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Four New Species of Earthworms (Oligochaeta: Megascolecidae: *Amynthas*) from Taiwan Based on Morphological and Molecular Evidence

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Yu-Hsi Wang and His-Te Shih (2017) Four new species of earthworms of the genus *Amynthas* are described from Taiwan. *Amynthas lalashan* n. sp. has two pairs of large genital markings medial to male porophores at the presetal portions on segments xviii and xix; the genital marking on segment xviii is closely adjacent to the male pore. *Amynthas fusing* n. sp. has a large C-shaped genital marking beside each male pore. *Amynthas lioujia* n. sp. has two large round genital markings beside each male pore. *Amynthas majia* n. sp. has a large V-shaped genital marking beside each male pore. The recognition of the four new species is also supported by the difference of the mitochondrial cytochrome oxidase subunit I (COI) marker.

Key words: Earthworm, New species, *Amynthas*, Taiwan, Cytochrome oxidase subunit I, COI.

BACKGROUND

Pheretimoid earthworms are largest group in the family Megascolecidae, and that mainly distribute in the Oriental region, *e.g.* Korea, Japan, China, Taiwan, Philippines, Vietnam (Blakemore et al. 2006; Aspe and James 2016; Nguyen et al. 2016). Earthworm studies of Taiwan have greatly accelerated since 1999, with the number of species increasing from 26 to 114, and new species are still being reported, which have covered nearly all areas of the main island of Taiwan and adjacent islets (Gueishandao, Lyudao, Lanyu, Siaoliouciou, Penghu, Kinmen and Matsu (= Mazu)) (Shih et al. 1999; James et al. 2005; Blakemore et al. 2006; Chang et al. 2009a, 2014; Tsai et al. 2009, 2010; Wang and Shih 2010; Shen et al. 2014, 2015, 2016). However, some areas near the Central Range (= Central Mountain Range) were not studied in detail before and are worth surveying further.

DNA barcoding by using the mitochondrial cytochrome oxidase subunit I (COI) provides a reliable solution to the current problem of species identification (Hebert et al. 2003a, b). And there are several studies used DNA barcoding to distinguish closely related species in earthworms successfully (Huang et al. 2007; Chang et al. 2009b; James et al. 2010; Porco et al. 2013; Hong and Csuzdi 2016).

In this study, we describe four new species of earthworms from Taiwan, which are also supported by the analyses of COI sequences.

MATERIALS AND METHODS

Specimens were collected by digging, anesthetized in 5%-10% alcohol, and then fixed in 70% alcohol. External and internal characteristics were examined and drawn under a stereomicroscope (Zeiss, model SV-6) with the help of a drawing tube. Specimens are deposited in the Zoological Collections of the Department of Life Science, National Chung Hsing University (NCHUZOOL), Taichung, Taiwan.

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Genomic DNA was extracted from the muscle tissue of body wall by using the GeneMark tissue and cell genomic DNA purification kit (Taichung, Taiwan). The COI gene was amplified with PCR using the primers LCO1490 (5'-GGTCAACA AATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). The PCR conditions for the above primers were 40 cycles of denaturation for 50s at 94°C, annealing for 70s at 45°C, and extension for 60 s at 72°C, followed by a 72°C extension for 10 min. Sequences were obtained by automated sequencing (Applied Biosystems 3730), after verification with the complementary strand. Sequences of different haplotypes have been deposited in the DNA Data Bank of Japan (DDBJ). Other published DNA sequences of species with similar morphology are also included for analyses. The pairwise estimates of Kimura (1980) two-parameter (K2P) distance for inter- and intraspecific genetic diversities were calculated by MEGA (vers. 6, Tamura et al. 2013).

RESULTS

Taxonomy

Family Megascolecidae Rosa, 1891

Genus *Amynthas* Kinberg, 1867

Amynthas lalashan n. sp.

(Fig. 1)

urn:lsid:zoobank.org:act:41C44139-8C07-41E2-BE46-4F8C92CC1A72

Material examined: Holotype: 1 clitellate (NCHUZOOL 13541), Lalashan, Fusing, Taoyuan, Taiwan, 24°41'17.5"N, 121°24'38.5"E, 1169 m, 7 September 2010, collected by Yu-Hsi Wang and Guan-Cyun Guo. Paratype: 1 clitellate (NCHUZOOL 14490) same collection data as for holotype.

Etymology: This species is named after the type locality. The name "La-La" is an aboriginal name from the Atayal Tribe of Taiwan which means very beautiful. The name is used as a noun in apposition.

Diagnosis: Size 157.2-171.9 mm. Segments 98-120. Setae regularly distributed around segmental equators; 10 setae between male pores. Prostomium, epilobic. First dorsal pore at 10/11 or 11/12. Clitellum annular, xiv-xvi, smooth. Female pore single on xiv. Male pores on xviii with many wrinkled edges around each pore, 2 pairs of large genital markings presetal on xviii and xix, with anterior genital marking closely adjacent to each pore on xviii. Spermathecal pores ventrally at 5/6-8/9, genital marking absent. Spermathecae four pairs in vi-ix. Seminal vesicles large in xi and xii. Prostates in xvii-xix. Prostatic duct long, C-shaped. Accessory gland sessile, large, corresponding to each external marking.

Description: External: Dimensions 157.2-171.9 mm by 7-7.23 mm in x, 5.6-6.9 mm in xxx, 6.8-7.5 mm in clitellum; body cylindrical throughout, segments 98-120. Setae regularly distributed around segmental equators; 10 setae between male pores; setal formula AA: AB: YZ: YZ = 1.4: 1: 1: 2.9 on xxv. Prostomium, epilobic. First dorsal pore at 10/11 or 11/12. Clitellum annular, xiv-xvi, smooth. Female pore single on xiv. Male pores on xviii (Fig. 1A).

Male pores with many wrinkled edges around each pore, 2 pairs of large genital markings presetal on xviii and xix, with anterior genital marking closely adjacent to each pore on xviii (Fig. 1A). Spermathecal pores ventrally at 5/6-8/9, genital marking absent.

