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Four new earthworm species and subspecies belonging to genus *Amynthas* and *Metaphire* (Oligochaeta: Megascolecidae) from Hainan Island, China

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Abstract

Three new species and one new subspecies of earthworms from Hainan Island, China, are described: *Amynthas dinganensis* sp. nov., *Amynthas tenuis* sp. nov., *Metaphire wuzhimontis* sp. nov. and *Metaphire magna minuscula* subsp. nov. *Amynthas dinganensis* and *Amynthas tenuis* have two pairs of spermathecal pores in 5/6–6/7, and *Metaphire wuzhimontis* and *Metaphire magna minuscula* have two pairs of spermathecal pores in 7/8–8/9. COI and 16S gene fragments of the new species taxa have been sequenced. We provide comparisons between these new species taxa and related species using morphological and molecular characters.

Key words: Earthworms, Clitellata, *Amynthas*, *Metaphire*, new species, Hainan Island, China

Introduction

Hainan Island (N 18°10'04"–20°9'40", E 108°36'43"–111°2'31") is the second largest island in China, separated from Leizhou Peninsula in Guangdong province to the north by the shallow and narrow Qiongzhou strait. To-date, and prior to our investigations, 45 species of earthworms were known from Hainan Island (Chen 1938; Quan 1985; Quan & Zhong 1989; Blakemore 2007; Zhao *et al.* 2009; Sun *et al.* 2009, 2010). From 2006 to 2011, we conducted four field surveys on earthworm diversity in Hainan Island. Our findings raise the number of species and subspecies from Hainan to 49. In this paper we describe three new species and one new subspecies of the genera *Amynthas* and *Metaphire* from Dongjiao Coco Forest and Wuzhi Mountain in Hainan Island, found in our last survey in May 2011. They are named *Amynthas dinganensis* sp. nov., *Amynthas tenuis* sp. nov., *Metaphire wuzhimontis* sp. nov. and *Metaphire magna minuscula* subsp. nov.

Wuzhi Mountain is in the center of Hainan Island. It is not only the highest mountain, but also with the highest original tropical forests on the island. Biodiversity is rich in this mountain, especially regarding rare and endemic animal species. Dongjiao Coco Forest lies in Dongjiao County, eastern Wenchang City. It has a northern tropical oceanic monsoon climate, with a mean annual temperature of 24.4°C and a mean annual precipitation of 1529.8mm. Soil is seashore sandy soil with high level of moisture (Tang *et al.* 2006).

Holotypes and paratypes are deposited in the Shanghai Natural History Museum, Shanghai, China (accession numbers starting with "C-") and at the Station Biologique, Université de Rennes I, France (accession numbers starting with "F-").

Material and methods

All earthworm species and subspecies described in this article were collected from Hainan Island in May 2011. Specimens were fixed and preserved in 95% ethanol solution, except the holotype of *Metaphire magna minuscula*

which was fixed and preserved in 4% formalin. All specimens were investigated with a dissecting microscope (10×1–6.5, Nikon SMZ800); species and subspecies were described including external characteristics (pigment, body length, body width, segments, setae, prostomium, clitellum, male pore region, female pore and spermathecal pore region) and internal structures (septa, gizzard, intestine, esophageal hearts, spermathecae, testis-sacs, seminal vesicles, prostate glands and accessory glands).

Fragments of the muscular body wall were cut from the tail of each individual for DNA extraction, the formalin-preserved holotype of *M. magna minuscula* sp. nov. excepted. All DNA extractions were performed using EZgene™ kits or Chelex, following the manufacturer's protocol for invertebrate tissue samples. The isolated DNA was stored at -20°C. Fragments of two mitochondrial genes (COI and 16S) were sequenced using published primers: COI: LCO1490, HCO2198, HCO2198 advanced (Folmer *et al.*, 1994; Bely & Wray 2004); 16S: Sar, Sbr (Hillis & Moritz 1990). The amplifications (total volume 50 µL) contained 1 µL DNA. The 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, reaction mixtures were subjected to electrophoresis in 1.5% agarose gels. All PCR products were sequenced by the Beijing Genomics Institute, Shanghai Branch, China, or in Roscoff, Geopôle Ouest, in France.

The sequence alignment was done among similar individuals by BLAST. Sequences of the COI and 16S gene fragments of the holotypes of the new species and of a paratype of the new subspecies were deposited in NCBI (Table 1). In addition, COI and 16S sequences of one specimen of *Metaphire magna magna* Chen, 1938 (HN201112-01) were obtained as well, and deposited in NCBI (Table 1). The specimen was collected in our fieldwork in 2011 in Qiongzong County of Hainan Island. NJ tree of combined 16S and COI sequences, K2P distances of COI sequences and combined 16S and COI sequences among *Metaphire* species (China mainland, Taiwan Island and Hainan Island) were calculated with the software MEGA 5.0 (Table 2).

TABLE 1. NCBI accession numbers of sequence fragments of earthworm species and subspecies generated or compared in this study.

