Zoosyst. Evol. 100 (3) 2024, 1061–1073 | DOI 10.3897/zse.100.119292

PENSOFT

MUSEUM FÜR NATURKUNDE BERLIN

Distribution and systematics of the cosmopolitan *Amynthas carnosus* complex (Crassiclitellata, Megascolecidae) from eastern Asia

Anne Charis N. Han[1,2], Yufeng Zhang[2], Pu Miao[3], Shaolong Wu[4], Nengwen Xiao[5], Mingyan Qin[1,2], Huifeng Zhao[2,6], Donghui Wu[1,6], Nonillon M. Aspe[7]

1 *State Environmental Protection Key Laboratory of Wetland Ecology and Vegetation Restoration, School of Environment, Northeast Normal University, Changchun 130117, China*
2 *Hebei Key Laboratory of Animal Diversity, College of Life Science, Langfang Normal University, Langfang 065000, China*
3 *Henan Province Tobacco Company, Luoyang 471000, China*
4 *Hunan Province Tobacco Company, Changsha 410004, China*
5 *State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100012, China*
6 *Key Laboratory of Wetland Ecology and Environment, State Key Laboratory of Black Soils Conservation and Utilization, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun 130102, China*
7 *College of Marine and Allied Sciences, Mindanao State University at Naawan, Naawan 9023, Misamis Oriental, Philippines*

https://zoobank.org/541660A7-7B6A-4432-AEF5-C689737C0A3C

Corresponding authors: Huifeng Zhao (zhaohf@lfnu.edu.cn); Donghui Wu (wudonghui@iga.ac.cn)

Academic editor: Pavel Stoev ♦ **Received** 23 January 2024 ♦ **Accepted** 17 June 2024 ♦ **Published** 1 August 2024

Abstract

Pheretimoid earthworms, *Amynthas carnosus*, were collected from Northeast and North China. An update on the distribution and systematics of the *A. carnosus* complex in eastern Asia using both morphological and molecular data is provided. Three subspecies, *A. carnosus carnosus*, *A. carnosus naribunji*, and *A. carnosus roki*, are confirmed. Comparisons of morphological characters between the subspecies of *A. carnosus* are provided. Our results support the subspecies assignment with an intraspecific K2P genetic distance of not greater than 10% using the mitochondrial cytochrome c oxidase subunit I (COI). In addition, a re-description of the morphology of *A. carnosus naribunji* is presented here.

Key Words

DNA barcoding, earthworm, morphological characters, K2P, Megascolecidae

Introduction

Pheretimoids are a group of earthworms belonging to the family Megascolecidae (Oligochaeta) characterized by having a perichaetine setal arrangement, a meronephridial excretory system, a single gizzard around segment VIII, a pair of racemose prostates opening through male pores in XVIII, and testes contained within testis sacs (Aspe 2016). They are known to be widely distributed and predominantly occur in East and Southeast Asia. *Amynthas* and *Metaphire*, two of the most speciose pheretimoid genera, have species with a wider range of distribution and have become well established outside their native ranges (McCay et al. 2020; Chang et al. 2021). In China, *Amynthas*, with around 450 species, and *Metaphire*, with around 130 species, account for 88.9% of the total number of earthworm species in the country (Aspe 2016; Jiang and Qiu 2018).

Amynthas carnosus Goto & Hatai, 1899 is known to be one of the cosmopolitan pheretimoid species (Blakemore 2009). They are characterized by four pairs of obvious spermathecal pores in segments 5/6–8/9, or occasionally three pairs in segments 6/7–8/9, with genital markings typically closely paired mid-ventral and presetal in VIII–

IX and often also in XVIII–XIX (Chen 1933; Blakemore 2012). Its distribution has been reported in Japan, from Kyushu to Tohoku and Hokkaido (Goto and Hatai 1899; Kobayashi 1936; Ohfuchi 1937; Easton 1981), in Nara and Hikone (Blakemore 2013a), and in South Korea, including Jeju Island and Dagelet Island (Ulleung-do) (Kobayashi 1938; Blakemore 2013b, c). In China, the species has been reported in several provinces (Xiao 2019), but such claims are deemed questionable because of the lack of information about where the specimens were collected. So far, the published record of *A. carnosus* in China is in Hainan (Sun 2013) and Shanghai (Zhang et al. 2016), which only provided molecular data. In the USA, the species was reported near Manhattan in Kansas (Carrera-Martínez and Snyder 2016). Therefore, the known distribution of this species is in South Korea, Japan, China, and North America.

