revisions of this speciose complex are urgently needed.

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1. Introduction

The Asiatic earthworm genus *Metaphire* is a member of the *Pheretima* complex, a speciose group of more than 800 described species within 12 genera belonging to the Megascolecidae (Blake-more, 2002; Easton, 1979, 1982; James, 2005a,b; Sims and Easton, 1972). This genus is widely distributed in East and Southeast Asia with more than 160 species belonging to 25 species groups (Blake-more, 2004; Sims and Easton, 1972). In pheretimoid earthworms, the number and position of the testes are considered to be important taxonomic characters. Generally, most species are holandric (two pairs of testes, one each in segments 10 and 11), but occasionally proandry (one pair of testes in segment 10) or metandry (one pair of testes in segment 11) occurs (Sims and Easton, 1972). These differences in testis condition are crucial in within-genus groupings as well as in species identification.

In *Metaphire*, octothecate species (species bearing four pairs of spermathecae) without secondary copulatory pouches and preclitellar genital markings are further divided into two species groups: the *ignobilis* species group (holandric) and the *stephensoni* species group (proandric) (Tsai et al., 2004; Sims and Easton, 1972). However, it has been remarked in many studies that 12 taxa belonging to these two groups share a number of morphological character

states (Chang and Chen, 2004, 2005a; James et al., 2005; Tsai et al., 2000b, 2004). These taxa are *Metaphire trutina* Tsai et al. (2003) and *Metaphire tahanmonta* Chang and Chen (2005a) of the former species group, and *Metaphire formosae* (Michaelsen, 1922), *Metaphire yuhsii* (Tsai, 1964), *Metaphire paiwana paiwana* Tsai et al. (2000b), *Metaphire paiwana liliumfordi* Tsai et al. (2000b), *Metaphire bununa bununa* Tsai et al. (2000b), *Metaphire bununa glareosa* Tsai et al. (2000b), *Metaphire taiwanensis* Tsai et al. (2004), *Metaphire feijani* Chang and Chen (2004), *Metaphire hengchunensis* (James et al., 2005), and *Metaphire nanaoensis* Chang and Chen (2005a) of the latter. These species all have large body sizes exceeding 30 cm in length and 10 mm in width, bluish-gray body coloration, male pores within copulatory pouches with one or two oval pads and four pairs of spermathecae in segments 6–9. Their burrowing behaviors and casts are also very similar. Morphologically, these species differ only in the condition of the testes, the distance between the paired spermathecal pores, and the structure of the male pores. Accordingly, considering the morphological similarity of the 12 taxa noted above, we herein included these taxa in a newly proposed *Metaphire formosae* species group, and defined this group as large octothecate *Metaphire* species without secondary copulatory pouches and preclitellar genital markings, but with oval pads in the male pores.

The *M. formosae* species group is endemic to Taiwan, an island between the Ryukyu Archipelago and the Philippines, and locally nicknamed “snake earthworms” for their large body size. *M. formosae* and *M. yuhsii* were described in 1922 and 1964 as *Pheretima*

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formosae and *Pheretima yuhsi*, respectively (Michaelsen, 1922; Tsai, 1964). The two species were re-assigned to *Amyntas* by Sims and Easton (1972) when the authors revised the systematics of the *Pheretima* complex. After that, re-inspection of specimens led Chang and Chen (2005b) to re-assign the two species to *Metaphire* due to the presence of copulatory pouches in the male pores. In addition, *M. yuhsii* was once regarded as a synonym of *M. formosae* (Tsai et al., 2000a), but was later resurrected as a valid species (Chang and Chen, 2005b) by the distinctive differences in the distances between the paired spermathecal pores and divergences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, the former being the only morphological difference between the two species.

The other 10 taxa of this species group were described recently (Chang and Chen, 2004, 2005a; James et al., 2005; Tsai et al., 2000b, 2003, 2004). *Metaphire trutina* was described as a sexthecate species (species bearing only three pairs of spermathecae) (Tsai et al., 2003), but as the octothecate *Metaphire yuanpowa* Chang and Chen (2005a) was regarded as a synonym of *M. trutina* (Blakemore et al., 2006), this species was proved to be an originally octothecate earthworm. *M. hengchunensis* was described as a member of the genus *Amyntas* (James et al., 2005). However, the assignment of this species to *Amyntas* was due to different criteria regarding the presence and absence of copulatory pouches (James et al., 2005), which is the only diagnostic character between *Metaphire* and *Amyntas* (for detailed discussion on this dispute, see James, 2005a,b and James et al., 2005). By using the same criteria as those used in *M. trutina*, *M. tahanmonta* and *M. taiwanensis*, we tentatively re-assigned this species to *Metaphire*. Considering the blooming of new taxa and names proposed in recent years, and the confusion that previous studies may lead to, a comprehensive taxonomic revision of this species group using molecular phylogenetic approaches is necessary for future systematic and biodiversity studies.

The morphological similarity among these earthworms suggests that they comprise a group of closely related species. Recently, Chang and Chen (2005a) hypothesized the monophyly of *M. nanaoensis*, *M. tahanmonta*, *M. formosae*, *M. paiwannana*, *M. paiwannana liliumfordi*, *M. bununa bununa*, *M. bununa glareosa*, *M. taiwanensis*, and *M. trutina* by comparing their morphology. Subsequently, the monophyly of *M. formosae*, *M. yuhsii*, *M. paiwannana*, *M. bununa bununa*, *M. trutina*, and *M. tahanmonta* was supported by molecular studies using the COI gene (Chang and Chen, 2005b). However, the relationships among these species were not unraveled due to insufficient sequence lengths analyzed. Using a molecular phylogenetic approach, we attempted to clarify the relationships.

The molecular phylogenetic analyses of these earthworms in a geographical context can also provide opportunities to test speciation hypotheses of these species. The *M. formosae* species group has been suggested to be derived from their mainland siblings in Southeast Asia or southeastern China (Chang and Chen, 2005a). Most of these species show an allopatric distribution in Taiwan (Chang and Chen, 2004, 2005a,b). This allopatric distribution suggests possible causal relationships between the speciation of these species and the geological history of this island. Recently, DNA barcoding has been used to evaluate morphologically similar earthworm species (Chang and Chen, 2005b; Chang et al., 2007; Pérez-Losada et al., 2005), and systematic revisions of some earthworm groups were also conducted using molecular phylogenetic analyses (Heethoff et al., 2004; James, 2005b; Jamieson et al., 2002; Pop et al., 2003, 2007). Nevertheless, no phylogeographical hypothesis concerning speciation of earthworms was inferred. In this study, we demonstrate the first case to use molecular phylogenetic approaches on the evolutionary and phylogeographical study of closely related earthworm species. We used mitochondrial DNA sequences to study the systematics and evolution of the

M. formosae species group. We revised the taxonomy of these species using the COI sequences, the DNA barcode as proposed by Herbert et al. (2003a,b), and evaluated the validity of the *M. formosae* species group proposed herein. We then hypothesized the phylogeny of the *M. formosae* species group and made phylogeographical inferences.