	COI	16S
Sequences generated in this study:		
<i>Amyntas dinganensis</i> sp. nov.		JQ904530
<i>Amyntas tenuis</i> sp. nov.		JQ904531
<i>Metaphire wuzhimontis</i> sp. nov.	JQ904537	JQ904532
<i>Metaphire magna minuscula</i> sp. nov.	JQ904536	JQ904533
<i>Metaphire magna magna</i> (Chen, 1938)	JQ904535	JQ904534
Sequences used for comparison:		
<i>Metaphire californica</i> (Kinberg, 1867)	AY739339	AY960823
<i>Metaphire schmardae schmardae</i> (Horst, 1883)	AY739340	AY960824
<i>Metaphire tschiliensis tschiliensis</i> (Michaelsen, 1928)	DQ835677	EF490525
<i>Metaphire formosae</i> (Michaelsen, 1922)	AY739334	AY960820
<i>Metaphire bununa</i> Tsai <i>et al.</i> , 2000	AY739337	AY960817
<i>Metaphire yuhsii</i> (Tsai, 1964)	AY739324	AY960812
<i>Lumbricus rubellus</i> Hoffmeister, 1843	HE611683	HE611675
<i>Lumbricus terrestris</i> Linnaeus, 1758	HQ024638	HE611669

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Descriptions

Amyntas dinganensis Qiu & Zhao, sp. nov.

(Fig. 1, Table 2)

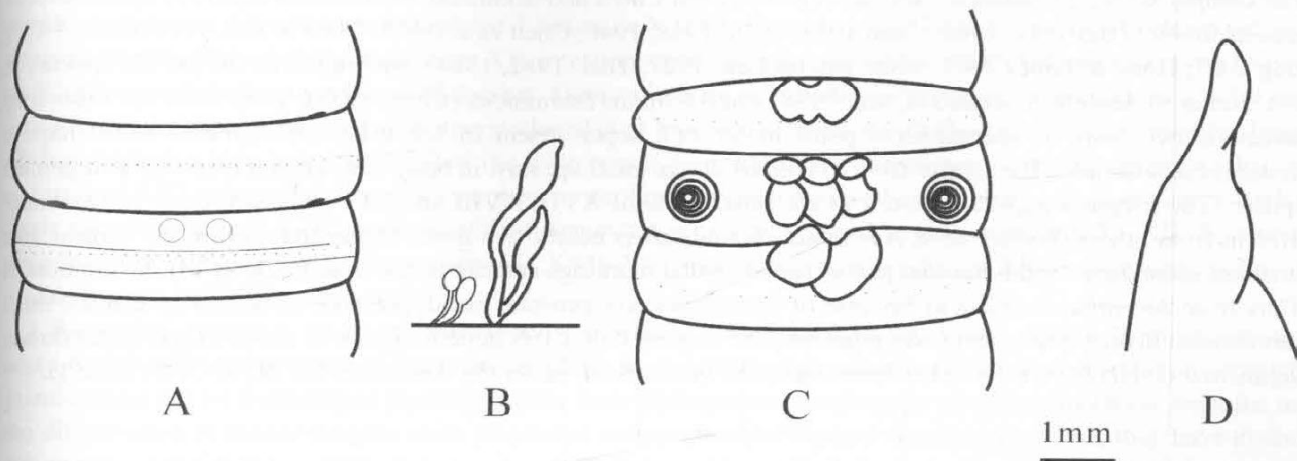


FIGURE 1. *Amyntas dinganensis* sp. nov. **A.** Ventral body surface with spermathecal pores. **B.** Spermatheca. **C.** Ventral body surface with male pores. **D.** Intestinal caeca.

Material. Holotype one clitellate (F-HN201116-04A), China, Hainan Province, Longmeng Town (19°26'44"N, 110°22'18"E), 739m a.s.l., soil, coll. J.P. Qiu, Q. Zhao, J.B. Jiang, D. Cluzeau, W.K. Zhang, 27 May 2011. Paratypes two clitellates (F-HN201116-04B, F-HN201116-05), same data as for holotype.

Locality and habitat. The specimens were collected under rubber plantation of Longmeng Town, Ding'an County, Hainan province, China.

Etymology. This species is named after its location.

Diagnosis. Dimensions 77–91.5 mm by 3.5–4.4 mm at clitellum, segments 107–138. Setae 40–56/III, 46–56/V, 48–60/VIII, 40–48/XX, 42–44/XXV; 10–13 between male pores; 30–32 between spermathecal pores. Spermathecal pores in 5/6–6/7, less than 0.5 circumferences ventrally apart. Male pores on XVIII, less than 0.5 body circumferences apart, ventrally placed and surrounded by several skin folds.

Description. Preserved specimens dorsally light brown before clitellum and brown behind clitellum, grey ventrally. One paratype specimen dorsally without pigmentation before clitellum and light red brown behind clitellum, pale ventrally. Dimensions 77–91.5 mm by 3.5–4.4 mm, segments 107–138; body cylindrical in cross-section, gradually tapered towards head and tail. Prostomium epilobous. Setae numbering 40–56/III, 46–56/V, 48–60/VIII, 40–48/XX, 42–44/XXV; 10–13 between male pores; 30–32 between spermathecal pores, setal formula AA=1.0–1.2AB, ZZ=1.0–1.5ZY. Clitellum annular XIV–XVI, grey or pale; setae not seen externally on clitellum. First dorsal pore 11/12. Two pairs of spermathecal pores in 5/6–6/7, ventral, eye-like, less than 0.5 body circumferences apart. A pair of round markings anterior to setae circle of VII, about 0.14 body circumferences apart. Genital markings absent in one paratype specimen. Male pores paired in XVIII, a little less than 0.5 body circumferences ventrally apart, surrounded by four to six circular folds. Irregular papillae present anterior to setae circles of XVII, XVIII and XIX, which constitute five to seven big gland protuberances. Female pore single in XIV.