Septa, 5/6-7/8 thin, 8/9/10 absent, 10/11-12/13 thick, 13/14-15/16 thin; large gizzard in x. Intestinal origin from xv; lymph glands absent; typhlosole simple from xxvii, extending forward as a small ridge to xv. Intestinal caeca with shallow saw-like ventral edge, originating in xxvii, extending anteriorly to xxii (Fig. 1D). Hearts in xi-xiii.

Male sexual system holandric, testes connected at ventral side, funnels ventrally joined to sacs in x and xi. Seminal vesicles large in xi and xii, with large dorsal lobe. Prostates in xviii, 3 main lobes, duct thick, muscular, C-shaped; without a copulatory pouch; joining vasa deferentia distal to glandular portion. Prostatic duct flanked by sessile glandular masses on body wall, corresponding to each external genital marking (Fig. 1C).

Ovaries in xiii. Spermathecae paired in vi-ix, ampulla large, heart-shaped in vi-ix; diverticulum axis shorter than ampulla axis (Fig. 1B).

Remarks: *Amynthas lalashan* n. sp. belongs to the *corticis* species-group (Sims and Easton 1972; Blakemore 2013), and there have been more than 100 species reported in this group according to Tsai et al. (2010). In this study, we compare species belonging to the *corticis* species-group from East Asia closely related to *A. lalashan*. *Amynthas lalashan* is similar with *A. biorbis* Tsai and Shen, in Tsai, Shen and Tsai, 2010, *A. hongyehensis* Tsai and Shen, in Tsai, Shen and Tsai, 2010, *A. hupeiensis* (Michaelsen,

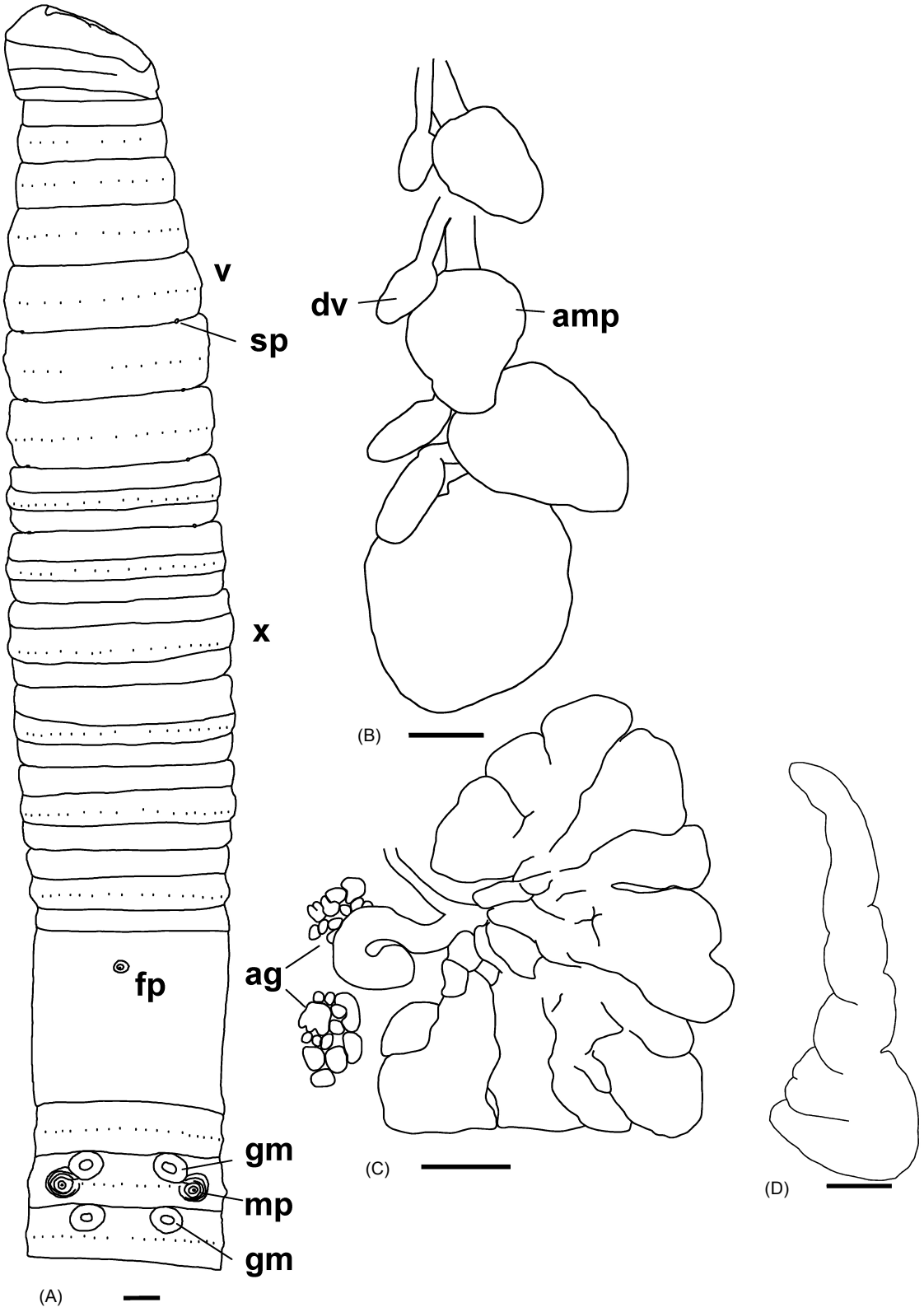

Fig. 1. *Amynthas lalashan* n. sp. holotype, NCHUZOOL 13541: (A) ventral view of spermathecal pores at intersegmental furrows; male pores with genital markings presetal on xviii and xix; (B) right spermathecae; (C) right prostate and small accessory glands; (D) right intestinal caeca. ag, accessory gland; amp, ampulla; dv, diverticulum; fp, female pore; gm, genital marking; mp, male pore; sp, spermathecal pore. All scale bars = 1 mm.