There has been an underlying confusion regarding *A. carnosus* morphology in the past due to its poor original account and successive misdescriptions. The problem with the original description by Goto and Hatai (1899) was that there were three pairs of spermathecal pores in 5/6/7/8, but the spermathecae were stated to be in 7, 8, and 9, suggesting they exited in 6/7/8/9 with the possibility of missing a pair. Nevertheless, Ohfuchi (1937), in a more detailed account, showed the species to have four pairs of spermathecal pores in 5/6/7/8/9. This then caused other character traits (e.g., dorsal pores, genital markings, segment count, and so on) to be misnumbered as well (Blakemore 2012). Not to mention that the species synonymy was caused by the erroneous assignment of names, which has added to the complexity of this species' identity. Further information about *A. carnosus'* provisional synonyms is listed and discussed by Blakemore (2012).

Amynthas pingi was previously considered a questionable synonym of *A. carnosus*, "as it is, on average, a larger worm with several other differences that presently exclude it from *A. carnosus*" (Blakemore 2012). However, Blakemore (2013a) re-examined the London type mature *A. pingi* specimen labeled as "*Pheretima pingi* 1924.11.29.5 HOLOTYPE (sic) Nanking, China Don. Prof. C. Ping" and refuted those differences (e.g., the supposed larger size in *A. pingi*, now known to be false as the type is only 132 mm long, and a later onset of intestine origin and septal glands, now also proven false). There, he also pointed out errors in Gates (1939) redefinition of *Pheretima pingi*, such as having "lower setal counts, mistaken septa, hearts, and spermathecal pores that he insisted were posterior in segments 5–8 (but that are now shown to be in the intersegmental furrows of 5/6/7/8/9 in the types of both *A. pingi* and *A. carnosus*). Moreover, the assumption that the 'characteristic' tubercles were nephridial (Goto and Hatai 1899) was more likely due to Monocystis infestation, as indicated by both Gates (1939) and Blakemore (2013a), hence dismissing the possible justification of retaining *A. pingi* as a separate species from *A. carnosus*. In which case, *A. carnosus* would likely suggest prevalence in China, from which *A. pingi* (= *A. carnosus*) was reported to have been abundantly distributed in Nanking (Stephenson 1931). Given this and with the incorporation of the new investigation of *A. carnosus* specimens in Northeast and North China (reported here), it may further support a possible indication of the prevalent range of *A. carnosus* in the country, as was suggested by Chen (1936) and concluded by Kobayashi (1936).

Preliminary attempts at using the DNA barcodes of *A. carnosus* specimens from Japan and South Korea were carried out (Blakemore 2013b). Two new subspecies have been established, namely, *A. carnosus naribunji* Blakemore 2013 from Naribunji, Ulleung-do (Dagelet Island, South Korea), and *A. carnosus roki* Blakemore and Lee 2013 from Incheon (South Korea). Preliminary DNA data for taxon identification and phylogenetic relationships were also explored, yet a rather deficient description of *A. carnosus naribunji*'s morphological characters by Blakemore (2013a) was presented, which then makes it more of a molecular taxon.

This paper provides an update on the taxonomic status of the *A. carnosus* complex in East Asia using both morphological and molecular data, as well as a report on the present distribution of this species in China. In addition, an update on the morphological diagnosis of *A. carnosus carnosus* and a re-description of *A. carnosus naribunji* are presented.

Materials and methods

Sampling

Earthworm specimens were collected during the summer of 2022 and 2023, around the months of May and July, in Northeast China and the neighboring provinces. The collection sites chosen were mainly based on three habitat types, including forests, farmlands, and urban parks (Table 1). Earthworm samples were also collected in a nature reserve area on Changbai Mountain. Earthworms were collected through digging and hand sorting. Collections near the sites with surface castings were also taken into account. The earthworms collected were preserved and stored in 100% ethanol.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from the muscle tissue of the posterior part using the TIANGEN Genomic DNA Kit (China) following the manufacturer's instructions. Regions of the cytochrome c oxidase subunit I (COI) were amplified using the polymerase chain reaction (PCR). The mixture (total volume 25 µl) contained 1 µl DNA and 17.25 µl sterile ddH_2O, 2.0 µl of dNTP, 2.5 µl of buffer, 0.25 µl TransGen EasyTaq-polymerase and 1.0 µl of Primer HCO1490 (5-GGTCAACAAATCATAAAGATATTGG-3) (Folmer et al. 1994), and 1.0 µl of Primer COIE (5-TATACTTCTGGGTGTC-

Table 1. Collection information for sampling areas and specimens.