Septa 5/6–7/8 comparatively thick and muscular, 10/11 thick (10/11 thin in one specimen of paratype), 11/12–12/13 thin, 8/9–9/10 absent. Dorsal blood vessel single, continuous onto pharynx; esophageal hearts in X–XIII, first pair small. Gizzard in IX–X, ball-shaped; intestine enlarged distinctly from XVI; intestinal caeca simple, originating in XXVII or XXVIII and extending forward to XXIV or XX, one or two big incisions on dorsal margins, smooth on ventrum. Spermathecae paired in VI–VII with a gradually tapering slender duct; ampulla elongated, heart-shaped, spermathecal duct as long as 0.67–0.75 main pouch; diverticulum as long as 0.66 to 0.8 of or a little longer than or equal to main pouch, terminal 0.33–0.5 enlarged as a clavate seminal chamber; stipitate

accessory glands present in holotype. Male sexual system holandric, testis sacs two pairs in X–XI; seminal vesicles paired in XI–XII, developed, separate from each other ventrally, seminal vesicles not developed in one paratype specimen; prostates in XVIII or XVII–XVIII, degenerate, closely adherent to body wall, prostatic duct stout and long, U-shaped. Four ovoid accessory glands present in segment XVII–XVIII, attached to the body wall, without stalk.

Remarks. *Amyntas dinganensis* sp. nov. keys to the *morrissi*-group according to Sims and Easton (1972). After comparison of 27 *Amyntas* species reported from China and Southeast Asia with two pairs of spermathecal pores in 5/6–6/7 (Bai 1982, 1984; Chen 1933, 1936, 1938, 1946; Chen *et al.* 1975; Chen & Hsu 1977; Gates 1935; Hong 2007; Hong & James 2001, 2009; Michaelsen, 1927; Thai, 1982, 1984), we found that the present species is most similar to *Amyntas varians* (Chen, 1938) and *Amyntas hainanicus* (Chen, 1938). They share the following characters: two pairs of spermathecal pores in 5/6–6/7, septa absent in 8/9–9/10, spermathecae heart-shaped, intestinal caeca simple. They differ from *Amyntas dinganensis* sp. nov. in body size, genital markings and genital papillae. The irregular papillae anterior to the setae circle of XVII, XVIII and XIX make *Amyntas dinganensis* different from others. Furthermore, *Amyntas dinganensis* is nearly two times bigger than *Amyntas varians* and *Amyntas hainanicus*, and it has one pair of round genital markings anterior to the setae circle in VII. In addition, it differs from *Amyntas hainanicus* because of its degenerative prostate gland, presence of accessory glands, short spermathecal diverticulum, and body pigment. The sequence of a 16S gene fragment of the holotype of *Amyntas dinganensis* (F-HN201116-04A) has been deposited in GenBank under the Accession No. JQ904530 (Table 1).

***Amyntas tenuis* Qiu & Jiang, sp. nov.**

(Fig. 2, Table 2)

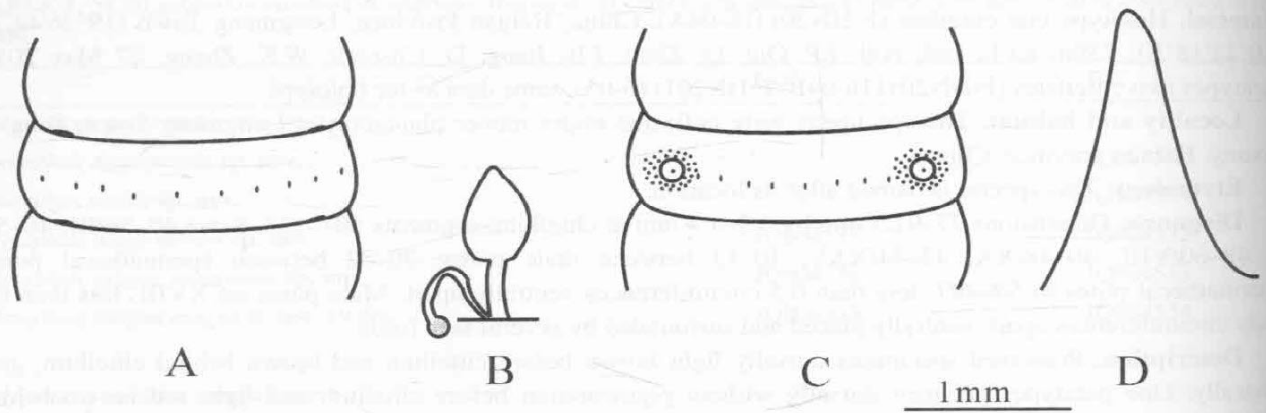


FIGURE 2. *Amyntas tenuis* sp. nov. A. Ventral body surface with spermathecal pores. B. Spermatheca. C. Ventral body surface with male pores. D. Intestinal caeca.