1895), *A. pavimentus* Tsai and Shen, in Tsai, Shen and Tsai, 2010 and *A. zhuya* Blakemore, 2013 (= *A. montanus* Qiu and Sun in Sun, Jiang and Qiu, 2012), but can be distinguished by several characters. *Amynthas hongyehensis* and *A. hupeiensis* are sexthecal, but *A. lalashan* is octothecal. In addition, *Amynthas hongyehensis* has genital markings located slightly medial to the male pore on presetal portions of xviii and xix, and male pores are surrounded by one or two shallow skin folds (Tsai et al. 2010: fig. 4B); *A. lalashan* has shallower skin folds surrounding the male pores, and genital markings on xviii are at the presetal portion closely adjacent to the male pore (Fig. 1A). *Amynthas hupeiensis* has genital markings at 17/18 and 18/19 (Chen 1933: fig. 19A), which differs from *A. lalashan* (Fig. 1A). *Amynthas biorbis*, *A. pavimentus* and *A. zhuya* are octothecal species and *A. biorbis* has paired genital markings at the postsetal portion on xviii closely adjacent to the male pore but lacks genital marking on xix (Tsai et al. 2010: fig. 3A); *A. pavimentus* has many small genital markings presetal on xviii and xix (Tsai et al. 2010: fig. 1A, B); *A. zhuya* has genital markings on xviii at the presetal portion closely adjacent to the male pore, without skin folds surrounding the male pores (Sun et al. 2012: fig. 2A), but *A. lalashan* has genital markings presetal on xviii and xix (Fig. 1A).

Lalashan, located in northern Taiwan, is part of the Hsuehshan Range and is famous for an area of large *Chamaecyparis* forest. The earthworm diversity is high in northern Taiwan with 29 species (10 endemic species), with some endemic species from the mountain range at an altitude of 500-1500 m (Tsai et al. 2007, 2010). The Hsuehshan Range and the Central Range have been suggested as barriers for the organisms of freshwater crabs and frogs in Taiwan (Shih et al. 2006; Lin et al. 2012) and even for earthworms (Chang and Chen 2005; Chang et al. 2008). However, *A. hongyehensis* is distributed in Hualien and Taitung (Shen 2012), which may be isolated from this new species by the Central Range.

***Amynthas fusing* n. sp.**

(Fig. 2)

urn:lsid:zoobank.org:act:08420304-0D0B-42B4-A624-267D33B7AB70

Material examined: Holotype: 1 clitellate (NCHUZOOL 13542), beside road no. 119, Fusing, Taoyuan, Taiwan, 24°50'07.4"N, 121°23'33.0"E, 736 m, 7 September 2010, collected by Y.-H. Wang and G.-C. Guo. Paratypes: 9 clitellates (NCHUZOOL 13543) same collection data as for holotype.

Others: 1 clitellate (NCHUZOOL 13544), Ronghua, Fusing, Taoyuan, Taiwan, 24°44'26.8"N, 121°20'35.8"E, 493 m, 7 September 2010, collected by Y.-H. Wang and G.-C. Guo; 9 clitellates (NCHUZOOL 13545), beside road no. 119, Fusing, Taoyuan, Taiwan, 24°49'40.9"N, 121°22'50.4"E, 704 m, 7 September 2010, collected by Y.-H. Wang and G.-C. Guo; 8 clitellates (NCHUZOOL 13546), beside road no. 119, Fusing, Taoyuan, Taiwan, 24°48'41.9"N, 121°21'43.0"E, 704 m, 7 September 2010, collected by Y.-H. Wang and G.-C. Guo.

Etymology: This species is named for its type locality, Fusing Township, Taoyuan, Taiwan. The name is used as a noun in apposition.

Diagnosis: Size 226.0-308.5 mm. Segments 112-143. Setae regularly distributed around segmental equators; 13-18 setae between male pores. Prostomium, epolibic. First dorsal pore at 12/13. Clitellum annular, xiv-xvi, smooth. Female pore single on xiv. Male pores on xviii with many wrinkled edges, with a large C-shaped genital marking close to each pore on xviii. Spermathecal pores 3 pairs laterally at 6/7-8/9, with genital markings present on vi, vii, and viii close to each pore. Spermathecae three pairs in vii-ix. Seminal vesicles large in xi and xii. Prostates large in xvii-xviii, ducts thick, muscular; without copulatory pouch.

Description: External: Dimensions 226.0-308.5 mm by 8.9-10.1 mm in x, 7.4-8.6 mm in xxx, 8.4-11.2 mm in clitellum; body cylindrical throughout, segments 112-143. Setae regularly distributed around segmental equators; 13-18 setae between male pores; setal formula AA: AB: YZ: YZ = 2: 1: 1: 1.8 on xxv. Prostomium, epolibic. First dorsal pore at 12/13. Clitellum annular, xiv-xvi, smooth. Female pore single on xiv. Male pores on xviii (Fig. 2A).

Male pores enlarged, with many wrinkled edges on xviii (Fig. 2A), with a large C-shaped genital marking close to each pore on xviii. Spermathecal pores 3 pairs laterally at 6/7-8/9, with genital markings present on vi, vii, and viii close to each pore, each pore with light swollen edge between segments (Fig. 2B).