Sampling ID	Location	Latitude, Longitude	Specimen number
362R	Liaoning Prov., Jinzhou Pref., Nanshan Park	41.0718°N, 121.1479°E	8
533R	Liaoning Prov., Dandong Pref., Jinjiang Mt. Park	40.1312°N, 124.3746°E	12
534R	Liaoning Prov., Dandong Pref., Kundian County, Beishan Park	40.7319°N, 124.7780°E	10
551R	Liaoning Prov., Huludao City, Longwan Park	40.7143°N, 120.8415°E	10
LFXH	Hebei Prov., Xianghe County, Zhuti Park	39.7774°N, 116.9816°E	7
LFSF	Hebei Prov., Langfang Pref., Anci Dist., Langfang Normal University	39.5222°N, 116.6654°E	1
E28, E29	Tianjin Municipality, Dongli Dist., Anonymous Park	39.0836°N, 117.3125°E	2
BJCY	Beijing Municipality, Chaoyang Dist., Lvfeng Park	39.8760°N, 116.5800°E	1
BJTZ	Beijing Municipality, Tongzhou Dist.,	39.8760°N, 116.7250°E	1
HNLNR2, HNLNGR, HNLNNG	Henan Prov., Luoyang Pref., Luoning County	34.4363°N, 111.6398°E	4
HNSQ	Henan Prov., Shangqiu Pref., Liangyuan Dist.	34.4291°N, 115.6183°E	3

CGAAGAATCA-3) (Bely and Wray 2004). The cycling profile was as follows: firstly, initial denaturation for 5 min at 95 °C; secondly, denaturation for 30 sec at 95 °C, annealing for 30 sec at 51 °C, and extension for 45 sec at 72 °C for 35 cycles; thirdly, final extension for 5 min at 72 °C. PCR amplifications were confirmed by electrophoresis in 1% agarose gel, which were visualized by SAGECREATION Gel Documentation and Image Analysis System Equipment, and Sage software was used for capturing the image. DNA samples were sent to Tianyi Huiyuan Biotechnology Co., Ltd. (Beijing) for Sanger sequencing using an ABI 3730 automated sequencer.

Data analysis

The raw data were corrected manually in BioEdit (Hall 1999), and the exported fasta files were aligned using Clustal W (Thompson et al. 1994). COI sequences from Genbank labeled as *A. carnosus* have also been included in the analysis (Suppl. material 1: table S1). A phylogenetic tree was constructed using the maximum likelihood method (ML) performed in RAxML 8.0 (Stamatakis 2014), using the default rapid hill-climbing algorithm and the GTRGAMMA model to search for the best tree. Clade support was assessed using 1,000 rapid bootstrap replicates. The tree was rooted using *Pontodrilus litoralis* as an outgroup. Pairwise distance analysis among *A. carnosus* subspecies and between COI sequences of the other 10 *Amynthas* species downloaded from GenBank was conducted using MEGA5 (Tamura et al. 2011) with the Kimura-2 parameter model (Kimura 1980).

Morphological examination and identification

Fixed specimens were brought to the laboratory for external and internal examination using a stereomicroscope (ZEISS) and ZEN 3.3. Pro software was used for image capture and to aid in identifying and measuring small organs and other characters. The generic diagnoses and taxonomic assignments to the subspecies level follow Blakemore (2012, 2013b) and Blakemore and Lee (2013).

References to figures from the cited papers are listed in lowercase (fig. or figs), and figures in this paper are noted with an initial capital (Fig. or Figs). The following abbreviations are used:

Ag accessory gland;
mp male pore;
re receptacle;
Amp ampula;
P prostomium;
sp spermathecal pore;
Gm genital marking;
prg prostate gland;
sv seminal vesicles.

Results and discussion

Molecular characterization

A total of 66 COI sequences had been sequenced and submitted to Genbank (Accession numbers: PP067669–PP067734). Results of the K2P analysis using COI show that the three intraspecific taxa of *A. carnosus* have inter-subspecific genetic distances that are between 7% and 10% (Table 2). Meanwhile, the genetic distance between *A. carnosus* and other species in the same genus is greater than 16% (16–22%). A study by Dong et al. (2019) revealed a genetic distance of 10.7–11.4% between two subspecies of *Amynthas shengtangmontis*: *A. s. shengtangmontis* and *A. s. minusculus*, which showed to be more than 1% and less than 15%. The intra-specific pairwise distances of subspecies *A. c. naribunji* and *A. c. roki* from *A. c. carnosus* are 7–8% and 9–10%, respectively (Table 2). In other studies, the interspecific distances in the same genus ranged between 17–23% (Sun 2013), 16–23% (Huang et al. 2007), 15–16% (Admassu et al. 2006), 16–22% (Novo et al. 2009), 15–28% (Chang et al. 2008), and 14.7–25% (Dong et al. 2019), which are all in agreement with our results.