2. Material and methods

2.1. Sample collection and preservation

Earthworms of the *M. formosae* species group were collected throughout Taiwan during 2000–2004. In our analyses (see below), *M. hengchunensis* was regarded as a subspecies of *M. paiwannana*, *M. bununa glareosa* was elevated to specific status, namely *M. glareosa*, and a cryptic species, *Metaphire* sp. was revealed. Therefore, 11 species in total of the *M. formosae* species group were used, including *M. formosae*, *M. yuhsii*, *M. paiwannana*, *M. bununa*, *M. taiwanensis*, *M. trutina*, *M. feijani*, *M. nanaoensis*, *M. tahanmonta*, *M. glareosa* and *Metaphire* sp.. Moreover, *M. paiwannana* is composed of three subspecies, namely *M. paiwannana paiwannana*, *M. paiwannana liliumfordi*, and *M. paiwannana hengchunensis*, and all three subspecies were included in the analysis. Samples were anesthetized in a 10% ethanol solution; some muscle tissues were isolated and preserved in a 70% or 95% ethanol solution for DNA extraction; the residual earthworm samples were fixed in 10% formalin and then preserved in a 70% ethanol solution. Some other pheretimoid earthworms collected in Taiwan were treated using the same procedures for the phylogenetic analysis (Table 1).

2.2. DNA extraction, polymerase chain reaction (PCR), and DNA sequencing

Muscle tissues were washed with distilled water, homogenized in liquid nitrogen, and digested in digestion buffer (10 mM Tris-HCl, 2 mM dihydrate EDTA, 10 mM NaCl, 10 mg/ml DTT, 1% SDS, and 0.4 mg/ml protease K) at 50 °C for 15–30 min. Total DNA was extracted from the digested tissue-buffer solution with a standard phenol/chloroform extraction method followed by ethanol precipitation (Palumbi et al., 1991). The ethanol-precipitated DNA was dissolved in distilled water, checked with 1.0% agarose gel electrophoresis, and stored at –20 °C.

Several DNA fragments from the earthworm mitochondrial genome were amplified by PCR, including COI, 16S ribosomal (r)RNA, and NADH dehydrogenase subunit 1 (ND1). All primer sequences were listed in Table 2. For COI, two partially overlapping fragments were amplified by LCO1490 and HCO2198 (Folmer et al., 1994) for the 5' fragment and by COIF0622 and COIR1117 for the 3' fragment. When the second primer pair failed to work, instead of COIR1117, COIR1294 was used for *M. trutina* and *Metaphire schmardae*, and COIR1102 was used for *M. feijani*. For 16S rRNA, the universal primers 16Sar and 16Sbr (Hillis and Moritz, 1990) were used. For ND1, the primers LeuND1F and IleND1R designed in this study were used. The amplifications were carried out in a 50- μ l total volume using one cycle at 94 °C for 1 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and extension for 90 s at 72 °C, with a final cycle at 72 °C for 10 min.

The PCR products were checked using 1.0% agarose gel electrophoresis and sequenced in both directions using the same primers as for PCR. Sequencing was performed with a BigDye Terminator Cycle Sequencing Ready Reaction Kit, V3.1 (Applied Biosystems, CA, USA). Products were analyzed on an ABI 3730 XL DNA Analyzer (Applied Biosystems). The sequence of each sample was verified through a comparison of complementary light and heavy strands and double-checked by eye.

Table 1
Samples used in the phylogenetic study and the corresponding GenBank Accession Numbers

Species	Locality	Sample no.	Voucher no.	Accession no. of haplotypes			
				COI	ND1	16S	
<i>Metaphire yuhsii</i>	Hsintien, Taipei County	B0665	Afo-65	AY739309 ^a			
	Wulai, Taipei County	B0650	Afo-50	AY739310 ^a			
	Shouyi, Taipei County	B0604	Afo-4	AY739311 ^a			
	Wanli, Taipei County	B0601	Afo-1	AY960799	AY960786	AY960812	
	Tamsui, Taipei County	B0612	Afo-12	AY739313 ^a			
	Sanchih, Taipei County	B0611	Afo-11	AY739314 ^a			
	Tuchen, Taipei County	B0663	Afo-63	AY739315 ^a			
	Sanshia, Taipei County	B0607	Afo-7	AY739316 ^a			
	Mucha, Taipei City	B0602	Afo-2	AY739317 ^a			
	Nangang, Taipei City	B0625	Afo-25	AY739318 ^a			
	Keelung City	B0624	Afo-24	AY739319 ^a			
	Taoyuan, Taoyuan County	B0660	Afo-60	AY739320 ^a			
	Lungtan, Taoyuan County	B0619	Afo-19	AY739321 ^a			
	Fushing, Taoyuan County	B0657	Afo-57	AY739322 ^a			
	Chudong, Hsinchu County	B0628	Afo-28	AY739323 ^a			
	Guanhsi, Hsinchu County	B0659	Afo-59	AY739324 ^a			
Jianshi, Hsinchu County	B0637	Afo-37	AY739325 ^a				
<i>M. formosae</i>	Yamgmei, Taoyuan County	B1801	AfoTII-1	AY960807	AY960794	AY960820	
	Baushan, Hsinchu County	B1808	AfoTII-8	AY739327 ^a			
	Sanwan, Miaoli County	B1811	AfoTII-11	AY739328 ^a			
	Sanyi, Miaoli County	B1847	AfoTII-47	AY739329 ^a			
	Nanchuang, Miaoli County	B1855	AfoTII-55	AY739330 ^a			
	Heping, Taichung County	B1875	AfoTII-75	AY739331 ^a			
	Guoshing, Nantou County	B1867	AfoTII-67	AY739332 ^a			
	Shinyi, Nantou County	B18105	AfoTII-105	AY739333 ^a			
	Mayshan, Chiayi County	B1899	AfoTII-99	AY739334 ^a			
	<i>M. tahanmonta</i>	Taoyuan, Kaohsiung County	B0830	sp40-2	AY739335 ^a		
Taoyuan, Kaohsiung County		B0831	sp40-3	AY960800	AY960787	AY960813	
Chunri, Pingtung County		B0807	sp40-1	AY962115			
Taoyuan, Kaohsiung County		B0874	sp40-4	AY962116			
<i>M. paiwana paiwana</i>	Liouguei, Kaohsiung County	B0827	Mpa-45	AY739336 ^a			
	Sandimen, Pingtung County	B1906	Mpa-6	AY962117			
	Majia, Pingtung County	B1911	Mpa-11	AY962118			
	Taiwu, Pingtung County	B1920	Mpa-20	AY962119			
	Taiwu, Pingtung County	B1930	Mpa-30	AY962120			
	Chunri, Pingtung County	B1941	Mpa-41	AY962121			
	Taimali, Taitung County	B08156	Mpa-55	AY962133			
	Taimali, Taitung County	B08157	Mpa-56	AY962135			
	Dazen, Taitung County	B08159	Mpa-57	AY962136			
	Dazen, Taitung County	B08174	Mpa-59	AY962137			
	Dawu, Taitung County	B08163	Mpa-58	AY962138			
	<i>M. paiwana hengchunensis</i> (<i>M. hengchunensis</i>)	Nanjenshan, Pingtung County	B1901	Mph-1	AY962122		
		Nanjenshan, Pingtung County	B08179	Mph-6	AY962123		
<i>M. paiwana liliumfordi</i>	Nanjenshan, Pingtung County	B08180	Mph-7	AY962124			
	Shulin, Hualien County	B0816	Mpl-3	AY962125			
	Guangfu, Hualien County	B0812	Mpl-2	AY962126			
	Juoshi, Hualien County	B0847	Mpl-18	AY962127			
	Juoshi, Hualien County	B0845	Mpl-16	AY962128			
	Yuli, Hualien County	B0857	Mpl-26	AY962129			
	Fengbin, Hualien County	B0814	Mpl-24	AY962130			
	Fengbin, Hualien County	B0853	Mpl-25	AY962131			
	Beinan, Taitung County	B08101	Mpl-27	AY960802	AY960789	AY960815	
<i>M. bununa</i>	Beinan, Taitung County	B0810	Mpl-1	AY962132			
	Taimali, Taitung County	B08154	Mpl-28	AY962134			
	Heping, Taichung County	B08145	Mbu-9	AY739337 ^a			
	Datong, Ilan County	B0815	Mbu-3	AY962139			
	Renai, Nantou County	B0802	Mbu-1	AY962140			
	Alishan, Chiayi County	B08143	Mbu-7	AY960804	AY960791	AY960817	
	<i>M. trutina</i>	Wulai, Taipei County	B0729	sp21-29	AY739338 ^a		
		Pinglin, Taipei County	B0704	sp21-4	AY962144		
Neihu, Taipei City		B0701	sp21-1	AY962145			
Hsiaochoachi, Ilan County		B0803	sp21-13	AY962146			
Jianshi, Hsinchu County		B08110	sp21-34	AY962147			
Wufeng, Hsinchu County		B0714	sp21-14	AY960808	AY960795	AY960821	
Nanchuang, Miaoli County		B0726	sp21-26	AY962148			
<i>M. nanaoensis</i>	Shulin, Hualien County	B1315	sp62-2	AY962149			
	Shulin, Hualien County	B1323	sp62-5	AY962150			
	Shulin, Hualien County	B1314	sp62-1	AY960805	AY960792	AY960818	
	Nanao, Ilan County	B1322	sp39-22	AY962151			
	Nanao, Ilan County	B1307	sp39-7	AY962152			
	Nanao, Ilan County	B1306	sp39-6	AY962153			
	Nanao, Ilan County	B1320	sp39-20	AY962154			
	<i>M. taiwanensis</i>	Renai, Nantou County	B1504	Mta-4	AY962155		
Renai, Nantou County		B1505	Mta-5	AY962156			