Septa, 5/6-7/8 thick, 8/9/10 absent, 10/11-13/14 thick, 14/15/16 thin; gizzard in x. Intestinal origin from xiv; lymph glands from xvi; typhlosole simple from xviii, extending forward as small ridge to xiv. Intestinal caeca simple, smooth margin, originating in xvii, extending anteriorly to xxiii, with

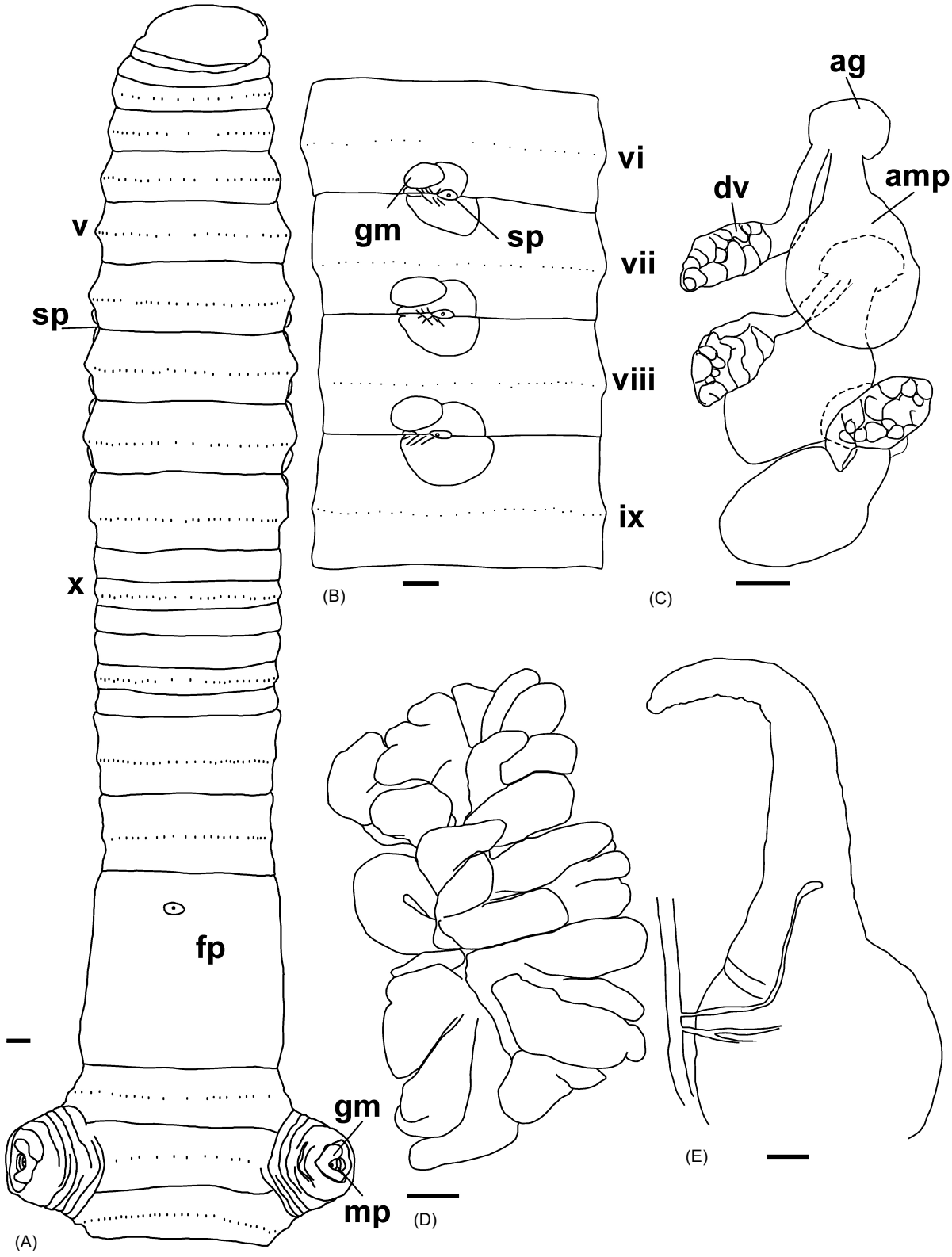

Fig. 2. *Amynthas fusing* n. sp. holotype, NCHUZOOL 13542: (A) ventral view of male pores with large C-shaped genital markings on xviii; (B) left lateral view of spermathecal pores at intersegmental furrows with genital markings; (C) right spermathecae and accessory glands; (D) right prostate; (E) right intestinal caeca with vasa. ag, accessory gland; amp, ampulla; dv, diverticulum; fp, female pore; gm, genital marking; mp, male pore; sp, spermathecal pore. All scale bars =1 mm.

links to dorsal vessel (Fig. 2E). Heart in xiii.

Male sexual system holandric, testes connected at ventral side, funnels ventrally joined to sacs in x and xi. Seminal vesicles large in xi and xii, with large dorsal lobe. Prostates large in xviii, extending to xvii and xx, with 3 main lobes, ducts thick, muscular; without copulatory pouch; joining vasa deferentia distal to glandular portion; vasa deferentia non-muscular (Fig. 2D).

Ovaries in xiii. Spermathecae paired in vii-ix, ampulla large, heart-shaped in vii-ix; diverticulum axis longer than ampulla axis, stalk thicker at ental portion, part of stalk and remainder of diverticulum coiled into closely compacted spherical mass; with accessory glands close to spermathecae in vi, vii, and viii (Fig. 2C).

Ecology and behavior: The vegetation of the habitat of this species is dense. The earthworm was found to spread out coelomic fluid from the dorsal pores and anus after being stimulated, a behavior similar to *Amynthas lioujia* n. sp. (see below). This behavior was also recorded for other species, including *Allolobophora chlorotica* (Savigny, 1826), *Didymogaster sylvaticus* Fletcher, 1886, *Eisenia fetida* (Savigny, 1826) and *Megascolides australis* McCoy, 1878 (Ressler et al. 1968; Edwards and Bohlen 1996; Płytycz et al. 2006).

Remarks: *Amynthas fusing* n. sp. is similar to *A. binoculatus* Tsai, Shen and Tsai, 1999, *A. lioujia* n. sp., *A. majia* n. sp., and *A. sexpectatus* Tsai, Shen and Tsai, 1999, but with some different characters. *Amynthas binoculatus* has a pair of large disk-like genital markings on viii and 3 large genital markings close to the male pore (Tsai et al. 1999: fig. 4A). *Amynthas sexpectatus* has a variable shape and number of genital markings with male pores (Tsai et al. 1999: fig. 3A-C). *Amynthas lioujia* has genital markings on viii and 2 large round genital markings close to the male pore (Fig. 3A); *A. majia* has a large V-shaped genital marking close to the male pore (Fig. 4A). *Amynthas fusing* lacks genital markings on viii, and has a large C-shaped genital marking close to the male pore (Fig. 2A). A comparison of characters is given in table 1.