Also, a specimen identified as "*A. carnosus carnosus*" in Hainan (China) by Sun (2013) (cf. Dong et al. 2019; KF205962) is seen to have diverged greatly from the *A. carnosus* taxa (Fig. 1), having a pairwise distance to the remaining *A. carnosus* taxa of 22–24% (Table 1), which could possibly suggest a misidentification of this species or subspecies.

Table 2. Percentage of K2P distance of the three subspecies of *A. carnosus* with inclusion of other pheretimoid species (values in %).

Species		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*A. carnosus carnosus*	0–1												
2	*A. carnosus naribunji*	7–8	0–1											
3	*A. carnosus roki*	9–10	8	0										
4	*A. carnosus carnosus* (KF205962)	23–24	22–23	24	0									
5	*A. daeari*	20	19	20	22	0								
6	*A. gageodo*	17–18	19–20	18–19	21	21	0							
7	*A. gracilis*	19	19–20	20–21	19	18–19	21	0						
8	*A. corticis*	16–20	17–19	19–20	19	18–19	18–20	18–20	0–7					
9	*A. fuscatus*	17–20	17–22	18–22	19–20	18–20	20–23	18–21	17–20	0–15				
10	*A. tokioensis*	20–21	19–21	22–23	23	16	20–21	20–21	18–21	18–22	0–1			
11	*A. maximus*	19	19	20	20	18	18	22	17–18	16–19	20–21	0		
12	*A. shengtangmontis*	20–21	19	20	21	20	20	20–21	18–19	19–21	22–23	23	0	
13	*A. robustus*	20–22	21–22	21–23	17–22	21–22	22–23	20	17–21	17–21	23–24	24	19–22	0–21