Table 1 (continued)

Species	Locality	Sample no.	Voucher no.	Accession no. of haplotypes		
				COI	ND1	16S
<i>M. feijani</i>	Renai, Nantou County	B1502	Mta-2	AY960806	AY960793	AY960819
	Yuanshan, Ilan County	B1605	sp22-5	AY962157		
	Yuanshan, Ilan County	B1607	sp22-7	AY962158		
	Majia, Pingtung County	B2401	sp92-1	AY960809	AY960796	AY960822
	Majia, Pingtung County	B2402	sp92-2	AY962159		
	Majia, Pingtung County	B2403	sp92-3	AY962160		
	Wutai, Pingtung County	B2404	sp92-4	AY962161		
<i>Metaphire</i> sp. (morphologically identified as <i>M. paiwana paiwana</i>)	Wutai, Pingtung County	B2405	sp92-5	AY962162		
	Taoyuan, Kaohsiung County	B0873	sp90-2	AY962163		
<i>M. glareosa</i> (<i>M. bununa glareosa</i>)	Taoyuan, Kaohsiung County	B08147	sp90-5	AY962164		
	Taoyuan, Kaohsiung County	B0833	sp90-1	AY960801	AY960788	AY960814
	Taoyuan, Kaohsiung County	B0832	sp90-3	AY962165		
	Taoyuan, Kaohsiung County	B0834	sp90-4	AY962166		
	Shitzi, Pingtung County	B08150	Mam-18	AY962167		
	Shitzi, Pingtung County	B08149	Mam-17	AY962168		
	Dazen, Taitung County	B08169	Mam-21	AY962169		
	Ruisui, Hualien County	B0813	Mam-2	AY962170		
	Ruisui, Hualien County	B0856	Mam-8	AY962171		
	Yuli, Hualien County	B0861	Mam-3	AY962172		
	Yuli, Hualien County	B0863	Mam-6	AY962173		
	Fuli, Hualien County	B08148	Mam-16	AY962174		
	Dunghe, Taitung County	B0872	Mam-4	AY962175		
	Luyee, Taitung County	B0875	Mam-9	AY962176		
	Beinan, Taitung County	B0879	Mam-10	AY962177		
	Taimali, Taitung County	B08108	Mam-12	AY962178		
	Taimali, Taitung County	B08151	Mam-19	AY962179		
	Taimali, Taitung County	B08106	Mam-11	AY960803	AY960790	AY960816
	Chupun, Taitung County	B08113	Mam-15	AY962180		
	Chupun, Taitung County	B08112	Mam-14	AY962181		
Chupun, Taitung County	B08111	Mam-13	AY962182			
Chupun, Taitung County	B08166	Mam-20	AY962183			
<i>M. californica</i>	Taipei City	B0106	Mca-6	AY960810	AY960797	AY960823
<i>M. schmardae</i>	Taipei City	B2502	Msc-2	AY960811	AY960798	AY960824
<i>M. posthuma</i>	Hsintien, Taipei County	B0203	Mpo-3			AY960825
<i>Amyntas binocularis</i>	Baushan, Hsinchu County	B2111	sp61-11	AY962184		AY968683
<i>A. carnosus</i>	Chaochi, Ilan County	B1201	sp34-1			AY960830
	Wulai, Taipei County	B1205	sp34-5	AY962185		
<i>A. aspergillum</i>	Guting, Taipei City	B0301	Aas-1			AY960826
<i>A. incongruus</i>	Wanli, Taipei County	B0503	Ain-3			AY960827
<i>A. robustus</i>	Yuanshan, Ilan County	B1101	Aro-1			AY960829
<i>A. gracilis</i>	Hsintien, Taipei County	B1005	Agr-5			AY960828
<i>Begemius queenslandicus</i>						AF406578 ^a
<i>Pontodrilus litoralis</i>				AF003256 ^a		
<i>Fletcherodrilus sigillatus</i>						AF406586 ^a
<i>Spenceriella cormieri</i>						AF406588 ^a
<i>Spenceriella</i> sp.						AF406589 ^a
<i>Diporochaeta</i> sp.						AF406572 ^a
<i>Perionychella kershawi</i>						AF406574 ^a
<i>Digaster lingi</i>						AF406567 ^a
<i>Dichogaster saliens</i>						AF406583 ^a
<i>Dic. samjamesi</i>						AF406573 ^a
<i>Terrisswalkerius grandis</i>						AF406571 ^a
<i>T. moritzi</i>						AF406566 ^a
<i>T. millamilla</i>						AF406560 ^a
<i>Perionyx excavatus</i>						AF406565 ^a
<i>Didymogaster sylvaticus</i>						AF406582 ^a
<i>Lumbricus terrestris</i>				U24570 ^a	U24570 ^a	U24570 ^a

^a Sequences retrieved from GenBank for analyses.

2.3. Sequence alignment, phylogenetic analysis, and topological tests

The sequences obtained were checked by aligning the sequencing results with the corresponding sequences of *Lumbricus terrestris* in GenBank using the default settings of Clustal X 1.81 (Thompson et al., 1997) and then submitted to GenBank (Table 1). All ingroup sequences used in Chang and Chen (2005b) and sequences of *L. terrestris* and some other megascolecid earthworms were retrieved from GenBank (Table 1) and used in the phylogenetic analysis. Alignments were performed using the default settings of Clustal X 1.81 and then manually adjusted using BioEdit

(Hall, 1999). Gaps and ambiguously aligned regions in the DNA sequences were eliminated in all of the following analyses. *Metaphire tschiliensis tschiliensis*, *Dichogaster samjamesi* or *L. terrestris* was used as the outgroup in the analyses. The 5' fragment of COI amplified using the primer pair, LCO1490 and HCO2198, was first used to reexamine the taxon status; then, one specimen for each species identified in the COI analysis was chosen and used in the following analyses. The 16S rRNA gene sequences were used to test the hypothesis of a monophyletic *M. formosae* species group. The 16S rRNA gene was chosen instead of COI because of the availability of sequences in GenBank and the slower evolutionary rate of this