***Amynthas lioujia* n. sp.**

(Fig. 3)
urn:lsid:zoobank.org:act:265FE1D5-8C28-40FE-9D79-B42C934BC689

Material examined: Holotype: 1 clitellate (NCHUZOOL 14491), Lioujia, Tainan, Taiwan, 23°13'51.2"N, 120°23'51.3"E, 139 m, 3 November 2010, collected by Y.-H. Wang et al. Paratypes: 8 clitellates (NCHUZOOL 13547) same collection data as for holotype.

Etymology: This species is named for its type locality, Lioujia District, Tainan City, Taiwan. The name is used as a noun in apposition.

Diagnosis: Size 167.2-244.9 mm. Segments 120-142. Setae regularly distributed around segmental equators; 17-25 setae between male pores. Prostomium, epolibic. First dorsal pore at 12/13 or 13/14. Clitellum annular, xiv-xvi, smooth. Female pore single on xiv. Male pores on xviii with many wrinkled edges, with 2 large round genital markings close to each pore on xviii. Spermathecal pores 3 pairs laterally at 6/7-8/9, with genital markings present in front of each pore or absent. Spermathecae three pairs in vii-ix. Seminal

Table 1. Morphological comparison among *Amynthas binoculatus* Tsai, Shen and Tsai, 1999, *A. fusing* n. sp., *A. lioujia* n. sp., *A. majia* n. sp., and *A. sexpectatus* Tsai, Shen and Tsai, 1999

	Length (mm)	Clitellum (mm)	No. of segments	Setae between male pores	Genital markings		Location	Reference
					before clitellum	around each male pore		
A. binoculatus	196.0	6.3	113	16	large, a pair on viii	large, disk-shaped, 3 on xviii	Taiwan (Taichung)	Tsai et al. (1999)
A. fusing	226.0-308.5	8.4-11.2	112-143	13-18	3 pairs on vi, vii, vii, beside spermathecal pores	large C-shaped, 1 on xviii	Taiwan (Taoyuan)	this study
A. lioujia	167.2-244.9	6.9-7.5	120-142	17-25	large, 1 pair on viii; 3 pairs on vi, vii, viii beside spermathecal pores or absent; sometimes additional 1 on vi	large, round, 2 on xviii	Taiwan (Tainan)	this study
A. majia	180.7-186.1	5.7-5.9	103-124	22	-	V-shaped, 1 on xviii	Taiwan (Pingtung)	this study
A. sexpectatus	193.0-258.0	6.6-12.2	102-140	23-24	-	variable shape and number on xviii	Taiwan (Nantou)	Tsai et al. (1999)