Material. Holotype one clitellate (C-HN201109-08), China, Hainan Province, Mt. Wuzhi (18°54'15"N, 109°41'02"E), 861m a.s.l., soil, coll. J.P. Qiu, Q. Zhao, J.B. Jiang, D. Cluzeau, W.K. Zhang, 25 May 2011. Paratype one clitellate (C-HN201110-04), same data as for holotype except that elevation is 858 m a.s.l.

Locality and habitat. The specimens were collected from core area of Wuzhishan National Nature Reserve, tropical rain forest, Hainan province, China.

Etymology. This species is named after its slim body shape.

Diagnosis. Dimensions 48–56 mm by 2 mm at clitellum, segments 72–89. Setae 12–28/III, 24–28/V, 30–36/VIII, 24–32/XX, 28–30/XXV; 8 between male pores; 10 between spermathecal pores. Spermathecal pores in 5/6–6/7, a little less than 0.33 circumferences ventrally apart. Male pores on XVIII, about 0.33 body circumferences apart, ventrally placed on an ovoid porophore.

Description. Preserved specimens from light purple to light yellow brown or pale on dorsum, no colour on ventrum. Dimensions 48–56 mm by 2 mm, segments 72–89; body cylindrical in cross-section, gradually tapered towards head and tail. Prostomium 1/2 epilobous. Setae numbering 12–28/III, 24–28/V, 30–36/VIII, 24–32/XX,

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28–30/XXV; 8 between male pores; 10 between spermathecal pores, setal formula AA=1.3–2AB, ZZ=1.3–1.5ZY. Clitellum annular XIV–XVI, no color or pale; 4 setae present in XVI or no setae ventrally. First dorsal pore in 12/13. Two pairs of spermathecal pores in 5/6–6/7, ventral, a little less than 0.33 body circumferences apart. Genital markings not present. Male pores paired in XVIII, about 0.33 body circumferences ventrally apart, placed on an ovoid porophore. Genital papillae not present in holotype, but two large ovoid genital papilla exist anterior and posterior of the ectal part of male pore in paratype. Female pore single in XIV.

Septa 5/6–6/7 comparatively thick, 7/8, 10/11–13/14 slightly thickened, 8/9–9/10 absent. Dorsal blood vessel single, continuous onto pharynx; esophageal hearts in X–XIII, first pair small. Gizzard in IX–X, elongated ball-shaped; intestine enlarged distinctly from XV; intestinal caeca simple, originating in XXVII and extending forward to XXIV, smooth both on ventrum and dorsum. Ovaries in XIII, spermathecae paired in VI–VII, about 1.5–1.9 mm; ampulla heart-shaped, about 1.1 mm, spermathecal duct long and straight, distinctly separate from ampulla; length of diverticulum a little less than or 2/3 of the main pouch, terminal 1/10 enlarged as an irregular spherical chamber or terminal 0.67 enlarged as virgulate chamber; no nephridia on spermathecal ducts. Male sexual system holandric, testis sacs two pairs in X–XI, well-developed, separated ventrally; seminal vesicles paired in XI–XII, developed, separate from each other ventrally; prostates in XVII–XX, developed, prostatic duct in XVIII, n-shaped. Accessory glands present in XVIII, with a stalk.

Remarks. *Amyntas tenuis* sp. nov. is most similar to *Amyntas infantilis* (Chen, 1938). Both species lack septa in 8/9–9/10 and first dorsal pores are in 12/13. Both of them have simple intestinal caeca and an accessory gland around the well-developed prostate glands. Both of them have an elongate ovoid spermathecal ampulla, and the diverticulum is shorter than the main pouch and with a spherical seminal chamber. However, they have distinct differences. *Amyntas tenuis* sp. nov. is more than 3 times bigger than *Amyntas infantilis*. It has two pairs of spermathecal pores in 5/6–6/7, while *Amyntas infantilis* has only one pair in 5/6. *Amyntas tenuis* sp. nov. has no genital markings, while *Amyntas infantilis* has both genital papillae and markings. The male pore is in XVIII in *Amyntas tenuis* while it is in XIX in *Amyntas infantilis*. The sequence of a 16S gene fragment of the holotype of *Amyntas tenuis* (C-HN201109-08) has been deposited in GenBank under the Accession No. JQ904531 (Table 1).

***Metaphire wuzhimontis* Qiu & Sun, sp. nov.**

(Fig. 3, Table 2)