Table 2
Primers used in the phylogenetic analyses

Primer	Sequence	Reference
LCO1490	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	Folmer et al., 1994
HCO2198	5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'	Folmer et al., 1994
COIF0622	5'-ACA GAT CGA AAC CTA AAT AC-3'	This study
COIR1117	5'-ATT CTC AAC ACG TAG TGG AAG TG-3'	This study
COIR1294	5'-TCA GAA TAT CGC CGA GGT ATA CC-3'	This study
COIR1102	5'-TGA AAA TGT GCT ACN ACA TAG TA-3'	This study
16Sar	5'-CGC CTG TTT ATC AAA AAC AT-3'	Hillis and Moritz, 1990
16Sbr	5'-CCG GTC TGA ACT CAG ATC ACG T-3'	Hillis and Moritz, 1990
LeuND1F	5'-CAA GAT GGC AGA GTG CCA-3'	This study
IleND1R	5'-TAA CGT CAT CAG AGT TAT CCG-3'	This study

gene. The combined sequence set of 16S rRNA, COI, and ND1 was then used to reconstruct the phylogeny of the *M. formosae* species group. Before combining the nucleotide sequences of the three genes, the incongruence length difference test (ILD; Farris et al., 1994) was conducted to check whether all of the sequences were suitable for combination. Since the result of the ILD test was not significant ($P = 0.38$), the three genes were combined.

In the phylogenetic analyses, the most-appropriate model of DNA substitution was chosen using hierarchical likelihood ratio tests with PAUP 4.0b10 (Swofford, 2000) and Modeltest 3.0 (Posada and Crandall, 1998). For the 5' fragment of COI, the TVM model (Rodríguez et al., 1990) with invariable sites of 0.5907 and a gamma shape parameter of 0.9298 (TVM + I + G) was chosen (Base frequencies: A, 0.3744; C, 0.2120; G, 0.1451; and T, 0.2684. Substitution rates: A–C, 1.2390; A–G, 11.5090; A–T, 0.6402; C–G, 0.2470; C–T, 11.5090; and G–T, 1.0000). For 16S rRNA, the general-time reversible model (Tavafé, 1986) with invariable sites of 0.3930 and a gamma shape parameter of 0.4588 (GTR + I + G) was chosen (Base frequencies: A, 0.4365; C, 0.1458; G, 0.1492; and T, 0.2684. Substitution rates: A–C, 1094.7264; A–G, 2152.7852; A–T, 1349.1843; C–G, 285.2955; C–T, 6401.8551; and G–T, 1.0000). The maximum likelihood (ML) analysis was performed using PAUP 4.0b10 with heuristic searches, starting trees obtained by neighbor joining (NJ), and TBR branch swapping. Three different approaches were used to evaluate the reliability of the inferred phylogenetic tree. First, Bayesian analysis was applied to generate a posterior probability distribution using the Metropolis-coupled Markov Chain Monte Carlo (MCMC) with MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The search was run for 1×10^6 generations, and every 500th tree was sampled after a burn-in of 10^5 generations. Posterior probabilities for each branch were calculated from the sampled trees. Second, an NJ analysis with 1000 bootstrap replicates was conducted using PAUP 4.0b10. Third, an unweighed maximum parsimony (MP) analysis with 1000

bootstrap replicates was performed using PAUP 4.0b10 with heuristic searches, random starting trees, 10 random additions of sequences, and TBR branch swapping. Because of the extensive computational time, the maximum number of trees saved was limited to 1000 in the COI dataset in this procedure.

For the combined dataset of COI, ND1, and 16S rRNA, Tamura–Nei's model (Tamura and Nei, 1993) with invariable sites of 0.5446 and a gamma shape parameter of 0.9102 (TrN + I + G) was the most-appropriate model of DNA substitution. Parameters were set to unequal base frequencies (A, 0.3473; C, 0.2200; G, 0.1294; and T, 0.3033), unequal transition rates (A–G, 9.4422; and C–T, 11.2124), and equal transversion rates (1.0000). The ML analyses were performed using random starting trees and 100 random additions of sequences with the other settings the same as those used in the COI and 16S rRNA analyses. In addition, the same three approaches used in the 16S rRNA analysis were employed to evaluate the reliability of the inferred phylogenetic tree except 100 instead of 10 random additions of sequences were used in the unweighed MP analysis.

The ML tree with the highest $\ln(L)$ score was compared to alternative tree hypotheses. The Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) was performed using PAUP 4.0b10 with 1000 bootstrap replications.

3. Results

3.1. Sequence characteristics

For the 5' fragment of COI, 94 haplotypes were observed from 104 individuals of the *M. formosae* species group (Table 1). All haplotypes were 535 bp in length, without insertions or deletions. Within the *M. formosae* species group, the mean interspecific sequence divergences ranged from 12.9% (*M. bununa* vs. *M. feijani*) to 27.7% (*M. trutina* vs. *M. yuhsii*) (Table 3).

Table 3
Mean interspecific sequence divergences of the *Metaphire formosae* species group calculated using the most-appropriate model of DNA substitution based on the COI sequences (lower left) and the combined sequences of COI, ND1, and 16S rRNA (upper right)

	MSP	FEI	PAI	BUN	TAH	TRU	TAI	GLA	NAN	YUH	FOR
MSP		0.086	0.087	0.095	0.102	0.105	0.109	0.110	0.114	0.109	0.125
FEI	0.151		0.086	0.088	0.095	0.110	0.107	0.107	0.111	0.116	0.120
PAI	0.164	0.147		0.080	0.086	0.091	0.096	0.100	0.104	0.105	0.115
BUN	0.192	0.129	0.156		0.086	0.095	0.097	0.100	0.099	0.110	0.115
TAH	0.201	0.155	0.156	0.152		0.093	0.105	0.102	0.095	0.112	0.121
TRU	0.241	0.241	0.190	0.184	0.174		0.097	0.109	0.098	0.113	0.125
TAI	0.256	0.230	0.225	0.206	0.217	0.241		0.105	0.099	0.110	0.122
GLA	0.224	0.226	0.197	0.204	0.216	0.222	0.253		0.095	0.108	0.116
NAN	0.232	0.178	0.234	0.199	0.192	0.232	0.228	0.200		0.109	0.117
YUH	0.216	0.205	0.223	0.193	0.197	0.277	0.275	0.222	0.224		0.122
FOR	0.226	0.222	0.193	0.207	0.215	0.249	0.233	0.242	0.239	0.228	

Abbreviations: MSP, *Metaphire* sp.; FEI, *M. feijani*; PAI, *M. paiwana*; BUN, *M. bununa*; TAH, *M. tahanmonta*; TRU, *M. trutina*; TAI, *M. taiwanensis*; GLA, *M. glareosa*; NAN, *M. nanaensis*; YUH, *M. yuhsii*; FOR, *M. formosae*.