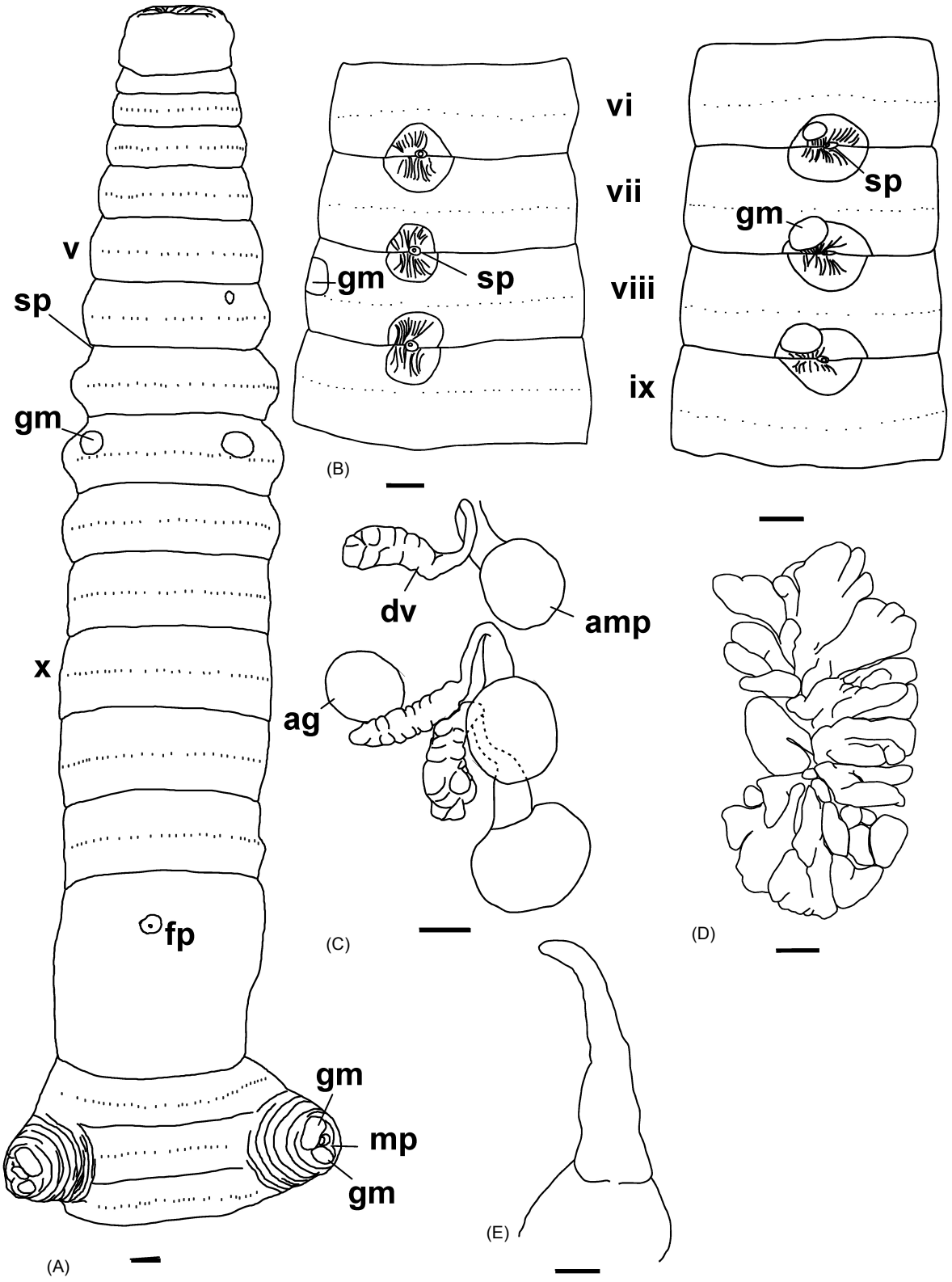

Fig. 3. *Amynthas lioujia* n. sp. holotype, NCHUZOOL 13547: (A) ventral view of male pores with two large genital markings on xviii; (B) lateral views of left spermathecal pores from two specimens (left, NCHUZOOL 13547; right, NCHUZOOL 13547 (no. 4)); (C) right spermathecae and accessory glands; (D) right prostate; (E) right intestinal caeca. ag, accessory gland; amp, ampulla; dv, diverticulum; fp, female pore; gm, genital marking; mp, male pore; sp, spermathecal pore. All scale bars = 1 mm.

vesicles large in xi and xii. Prostates large in xvii-xviii, ducts thick, muscular; without copulatory pouch.

Description: External: Dimensions 167.2-244.9 mm by 7.5-9.0 mm in x, 6.9-7.5 mm in xxx, 7.3-8.8 mm in clitellum; body cylindrical throughout, segments 120-142. Setae regularly distributed around segmental equators; 17-25 setae between male pores; setal formula AA: AB: YY: YZ = 2.2: 1: 5: 1.2 on xxv. Prostomium, epilobic. First dorsal pore at 12/13 or 13/14. Clitellum annular, xiv-xvi, smooth. Female pore single on xiv. Male pores on xviii (Fig. 3A).

Male pores enlarged, with many wrinkled edges, with 2 large round genital markings close to each pore on xviii (Fig. 3A). Spermathecal pores laterally at 6/7-8/9, with genital markings present in front of each pore or absent; 1 pair of large genital marking ventrally on viii, each pore with a slightly swollen edge between segments (Figs. 3A, B).

Septa, 5/6-7/8 thick, 8/9 thin, 9/10 absent, 10/11-12/13 thick, 13/14-15/16 thin; large gizzard in x. Intestinal origin from xv; lymph glands absent; typhlosole simple from xxvii, extending forward as a small ridge to xv. Intestinal caeca simple, originating in xxvii, extending anteriorly to xxiii (Fig. 3E). Hearts in xi-xiii.

Male sexual system holandric, testes connected at ventral side, funnels ventrally joined to sacs in x and xi. Seminal vesicles large in xi and xii, with large dorsal lobe. Prostates in xviii, 3 main lobes, prostate ducts thick, muscular; without copulatory pouch; joining vasa deferentia distal to glandular portion; vasa deferentia non-muscular (Fig. 3D).

Ovaries in xiii. Spermathecae paired in vii-ix, ampulla large, ball-shaped in vii-ix; diverticulum axis shorter than ampulla axis, twisted and coiled on same plane, its limbs inseparable; with mass accessory glands in viii (Fig. 3C).

Ecology and behavior: The vegetation of the habitat was dense and with hard loess. When stimulated, the earthworm secreted coelomic fluid from its dorsal pores as mentioned in *Amynthas fusing* n. sp.

Remarks: This species is similar to *Amynthas binoculatus*, *A. fusing* n. sp., and *A. majia* n. sp., but with some different characters. *Amynthas binoculatus* has a pair of large disk-like genital markings on viii and 3 large genital markings with the male pore (Tsai et al. 1999: fig. 4A). *Amynthas fusing* and *A. majia* lack genital markings on viii, *A. fusing* has large C-shaped genital markings close to the male pore (Fig. 2A), and *A. majia* has a large V-shaped genital marking close to the male pore (Fig. 4A). Although *Amynthas lioujia* n. sp. has large disk-like genital markings on viii, it has 2 large round genital markings close to each male pore (Fig. 3A). A comparison of characters is given in table 1.

***Amynthas majia* n. sp.**

(Fig. 4)

urn:lsid:zoobank.org:act:831BFDE1-BD35-450A-A14A-0DF7CEB891D9

Material examined: Holotype: 1 clitellate (NCHUZOOL 14492), Liangshan Waterfall, Majia, Pingtung, Taiwan, 22°40'58.1"N, 120°38'38.7"E, 95 m, 18 August 2010, collected by Y.-H. Wang et al. Paratypes: 2 clitellates and 2 aclitellates (NCHUZOOL 13548) same collection data as for holotype.

Etymology: This species is named for it type locality, Maja, a village inhabited by the aboriginal Paiwan Tribe in southern Taiwan. The name is used as a noun in apposition.

Diagnosis: Size 180.7-186.1 mm. Segments 103-124. Setae regularly distributed around segmental equators; 22 setae between male pores. Prostomium, epolibic. First dorsal pore at 12/13. Clitellum annular, xiv-xvi, with setae ventrally on xiv, xvi. Female pore single on xiv. Male pores on xviii with wrinkled edges, with large V-shaped genital marking close to male pore. Spermathecal pores 3 pairs laterally at 6/7-8/9, pore eye-shaped, without genital markings. Spermathecae three pairs in vii-ix. Seminal vesicles large in xi and xii. Prostates large in xvii-xviii, ducts thick, muscular; without copulatory pouch.

Description: External: Dimensions 180.7-186.1 mm by 5.0-5.2 mm in x, 5.7-5.9 mm in xxx, 5.3-5.5 mm in clitellum; body cylindrical throughout, segments 103-124. Setae regularly distributed around segmental equators; 22 setae between male pores; setal formula AA: AB: YZ: YZ = 1.6: 1: 1: 1.6 on xxv. Prostomium, epilobic. First dorsal pore at 12/13. Clitellum annular, xiv-xvi, with setae ventrally on xiv, xvi. Female pore single on xiv, 1 paratype with a pair of female pores. Male pores on xviii (Fig. 4A).