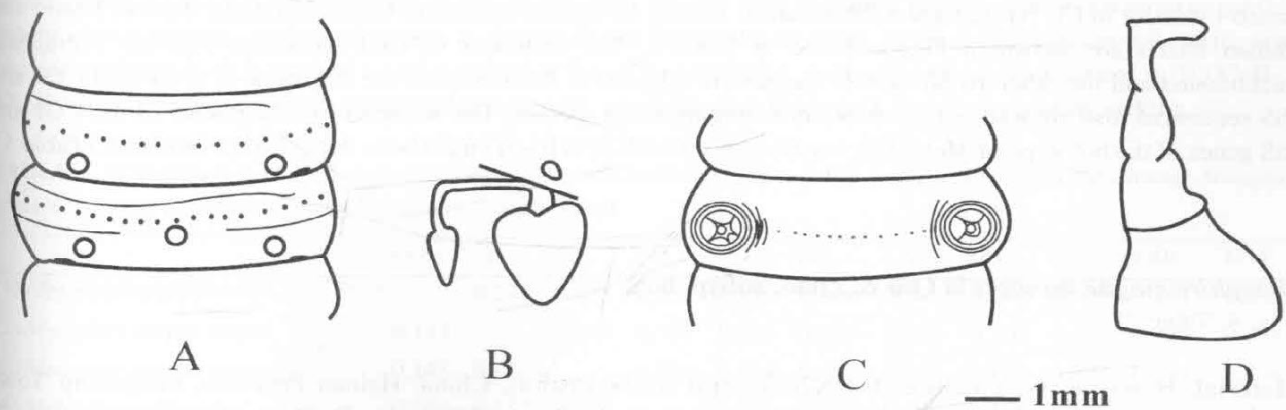


FIGURE 3. *Metaphire wuzhimontis* sp. nov. **A.** Ventral body surface with spermathecal pores. **B.** Spermatheca. **C.** Ventral body surface with male pores. **D.** Intestinal caeca.

Material. Holotype one clitellate (F-HN201110-01), China, Hainan Province, Mt. Wuzhi (18°54'15" N, 109°41'02" E), 858m a.s.l., soil, coll. J.P. Qiu, Q. Zhao, J.B. Jiang, D. Cluzeau, W.K. Zhang, 25 May 2011.

Locality and habitat. The single specimen was collected from the core area of the Wuzhishan National Nature Reserve, tropical rain forest, Hainan province, China.

Etymology. This species is named after its location.

Diagnosis. Dimensions 163 mm by 5.5 mm at clitellum, segments 94. Setae 20/III, 24/V, 40/VIII, 56/XX, 40/

XXV; 13 between male pores; 13–16 between spermathecal pores. Spermathecal pores in 7/8–8/9, less than 0.5 circumferences ventrally apart. Male pores on XVIII, less than 0.5 body circumferences apart, ventrally placed in a copulatory pouch, surrounded by five to six circular folds.

Description. Preserved specimen dark brown on dorsum, gradually lighter after clitellum, pale ventrally. Dimensions 163 mm by 5.5 mm, segments 94; body cylindrical in cross-section, gradually tapered towards head and tail. Prostomium epilobous. Setae numbering 20/III, 24/V, 40/VIII, 56/XX, 40/XXV; 13 between male pores; 13–16 between spermathecal pores, setal formula AA=1–2AB, ZZ=2ZY. Clitellum annular XIV–XVI, finger-shaped, markedly glandular, dark brown; setae cannot be seen externally in clitellum. First dorsal pore 9/10. Two pairs of spermathecal pores in 7/8–8/9, ventral, eye-like and conspicuous, less than 0.5 body circumferences apart. Each pore with a small round marking placed between the pore and the setae circle of VII and VIII, another marking with the same size placed ventro-medially on VIII. Male pores paired in XVIII, less than 0.5 body circumferences ventrally apart, each in a copulatory pouch, surrounded by five to six circular folds. Genital papillae not present. Female pore single in XIV.

Septa 5/6–7/8 comparatively thick, 10/11–11/12 slightly thickened, 8/9–9/10 absent. Dorsal blood vessel single, continuous onto pharynx; esophageal hearts in X–XIII, the first pair is small. Gizzard in IX–X, ball-shaped; intestine enlarged distinctly from XV; intestinal caeca simple, originating in XXVI and extending forward to XXIV, three distinct incisions on dorsal margins, smooth ventrally. Spermathecae paired in VIII–IX, large; ampulla peach-shaped, spermathecal duct short, as long as 0.14 main pouch; diverticulum equal to main pouch, straight, terminal 0.5 enlarged, ectal 0.33 served as an elongated seminal chamber; accessory glands present. Male sexual system holandric, testis sacs two pairs in X–XI; seminal vesicles paired in XI–XII; prostates in XV–XIX, well-developed, closely adhering to body wall, prostatic duct stout, U-shaped. Accessory glands lacking.

Remarks. *Metaphire wuzhimontis* sp. nov. keys to the *javanica*-group according to Sims and Easton (1972). There are only 5 species in the *javanica*-group in China and Southeast Asia. They are *Metaphire californica* (Kinberg, 1867), *Metaphire javanica* (Kinberg, 1867), *Metaphire longipenis* (Chen, 1946), *Metaphire magna* (Chen, 1938) (including *Metaphire magna minuscula* subsp. nov., see below), and *Metaphire prava* (Chen, 1946) (Sims & Eastern 1972; Chen 1946; Chang et al. 2009). Genetic distances of these species are compared in Table 2. *Metaphire wuzhimontis* is a moderately large species, obviously 2 to 4 times smaller than *Metaphire magna* (Chen, 1938), but nearly 3 times larger than *Metaphire longipenis* and *Metaphire prava*. Its first dorsal pore is in 9/10, different from the others. Only *Metaphire wuzhimontis* and *Metaphire magna magna* have genital markings. However, 3–6 markings in *Metaphire magna* are encircling the spermathecal pores, while in *Metaphire wuzhimontis* one pair of markings is placed between the pore and the setae circle of VII and VIII, and another one ventro-medially in IX. NJ tree and K2P distances among *Metaphire* species in China mainland, Taiwan Island and Hainan Island are shown in Figure 5 and in Table 2. The distances of COI sequences between *Metaphire wuzhimontis* and the other 10 *Metaphire* species are all > 20%. Furthermore, the distances of combined COI and 16S sequences also showed > 13% differences among these species. The sequences of fragments of the COI and 16S genes of the holotype of *Metaphire wuzhimontis* (C-HN201110-01) have been deposited in GenBank (Table 1).