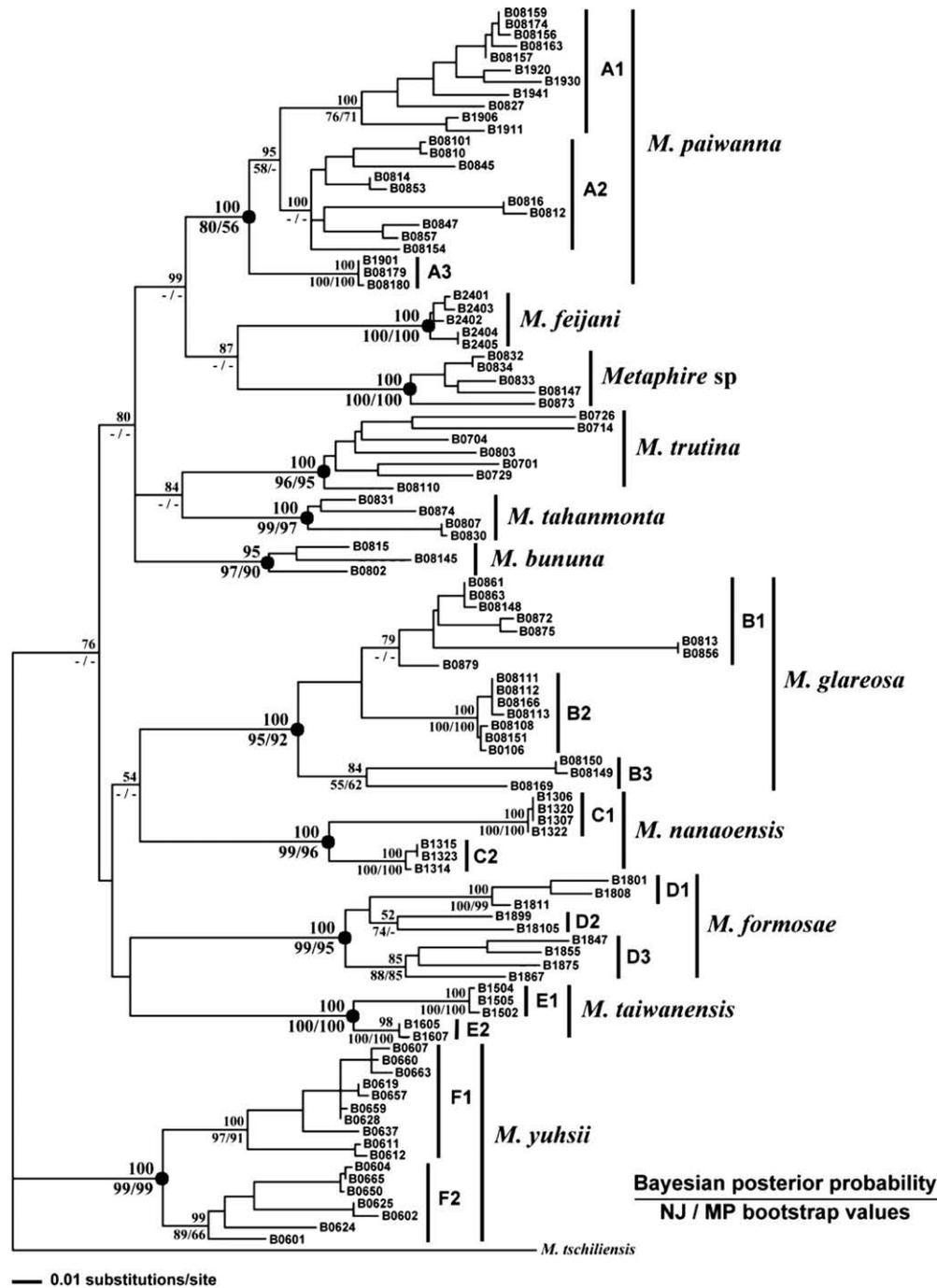


Fig. 1. One of the two maximum likelihood trees of the *Metaphire formosae* species group based on the cytochrome c oxidase subunit I (COI) gene. The two trees differed only slightly in arrangements of specimens within *M. paiwana liliumfordi*, and hence only one tree is shown. Nodes for each species identified are emphasized with black dots. Intraspecific clades with allopatric distributions are identified in *M. paiwana* (A1~A3), *M. glareosa* (B1~B3), *M. nanaoensis* (C1 and C2), *M. formosae* (D1~D3), *M. taiwanensis* (E1 and E2), and *M. yuhsii* (F1 and F2), and their supporting values are presented.

The lengths of the *M. formosae* species group 16S rRNA gene haplotypes ranged from 385 to 388 bp. The lengths of the pheretimoid earthworm 16S rRNA gene haplotypes ranged from 381 to 388 bp. The total aligned sequences were 395 bp, with insertions or deletions of 1~2 bp.

The lengths of the combined sequences ranged from 2435 to 2442 bp, including 456~464 bp from the 16S rRNA gene, 1,056 bp from the COI gene, and 922 or 925 bp from the ND1 gene. The aligned sequences were 2453 bp, with insertions or deletions of 1~4 bp due to a 3-bp insertion of the *L. terrestris* ND1 gene (corresponding to an amino acid) and variations in the 16S rRNA gene fragments. Within the *M. formosae* species group, interspecific sequence

divergences ranged from 8% (*M. paiwana* vs. *M. bununa*) to 12.5% (*M. formosae* vs. *M. trutina* and *M. formosae* vs. *Metaphire* sp.) (Table 3).

3.2. Phylogenetic analyses

In the analyses using the 5' fragment of COI, the ML analysis resulted in two trees with the highest $\ln(L)$ score of -6202.47. The two trees differed only slightly in arrangements of specimens within *M. paiwana liliumfordi*, and hence only one tree is shown (Fig. 1). The posterior probabilities from the Bayesian analysis and the bootstrap values from the MP and NJ analyses were plotted on the ML tree (Fig. 1). The phylogenetic analysis based on the 5'

Table 4

Mean intraspecific sequence divergences between clades within *Metaphire paiwana*, *M. glareosa*, *M. nanaoensis*, *M. formosae*, *M. taiwanensis* and *M. yuhsii* calculated using the most-appropriate model of DNA substitution based on the COI sequences

Species	Clade pair	Sequence divergence
<i>M. paiwana</i>	A1 and A2	0.083
	A1 and A3	0.095
	A2 and A3	0.094
<i>M. glareosa</i>	B1 and B2	0.081
	B1 and B3	0.118
	B2 and B3	0.135
<i>M. nanaoensis</i>	C1 and C2	0.090
<i>M. formosae</i>	D1 and D2	0.093
	D1 and D3	0.118
	D2 and D3	0.106
<i>M. taiwanensis</i>	E1 and E2	0.057
<i>M. yuhsii</i>	F1 and F2	0.120

fragment of COI revealed that there are 11 monophyletic groups, corresponding to 11 species, within the *M. formosae* species group (Fig. 1). These monophyletic groups were largely congruent with morphological species previously identified but with a few exceptions. A cryptic species morphologically identified as *M. paiwana* was discovered, namely *Metaphire* sp. *M. bununa glareosa* was elevated to a specific status, namely *M. glareosa* (see Discussion). In addition, intraspecific clades for *M. paiwana*, *M. taiwanensis*, *M. nanaoensis*, *M. formosae*, *M. yuhsii*, and *M. glareosa* were defined (Fig. 1), and their genetic divergences were estimated (Table 4) for further discussion. However, although the COI

fragment proved to be useful in species clustering, it showed poor resolution for interpreting the interspecific relationships.

In the analyses using 16S rRNA, the ML analysis resulted in a tree with the highest ln(L) score of –3657.68 (Fig. 2). The posterior probabilities from the Bayesian analysis and bootstrap values from the MP and NJ analyses were plotted on the ML tree (Fig. 2). The analysis of the 16S rRNA gene sequence supported the *M. formosae* species group being monophyletic within the *Pheretima* complex. Furthermore, the monophyly of the *Pheretima* complex within the Megascolecidae was also supported (Fig. 2).