Male pores enlarged, with wrinkled edges, with large V-shaped genital marking close to male pore (Fig. 4A). Three pairs of spermathecal pores laterally at 6/7-8/9, pore eye-shaped, without genital markings, each pore with a slightly swollen edge between segments (Fig. 4A).

Septa, 5/6-7/8 thin, 8/9/10 absent, 10/11 thin, 11/12/13 thick, 13/14-15/16 thin; gizzard in

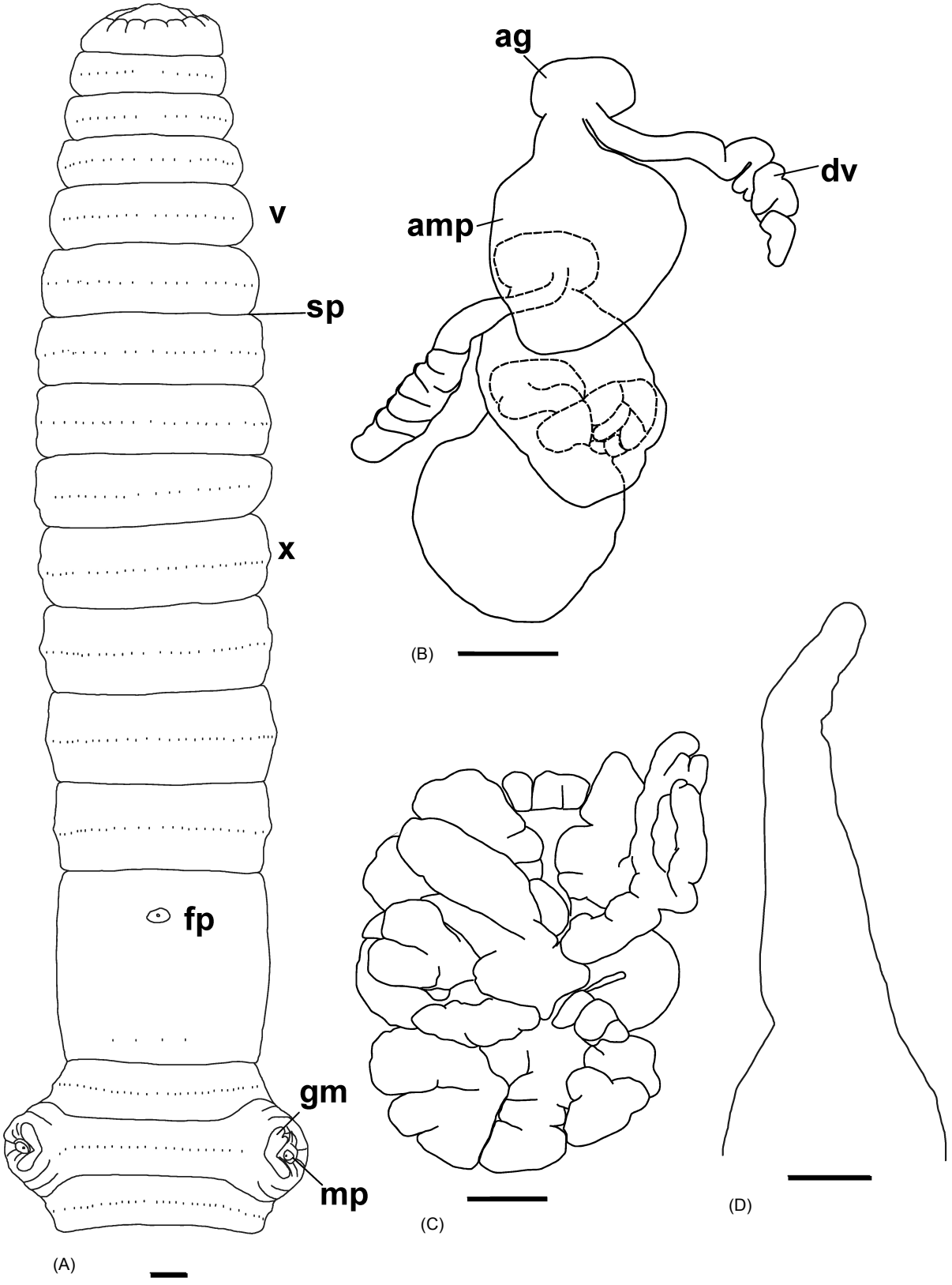

Fig. 4. *Amynthas majia* n. sp. holotype, NCHUZOOL 13548: (A) ventral view of male pores with large V-shaped genital markings on xviii; (B) left spermathecae; (C) left prostate; (D) right intestinal caeca. ag, accessory gland; amp, ampulla; dv, diverticulum; fp, female pore; gm, genital marking; mp, male pore; sp, spermathecal pore. All scale bars = 1 mm.

x. Intestinal origin from xv; lymph glands in xxvi-xxix; typhlosole simple from xxii, extending forward as a small ridge to xv. Intestinal caeca with small incisions on ventral margin, originating in xxvii, extending anteriorly to xxii (Fig. 4D). Hearts in xi-xiii.

Male sexual system holandric, testes connected at ventral side, funnels ventrally joined to sacs in x and xi. Seminal vesicles large in xi and xii, with large dorsal lobe. Prostates in xviii, 4 main lobes, ducts thick, muscular; without copulatory pouch; joining vasa deferentia distal to glandular portion; vasa deferentia non-muscular (Fig. 4C).

Ovaries in xiii. Spermathecae paired in vii-ix, ampulla large, heart-shaped in vii-ix; diverticulum axis longer than ampulla axis, variously irregularly looped; with sessile accessory glands close to spermatheca in vi-viii, corresponding to each external swollen edge (Fig. 4B).