***Metaphire magna minuscula* Qiu & Zhao, subsp. nov.**
(Fig. 4, Table 2)

Material. Holotype one clitellate (F-HN201116fm) (fm=formalin), China, Hainan Province, Longmeng Town (19°26'44" N, 110°22'18"E), 101m a.s.l., soil, coll. J.P. Qiu, Q. Zhao, J.B. Jiang, D. Cluzeau and W.K. Zhang, 27 May 2011. Paratype one clitellate (C-HN201116-01), same data as for holotype.

Locality and habitat. The specimens were collected under rubber plantation of Longmeng Town, Tunchan City, Hainan province, China.

Etymology. This subspecies' name indicates that it is smaller than *Metaphire magna magna*.

Diagnosis. Dimensions ?–297 mm by 10–12 mm at clitellum, segments ?–137. Setae 36–46/III, 44–46/V, 52–56/VIII, 40–60/XX, 52–78/XXV; 0–11 between male pores; 12–20 between spermathecal pores. Spermathecal pores in 7/8–8/9, about 0.4 circumferences ventrally apart. Male pores on XVIII, a little longer than 0.3 body circumferences apart, ventrally placed in a copulatory pouch, surrounded by three to four circular folds.



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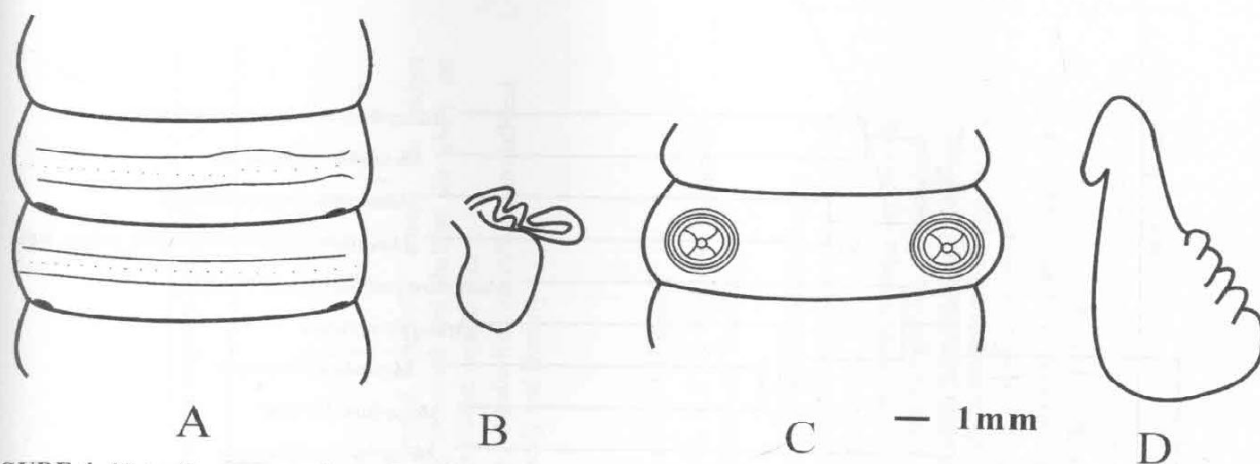


FIGURE 4. *Metaphire magna minuscula* subsp. nov. **A.** Ventral body surface with spermathecal pores. **B.** Spermatheca. **C.** Ventral body surface with male pores. **D.** Intestinal caeca.

Description. Preserved specimens dark brown on dorsum, light brown ventrally. Dimensions ? –297 mm by 10–12 mm, segments ? –137; body cylindrical in cross-section, gradually tapered towards head and tail. Prostomium epilobous. Setae numbering 36–46/III, 44–46/V, 52–56/VIII, 40–60/XX, 52–78/XXV; 0 between male pores; 12–20 between spermathecal pores, setal formula AA=1.2–1.5AB, ZZ=1–2ZY. Clitellum annular XIV–XVI, markedly glandular, dark brown; setae cannot be seen externally in clitellum. First dorsal pore in 11/12. Two pairs of spermathecal pores in 7/8–8/9, ventral, eye-like and obvious, about 0.4 body circumferences apart. Genital markings not present. Male pores are paired in XVIII surrounded by three to four circular folds, a little longer than 0.3 body circumferences ventrally apart, each in a copulatory pouch. Genital papillae not present. Female pore single in XIV.