In the combined analyses using COI, ND1, and 16S rRNA, the ML analysis resulted in a tree with the highest ln(L) score of –13389.88 (Fig. 3). The posterior probabilities from the Bayesian analysis and the bootstrap values from the MP and NJ analyses were plotted on the ML tree (Fig. 3). In addition, three groups within the *M. formosae* species group were defined for further discussion: at the basal part of the inferred phylogenetic tree is the western hill group, including *M. formosae* and *M. yuhsii*; the monophyletic eastern mountain group includes *M. nanaoensis* and *M. glareosa*; the monophyletic western mountain group includes *M. paiwana*, *M. bununa*, *M. trutina*, *M. taiwanensis*, *M. tahanmonta*, *M. feijani* and *Metaphire* sp. (Fig. 3).

To test if *M. paiwana* and *Metaphire* sp. comprise a monophyletic group, the SH test was applied. In addition, the monophyly of species with dorsally positioned spermathecal pores, *M. formosae* and *M. yuhsii*, was also tested using the SH test. The constrained topologies inferred from these monophyly hypotheses were compared with the topology of the ML tree derived from the combined

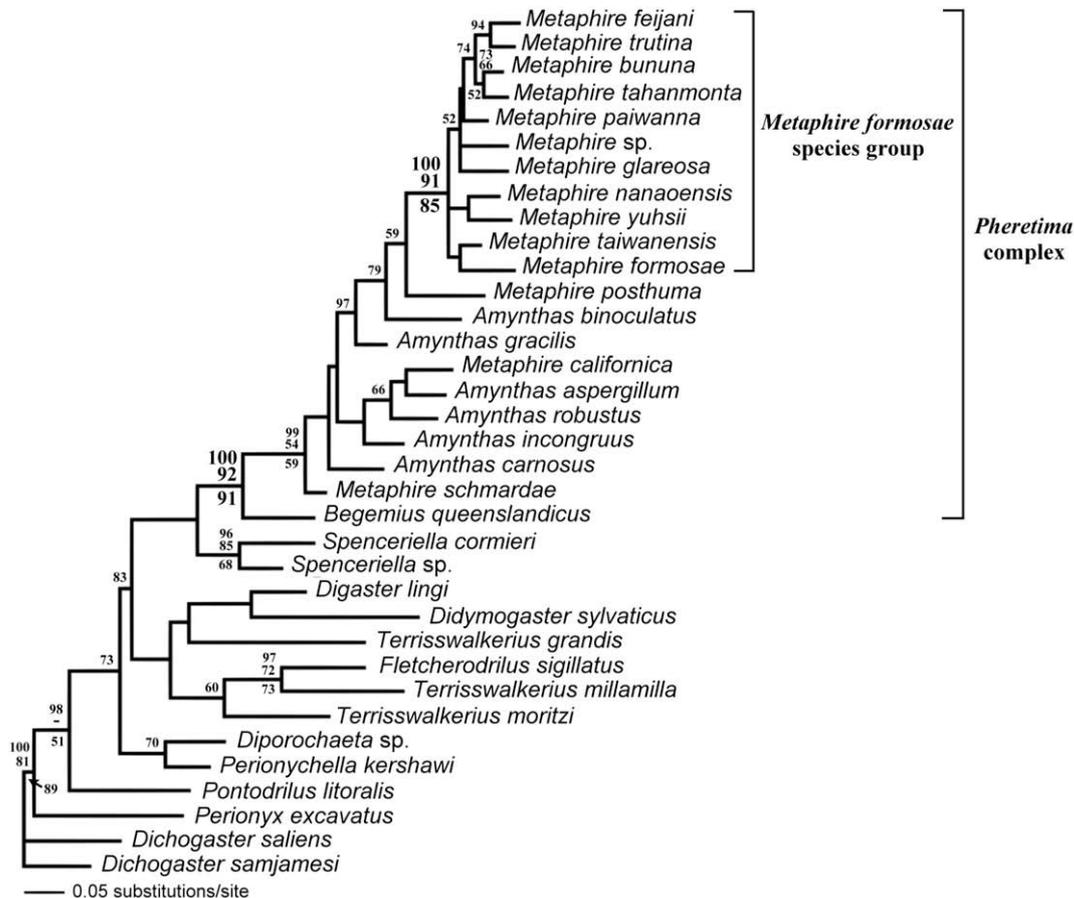


Fig. 2. Maximum likelihood tree of *Metaphire*, *Amynthus*, and *Begemius* of the *Pheretima* complex and some other megascolecoid earthworms based on the 16S rRNA gene. The *Pheretima* complex forms a monophyletic group within the Megascolecidae, and the monophyly of the *M. formosae* species group is also supported. When three numbers are shown around the nodes, the numbers above the branches are Bayesian posterior probabilities followed by neighbor joining bootstrap values, and the numbers below branches are maximum parsimony bootstrap values; values <50 are marked as '-'. When only one number is shown above the branch, this number is the Bayesian posterior probability, and the neighbor joining and maximum parsimony bootstrap values were both <50. For some nodes, the three values were all <50, and no numbers are presented.

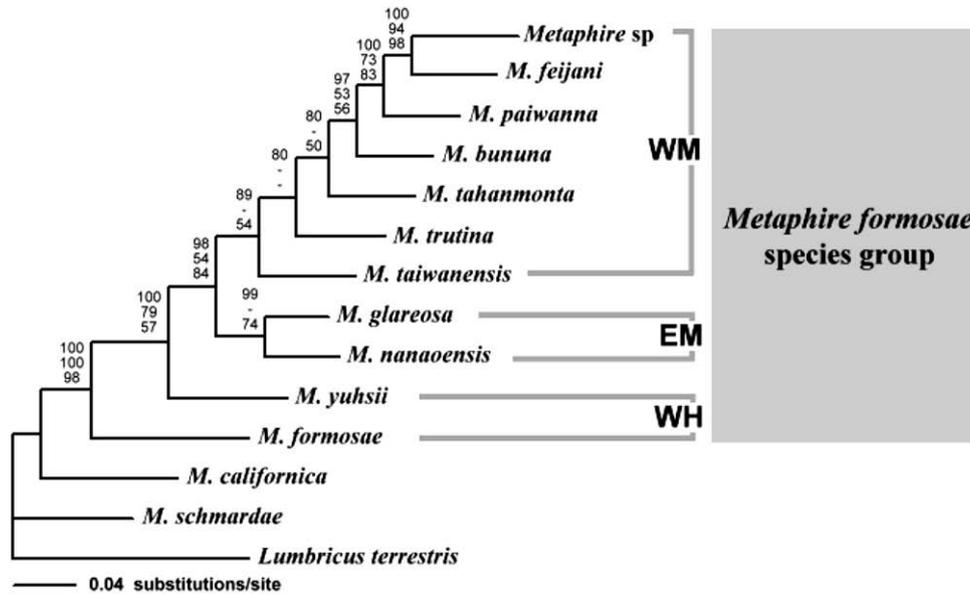


Fig. 3. Maximum likelihood tree of the *Metaphire formosae* species group based on the combined sequences of cytochrome c oxidase subunit I (COI), NADH dehydrogenase subunit 1 (ND1), and 16S rRNA. Numbers above the branches are Bayesian posterior probabilities, neighbor joining bootstrap values, and maximum parsimony bootstrap values. Values <50 are marked as '-'. Three groups were defined for further discussion: WM, western mountain group; EM, eastern mountain group; WH, western hill group.

Table 5

Comparison of the ML tree ($\ln(L)$ score = -13389.88) with the constraint trees using the Shimodaira–Hasegawa test

Topological constraint	$-\ln L$	Diff. $-\ln L$	p value
Monophyly of <i>M. paiwana</i> and <i>Metaphire sp.</i>	13406.37	16.49	0.02 ^a
Monophyly of <i>M. formosae</i> and <i>M. yuhsii</i>	13398.76	8.88	0.039 ^a

^a Topologies significantly worse than the ML tree ($\alpha = 0.05$).

analyses. The result indicated that all of the constrained topologies were significantly worse than those of the ML tree (Table 5).