Ecology: The habitat was humid, covered with a thick layer of fallen leaves.

Remarks: This species is similar to *Amynthas binoculatus*, *A. fusing* n. sp., and *A. lioujia* n. sp., but with some different characters. *Amynthas binoculatus* and *A. lioujia* have large round genital markings on viii, *A. binoculatus* has 3 large genital markings with the male pore (Tsai et al. 1999: fig. 4A), and *A. lioujia* has 2 large round genital markings closely adjacent to the male pore (Fig. 3A). *Amynthas fusing* has a large C-shape genital marking close to the male pore (Fig. 2A). *Amynthas majia* n. sp. has a large V-shaped genital marking close to the male pore and no genital markings on viii (Fig. 4A). A comparison of characters is given in table 1.

DNA analyses and discussion

A total of ten specimens of the four new species were used for molecular study. A 535-bp segment of COI was amplified, resulting in nine different haplotypes (Table 2). The studied segment of the COI sequences was AT-rich (60.2%) (T, 31.0%; A, 29.2%; G, 17.6%; C, 22.2%). According to the analysis of DNA barcoding, *A. lalashan* n. sp., *A. micronarius* (Goto and Hatai, 1898) (from main islands of Japan) and *A. mutabilitas* Shen, 2012 (from Taiwan) form a monophyletic clade (NJ tree data not show), which *A. lalashan* and *A. mutabilitas* are as sister species. The pairwise nucleotide divergences for COI with K2P distance are shown in table 3. The mean interspecific K2P

Table 2. Ten haplotypes of the COI gene of *Amynthas* collected from Taiwan

Species identified	Locality	Sample size	No. of haplotypes of COI	GenBank Accession no.
A. lalashan	Taoyuan, Taiwan	2	1	LC306643, LC306644
A. fusing	Taoyuan, Taiwan	3	3	LC306645, LC306646, LC306647
A. lioujia	Tainan, Taiwan	2	2	LC306648, LC306649
A. majia	Pingtung, Taiwan	3	3	LC306650, LC306651, LC306652

Table 3. Matrix of percentage pairwise nucleotide divergences with K2P distance on cytochrome *c* oxidase subunit I (COI) within and between species of *Amynthas* used in this study, and other related species from GenBank. Values of range are shown in parentheses

	Intraspecific	Interspecific						
	Nucleotide divergence	*A. lalashan*	*A. mutabilitas* (JX290402, 290405, 290412, 290418, 290420)	*A. micronarius* (AB542498, 542502, 542503, 542505)	*A. binoculatus* (AY962184)	*A. fusing*	*A. lioujia*	*A. majia*
A. lalashan	-							
A. mutabilitas	3.1 (0.4-4.3)	14.6 (13.7-15.2)						
A. micronarius	0.3 (0.0-0.6)	15.3 (15.2-15.4)	17.1 (16.1-18.0)					
A. binoculatus	0.0	20.7	21.1 (19.9-21.5)	20.2 (19.9-20.4)				
A. fusing	1.1 (0.0-1.7)	22.7 (22.5-23.1)	22.7 (21.5-23.6)	20.9 (20.4-21.2)	4.3 (4.1-4.7)			
A. lioujia	0.4	19.6 (19.3-19.8)	22.6 (22.2-23.0)	22.6 (22.1-23.1)	12.0 (11.9-12.1)	12.5 (12.1-13.1)		
A. majia	1.7 (0.2-2.5)	21.5 (20.8-21. 9)	22.6 (21.6-23.4)	21.7 (21.0-22.3)	11.4 (11.2-11.6)	12.8 (12.7-13.0)	13.2 (13.0-13.4)	

distance of *A. lalashan* is 14.6% with the closest *A. mutabilitas*, which is 4.7 times greater than the mean intraspecific distance of *A. lalashan*, 3.1% (Table 3). In addition, the lowest interspecific K2P distance of *A. lalashan* is 13.7% with *A. mutabilitas* and the largest intraspecific distance of *A. lalashan* is 0.0%. From table 3, *A. lalashan* is close to *A. micronarius* and *A. mutabilitas*, but morphological characters (including number and position of spermathecal pores) are different. *Amynthas micronarius* has 4 pairs spermathecal pores at 5/6-8/9 and 2 pairs of genital markings between 17/18 and 18/19 segments (Goto and Hatai 1898: 74), but which is small-sized earthworm and distributed in Miyagi, main islands of Japan. *Amynthas mutabilitas* has 2 pairs spermathecal pores at 5/6/7 and the genital markings are variation close around to male pore and found in Taitung from Taiwan (Shen 2012: fig. 2A-E). *Amynthas lalashan* has 4 pairs spermathecal pores at 5/6-8/9 and 2 pairs GMs on 18 and 19 segments (Fig. 1A) and is medium size worm which was collected in Taoyuan from Taiwan that was different to *A. micronarius* and *A. mutabilitas*.

The genetic analysis of DNA barcoding, *A. binoculatus*, *A. fusing* n. sp., *A. lioujia* n. sp. and *A. majia* n. sp. form a monophyletic clade (NJ tree data not show), with the front two as sister species. The mean interspecific K2P distance of *A. binoculatus* is 4.3% with the closest *A. fusing*, which is greater than the mean intraspecific distance of *A. binoculatus*, 0.0% (Table 3). In addition, the lowest interspecific K2P distance of *A. binoculatus* is 4.1% with *A. fusing*, which is greater than the mean intraspecific distance of *A. binoculatus*, 0.0%. The mean interspecific genetic distance of other earthworm DNA barcoding studies were variations: 15.4% (Huang et al. 2007); 18.7% (Chang et al. 2009b); 19.8% (James et al. 2010); 13.7% (Jiang et al. 2015), which could support genetic distance of *A. lalashan* and *A. mutabilitas*. However, the mean interspecific genetic distance of *Amynthas binoculatus* and *A. fusing* is smaller than above values, but NJ tree (not show) and morphological characters are enough to support that are different species. In conclusion, morphological characters and COI sequences analysis support four species are different species.

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