Septa 5/6–7/8 comparatively thick and muscular, 10/11–12/13 thin, 8/9 thin, membranous, 9/10 absent. Dorsal blood vessel single, continuous onto pharynx; esophageal hearts in X–XIII, the first pair is small. Gizzard in IX–X, ball-shaped; intestine enlarged distinctly from XVI; intestinal caeca simple, originating in XXVII and extending forward to XXIV, with distinct teeth-shaped incision on dorsum, smooth ventrally. Spermathecae paired in VIII–IX, 3 mm long; ampulla heart-shaped, about 2.5 mm long, spermathecal duct as long as 0.2 main pouch; diverticulum a little longer than main pouch by 0.2 mm, weakly bent in zigzag fashion or U-curved, terminal 0.67 enlarged as a clavate seminal chamber; no nephridia on spermathecal ducts. Male sexual system holandric, testis sacs two pairs in X–XI; seminal vesicles paired in XI–XII, developed, ventrally separated; prostates in 2/3XVII–2/3XIX, small but stout, closely adherent to body wall, prostatic duct U-shaped. Accessory glands lacking.

TABLE 2. K2P distances of COI sequences (top right) and combined COI and 16S sequences (bottom left) among *Metaphire* species in China mainland, Taiwan Island and Hainan Island.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
(1) <i>Metaphire wuzhimontis</i>		0,218	0,208	0,231	0,199	0,184	0,216	0,202	0,209	0,239	0,238
(2) <i>Metaphire magna magna</i>	0,153		0,163	0,197	0,224	0,185	0,213	0,200	0,191	0,226	0,248
(3) <i>Metaphire magna minuscula</i>	0,145	0,124		0,224	0,214	0,193	0,213	0,198	0,203	0,234	0,266
(4) <i>Metaphire californica</i>	0,159	0,152	0,163		0,254	0,190	0,192	0,195	0,175	0,241	0,249
(5) <i>Metaphire schmaridae</i>	0,131	0,155	0,147	0,174		0,192	0,213	0,184	0,195	0,257	0,252
(6) <i>Metaphire tschiliensis tschiliensis</i>	0,137	0,149	0,143	0,154	0,136		0,141	0,120	0,132	0,210	0,241
(7) <i>Metaphire formosae</i>	0,159	0,168	0,165	0,148	0,168	0,132		0,126	0,108	0,208	0,238
(8) <i>Metaphire bununa</i>	0,154	0,149	0,152	0,156	0,149	0,120	0,100		0,129	0,229	0,244
(9) <i>Metaphire yuhsii</i>	0,157	0,155	0,155	0,141	0,150	0,127	0,094	0,095		0,206	0,234
(10) <i>Lumbricus rubellus</i>	0,219	0,221	0,222	0,223	0,225	0,205	0,209	0,223	0,206		0,211
(11) <i>Lumbricus terrestris</i>	0,215	0,234	0,240	0,225	0,225	0,229	0,233	0,240	0,231	0,161	

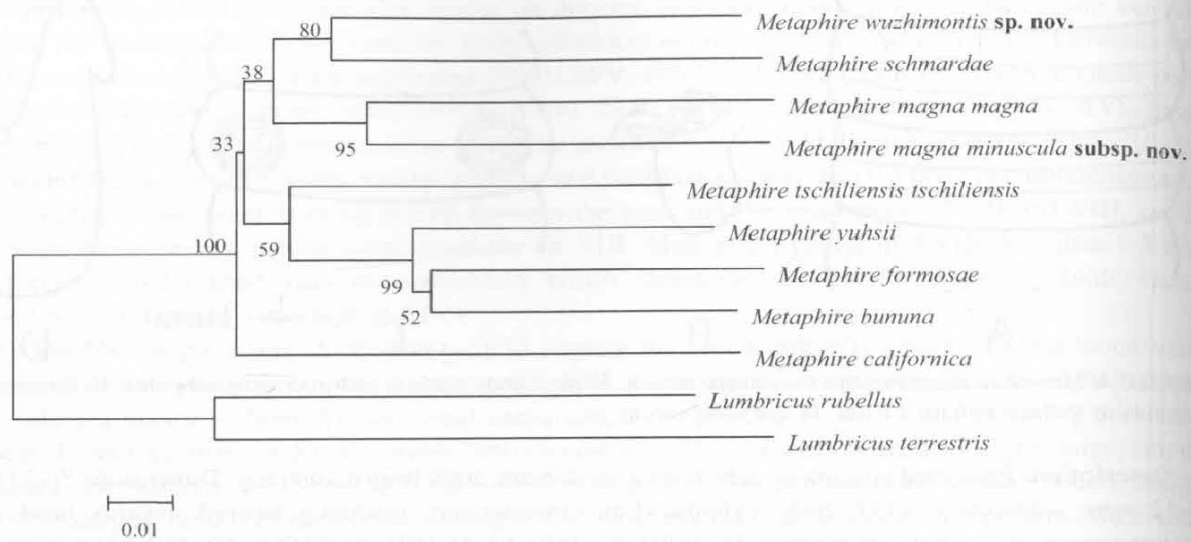


FIGURE 5. Neighbour joining (NJ) tree of *Metaphire* species based on combined COI and 16S genes.