4. Discussion

4.1. Systematics of the *M. formosae* species group and the *Pheretima* complex

According to the phylogenetic analyses, the *M. formosae* species group is composed of at least 11 species, including ten described species and a cryptic species previously identified as *M. paiwana paiwana*. In addition, the monophyly of the 11 species was strongly supported. Among them, *M. formosae* and *M. yuhsii* have spermathecal pores near the central dorsal lines, a rare feature in the *Pheretima* complex (Chang and Chen, 2005b). This unique feature, together with other similarities in morphology, resulted in our hypothesizing the monophyly of these two species. However, this hypothesis was rejected by the SH test. Similarly, although *Metaphire sp.* is a cryptic species morphologically similar to *M. p. paiwana*, the monophyly of the two species was also rejected by the SH test.

Metaphire sp. is morphologically similar to *M. p. paiwana*. However, this species has a smaller body size, less apparent horizontal ridges in the male pore areas, and more regularly coiled spermathecal diverticulum stalks. In addition, the two species require different habitats: *M. p. paiwana* lives in evergreen broadleaf forests, while *Metaphire sp.* lives in deciduous broadleaf forests at higher elevations where *M. p. paiwana* has never been found (Fig. 4).

In the original description, *M. bununa glareosa* was described based on four specimens, but these specimens were destroyed in a strong earthquake on 21 September 1999 that devastated central Taiwan. Our phylogenetic analyses do not support the monophyly of *M. b. bununa* and *M. b. glareosa*. On the contrary, two independent monophyletic groups corresponding to *M. b. bununa* and *M. b. glareosa* are supported (Figs. 1 and 3). Moreover, the average genetic distances between the two taxa are equivalent to those among species (Table 3). Therefore, *M. b. glareosa* should be elevated to specific status, namely *M. glareosa*.

According to the phylogenetic analysis, *M. hengchunensis*, *M. p. paiwana* and *M. p. liliumfordi*, which correspond to clades A1, A2 and A3 in Fig. 1, respectively, are supported to form a monophyletic group (Fig. 1). The genetic distances between *M. hengchunensis* and each of *M. p. paiwana* and *M. p. liliumfordi* (9.5% and 9.4%, respectively), are almost equivalent to that between *M. p. paiwana* and *M. p. liliumfordi* (8.3%), and are obviously lower than interspecific distances within the *M. formosae* species group (Table 3). Morphologically, the type specimens of *M. hengchunensis* are almost indistinguishable from specimens of *M. p. paiwana* examined in this study, except that the seminal grooves and oval pads in the male pore areas of *M. hengchunensis* are slightly degenerated. In addition, the three taxa show an allopatric distribution. Altogether, we strongly suggest that *M. hengchunensis* should be regarded as one of the subspecies of *M. paiwana*, namely *M. paiwana hengchunensis*.

In the *M. formosae* species group, *M. trutina* and *M. tahanmonta* are holandric (with two pairs of testes, one each in segments 10 and 11), while other species are proandric (with only one pair of testes in segment 10). There are two hypotheses with two steps of changes that may explain this character evolution. In one hypothesis, *M. tahanmonta* and *M. trutina* independently acquired the second pair of testes (Fig. 5, left). Alternatively, in the other equally parsimonious hypothesis, the common ancestor of *M. tahanmonta* and *M. trutina* acquired the second pair of testes, and then the common ancestor of *M. bununa*, *M. paiwana*, *M. feijani* and *Metaphire sp.* lost it (Fig. 5, right). Although the predominance of holandry in pheretimoid earthworms implies that this character state may be plesiomorphic, a hypothesis generally accepted

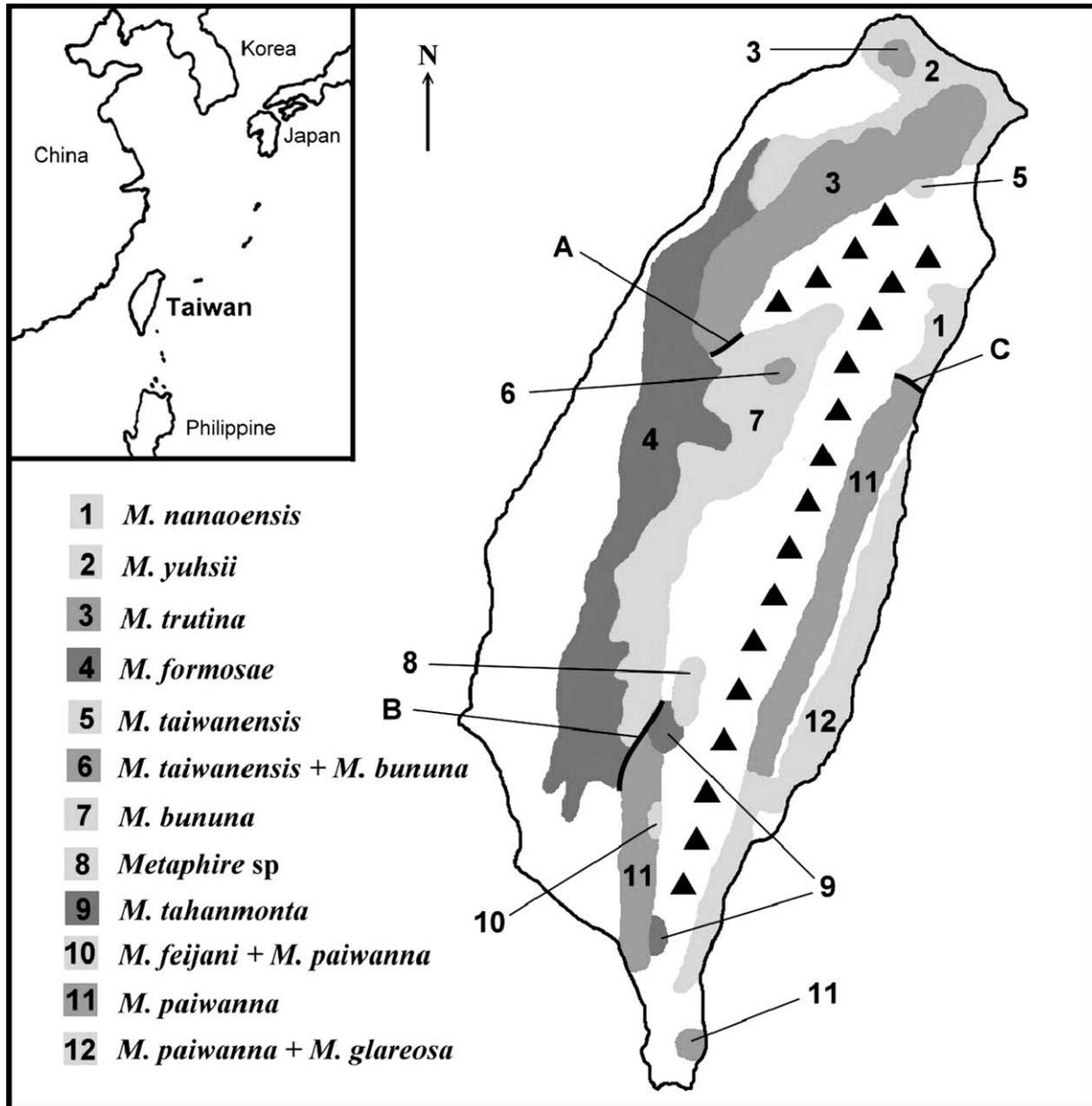


Fig. 4. Location of Taiwan and the distribution of the *Metaphire formosae* species group on the island. Closed triangles indicate the general positions of the higher parts of the Central Mountain Range. Neighboring species not separated by a black line have a contact zone, which is not shown for clarity. The black lines indicate geographic barriers between species: A, Shuei-Shan Mountain Ridge; B, Launong River; C, Liwu River.

among earthworm taxonomists, the case of the *M. formosae* species group suggests that holandry can also be an apomorph. Moreover, the fact that testis condition changes even among closely related species indicates that this character is highly variable and cannot be used in grouping earthworm species within a genus. Therefore, the sub-grouping within a genus of the *Pheretima* complex, which is partly based on testis condition (Sims and Easton, 1972), may be inconsistent with the phylogeny. In addition, based on morphological phenetic analyses conducted about 30 years ago (Easton, 1979, 1982; Sims and Easton, 1972), the generic divisions in the *Pheretima* complex were recently challenged by overlapping diagnostic characters and serious homoplasy (Blakemore, 2002; James, 2005a). This opinion is further supported by the non-monophyly of *Amyntas* and *Metaphire* as revealed in the present study, as well as some other pheretimoid genera in previous DNA analyses (James, 2005b). All the evidence suggests the urgent need to revise the systematics of the *Pheretima* complex.

4.2. Phylogeography

Taiwan is a mountainous island about 170 km off the southeastern coast of China. The Central Mountain Range (CMR) runs north-south throughout the center of the island, with more than 200 mountain peaks exceeding 3000 m; along the southeast coast lies the Coastal Mountain Range, in which the majority of the mountain peaks are about 1000 m. The main island of Taiwan is the result of a collision between the Luzon Volcanic Arc and the Eurasian Continental Margin between 5 and 2.5 million years ago (Ma), a geological event known as Penglai Orogeny (Huang et al., 1997, 2000; Teng, 1990;), and is still rising at present (Huang et al., 1997, 2000).

The mountains and the rivers in the CMR have long been considered as major factors resulting in intraspecific genetic differentiation of many terrestrial and freshwater animals in Taiwan, such as mice (Hsu et al., 2001), lizards (Liu, 1995), frogs

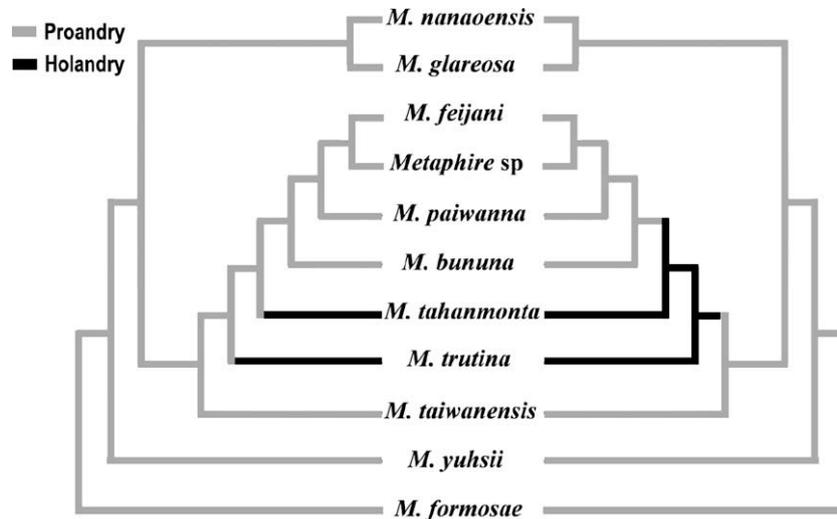


Fig. 5. Two equally parsimonious hypotheses of evolution of the testis condition in the *Metaphire formosae* species group. The two alternative hypotheses demonstrate how holandry (two pairs of testes, one each in segment 10 and 11) derives from proandry (one pair of testes in segment 10) and *vice versa*: in the first hypothesis, *M. tahanmonta* and *M. trutina* independently acquired the second pair of testes (left); alternatively, the common ancestor of *M. tahanmonta* and *M. trutina* acquired the second pair of testes, and then the common ancestor of *M. bununa*, *M. paiwana*, *M. feijani* and *Metaphire* sp. lost it (right).

(Yang et al., 1994; Toda et al., 1997), spiders (Lin et al., 1999), crabs (Shih et al., 2006), and earthworms (Chang and Chen, 2005b). For instance, Chang and Chen (2005b) proposed that the formation of the ancient Tamsui River about 2.5 Ma in northern Taiwan caused the divergence between the two populations of *M. yuhsii* (re-revealed as clades F1 and F2 in Fig. 1). Similarly, clades C1 and C2 of *M. nanaoensis* isolated by the Heping River in northeastern Taiwan shows another case of river-driven genetic differentiation. Except the two cases of *M. yuhsii* and *M. nanaoensis*, intraspecific genetic differentiation has been observed among geographically isolated populations of four more species in the *M. formosae* species group, including *M. paiwana* (clades A1~A3), *M. glareosa* (clades B1~B3), *M. formosae* (clades D1~D3), and *M. taiwanensis* (clades E1 and E2). This genetic structure is clear evidence that the formation of the mountains and the rivers in the CMR has resulted in intraspecific genetic differentiation in this species group.

In Taiwan, most species of the *M. formosae* species group are allopatrically distributed (Fig. 4). Furthermore, in the inferred phylogenetic tree, species of the eastern mountain group are distributed east of the CMR, those of the western mountain group are distributed west of the CMR, except *M. paiwana*, and those of the western hill group are distributed in the western foothills to the west of the CMR (Figs. 3 and 4). This pattern suggests that allopatric speciation may be the major mechanism driving the species diversity of this group. Using the estimated formation time of the ancient Tamsui River and the genetic divergence within *M. yuhsii* (Table 4), we can roughly estimate the evolutionary rate of earthworm COI genes as 4.8% per million years, and consequently, the speciation events of the *M. formosae* species group are estimated to have occurred between 5.8 and 2.7 Ma. This inferred time range is congruent with the period of the Penglai Orogeny, during which the rapidly formed mountains and rivers were geographical barriers for many flightless invertebrates. Therefore, by combining the geological and phylogenetic evidence, we herein propose a vicariance hypothesis to explain the allopatric speciation events of the *M. formosae* species group. In this hypothesis, the ancestors of these species arrived in Taiwan before the rapid uplift of this island, probably during the late Miocene, and then dispersed throughout this island. During the period of rapid uplift between 5.0 and 2.5 Ma, different populations of the ancestral species were

rapidly isolated by the mountains and the rivers that formed due to orogenesis. This isolation resulted in genetic differentiation and ultimately caused speciation of the *M. formosae* species group.

The endemic land fauna diversity in Taiwan was generally attributed to be consequences of multiple dispersal-isolation events between Taiwan and the surrounding regions (Lin et al., 2002; Ota, 1997; Ota et al., 2002; Tu et al., 2000; Yeh et al., 2004), while the contribution of the CMR and vicariance events during Penglai Orogeny to speciation of animals on this island has never been well investigated before. Our present study is the first case that demonstrates within-island speciation of animals through vicariance events caused by orogenesis in Taiwan. Considering the diverse endemic invertebrate fauna awaiting being discovered and investigated, the present case may be one of the tremendous amounts of similar stories among the endemic fauna on this island.

Acknowledgments

We are grateful to the persons who kindly assisted us in the collection of earthworm samples. We are also grateful to Drs. S. James, C.-F. Tsai and H.-P. Shen and Mr. C.-C. Huang for their helpful comments on the manuscript. This study was supported by the National Science Council of Taiwan (NSC92-2621-B-002-019) to J.-H. Chen.

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