Remarks. Our specimens agree with the diagnosis of *Metaphire magna* (Chen, 1938) in the following characteristics: body colour dark, setal formula, two pairs of spermathecal pores in 7/8–8/9, septa in 8/9 membranous, distinct teeth-shaped intestinal caeca, and type of spermathecae. However, our specimens are much smaller than described for *M. magna*, setae are fewer, and there is no accessory gland. In view of the slight but nonetheless clearcut differences, we decided to give subspecies status to our specimens. Our result is confirmed by the NJ tree and K2P distances of all the *Metaphire* species in China mainland, Taiwan Island and Hainan Island (Figure 5, Table 2). Distance of COI between *Metaphire magna minuscula* and *Metaphire magna magna* was 16%, and those between *Metaphire magna minuscula* and other *Metaphire* species were nearly or more than 20%. The same pattern appeared in the distances of combined COI and 16S sequences among *Metaphire* species: 12% between *Metaphire magna minuscula* and *Metaphire magna magna*, and nearly or more than 15% between *Metaphire magna minuscula* and other *Metaphire* species (Table 2). The sequences of COI and 16S gene of both *Metaphire magna minuscula* subsp. nov. (C-HN201110-01) and *Metaphire magna magna* (C-HN201112-01) have been deposited in GenBank (Table 1). A morphological comparison of *Metaphire magna minuscula* with other *Metaphire* species with spermathecal pores in 7/8–8/9 from China and Southeast Asia is given in Table 3.

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TABLE 3. Comparison of earthworm species with two pairs of spermathecal pores (7/8, 8/9) in the genus *Metaphire* in China.

Species name	<i>Metaphire wuzhimonitis</i> Qiu & Sun sp. nov.	<i>Metaphire magna minuscula</i> Qiu & Zhao subsp. nov.	<i>Metaphire magna magna</i> (Chen, 1938)
Length [mm]	163	297	680
Width [mm]	5.5	12	22
Segments	94	137	178
First dorsal pore	09/10	11/12	12/13
Genital papillae	lacking	lacking	1, small, round, medially among the ridges surrounding the small and shallow slit where the male pore is placed, on setal line
Genital markings	5, round, all of same size, two pairs, one unpaired: one pair each between spermathecal pores and the setae circle of VII and VIII, respectively; unpaired marking ventro-medially on IX	lacking	3-6, minute, ampulla-like, encircling spermathecal pores anteriorly and laterally
Septa	8/9-9/10 absent	8/9 thin, membranous, 9/10 absent	8/9 membranous, 9/10 absent
Prostate glands	in XV-XIX, well-developed, closely adherent to body wall, prostatic duct stout, U-shaped	in 23XVII-23XIX, small but stout, closely adherent to body wall, prostatic duct U-shaped	large, granular, in XVII-XIX, duct uniform in diameter, slender only at ectal end
Accessory glands around prostate gland	lacking	lacking	3, distinct, each roundish and enclosed in peritoneum, close to base of prostatic duct, duct cord-like, fairly thick
Spermathecae	large; ampulla peach-shaped, duct short, as long as 0.14 main pouch; diverticulum equal to main pouch, straight, terminal 0.5 enlarged, ental 0.33 as elongate seminal chamber	ampulla large, heart-shaped, duct as long as 0.2 main pouch; diverticulum slightly longer than main pouch by 0.2 mm, weakly in zigzag fashion or U-curved, terminal 0.67 enlarged as a clavate seminal chamber	comparatively large, ampulla large, sac-like, thin-walled, duct about 0.3x as long as main pouch, diverticulum when extended longer than main pouch; seminal chamber thumb-shaped, weakly U-curved, 0.75x as long as diverticulum
Accessory glands around spermathecae	present	lacking	present, each consisting of a gland mass and a cord-like duct leading to each papilla

.....continued on the next page

TABLE 3. (Continued)

Species name	<i>Metaphire longipennis</i> (Chen, 1946)	<i>Metaphire javanica</i> (Kingberg, 1867)	<i>Metaphire californica</i> (Kingberg, 1867)	<i>Metaphire prava</i> (Chen, 1946)
Length [mm]	45-30	50-130	50-156	45-50
Width	3.0-3.2	3-5	3-5	2.5
Segments	71-81	80-110	55-115	80-110
First dorsal pore	10/11	06/07	11/12	11/12
Genital papillae	lacking	lacking	lacking	lacking
Genital markings	lacking	lacking	lacking	lacking
Septa	8/9-9/10 absent	8/9-9/10 absent.	8/9-9/10 absent	8/9-9/10 absent
Prostate glands	well-developed in XVII-XXI, duct twisted	from XVI, XVII to XX, XXI, XXII, ducts paired, fairly stout, straight to S- or U-shaped passing antero-laterally into the copulatory pouch of its side	paired in XVIII, extending anteriorly to XVII and posteriorly to XX or XXI, racemose	coarse lobules, in XVII-XXII, duct slender in its ental and ectal portions, crooked, middle portion straight, very stout
Accessory glands around prostate gland	lacking	lacking	lacking	present, diffuse, sessile, surrounding ectal end of prostatic duct
Spermathecae	very large, posterior pair larger than anterior, ampulla kidney-shaped, with very short duct, diverticulum shorter than main pouch, with a globular or date-shaped seminal chamber which may be comparatively long but never zigzag twisted	duct shorter than ampulla, diverticulum slender, longer than their combined lengths, duct narrow ectally, diverticulum "C" shape, usually long and highly convoluted	ampulla large, heart-shaped, stalk short and stout; diverticulum tubular, slender, coiled	very large, ampulla rounded, with a comparatively short duct; diverticulum zigzag twisted, its ental 0.2 serving as seminal chamber; diverticular duct short and not well-marked
Accessory glands around spermathecae	lacking	lacking	lacking	lacking

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