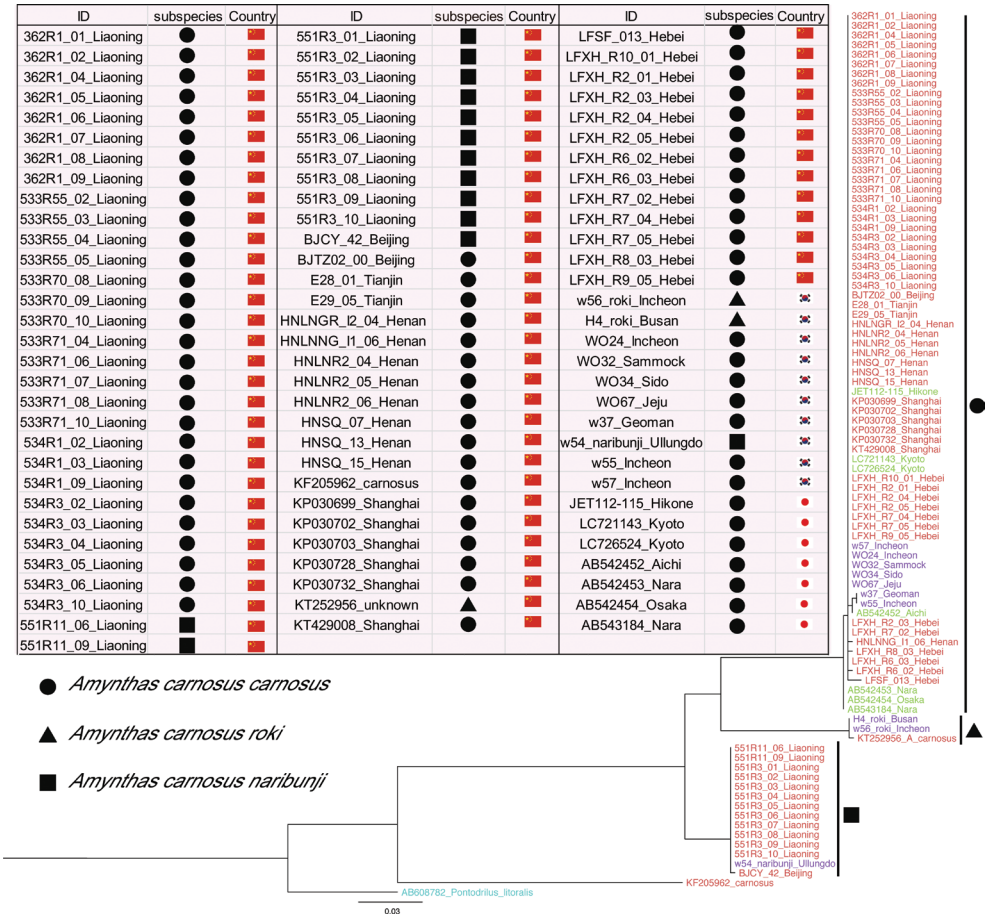

Figure 1. The geographical distribution of the *A. carnosus* complex in eastern Asia and its corresponding phylogenetic tree based on COI using the maximum likelihood method. Color coding: red for China, purple for South Korea, and green for Japan.

Molecular data show a divergence among the subspecies of *A. carnosus* (Fig. 1), which comprise the DNA samples provided from China, Japan, and South Korea. It also shows little genetic variation of *A. c. carnosus*, as shown by the absence or having of very short branches within the clade that is composed of the three countries. The same is observed in the other two subspecies that occur in China and South Korea. Here, the geographic representation shows a wide distribution of the species in eastern China (Fig. 1).

On the one hand, *A. c. naribunji*'s current distribution pattern has been expanded because of its new record in northern China (Beijing and Liaoning). Still, future investigations and additional sampling sites must be explored to be able to have a thorough understanding of the origin of this species and its migration pattern across countries.

Moreover, the specimen of *A. c. roki* (NIBR-IV0000261264 providing DNA w56) from Incheon Great Park (Blakemore and Lee 2013) has inter-subspecific distance values of 8.1–10% from *A. c. carnosus* and

7.2–7.5% from *A. c. naribunji*, respectively. Alternatively, a specimen labeled "*A. carnosus*" from China (KT252956; the detailed location is unknown) was grouped with the *A. c. roki* specimens from South Korea, with a 100% bootstrap value. Intersubspecific distances among *A. c. carnosus* and *A. c. naribunji* are 9–10% and 8%, respectively. As no data on the exact location and morphological descriptions of *A. c. roki* in China have been reported, further sampling of *A. c. roki* needs to be done in the future.

Morphological characterization

Family Megascolecidae Rosa, 1891
Genus *Amynthas* Kinberg, 1867

Amynthas carnosus carnosus Goto & Hatai, 1899

Perichaeta carnosa Goto & Haitai, 1899: 15.

Pheretima carnosa – Kobayashi 1936: 115.

Amynthas carnosus – Sims and Easton 1972: 235. Blakemore 2012: 36; 2013a: 58; 2013c: 101. Carrera-Martínez and Snyder 2016: 297. Chang et al. 2016: 505.

Amynthas pingi (Stephenson, 1925) – Sims and Easton 1972: 235. Blakemore 2013c: 112.

Material examined. Specimen IDs: 362R1_01, 02, 04, 05, 06, 07, 09, seven matures from Nanshan Park, Jinzhou, Liaoning; 533R70_08, 09, 10, three matures from Jinjiang Mt. Park, Dandong, Liaoning; LFXHR7_02, 04, 05, three matures from Zhuti Park, Xianghe County, Langfang, Hebei; LFSF_013, one mature from Langfang Normal University, Anci District Langfang, Hebei; E29_05, one mature from an anonymous park in Dongli District, Tianjin Municipality; HNLNR2_04, 05, 06, three matures from the tobacco field in Xiaojie Town, Luoning County, Luoyang, Henan; HNSQ_07, 13, 15, three matures under the bushes in Shangqiu Normal University, Liangyuan District, Shangqiu, Henan.

Diagnosis. Length 105–210 mm. Spermathecal pores having four pairs in 5/6/7/8/9, rarely 3 pairs in 6/7/8/9, with pre-intersegmental hemispherical arc (spermathecal papillae). Dorsal pores typically from 12/13. Pre-clitellar genital markings typically with two pairs, pre-setal in VIII and IX; these genital markings paired either widely or closely apart (B1 and B2, Fig. 3); Post-clitellar genital markings prominent, up to three pairs median to male pores; first pair pre-setal on XVIII, slightly median to male pores; second pair post-setal and more medial than the first; third pair pre-setal in XIX (Fig. 4). Male pores superficially paired in XVIII close to the lateral margin on round or elliptical porophores (Fig. 4). Ampulla ovate to narrowly ovate (Fig. 2D–F). Intestinal caeca simple at XXVII.

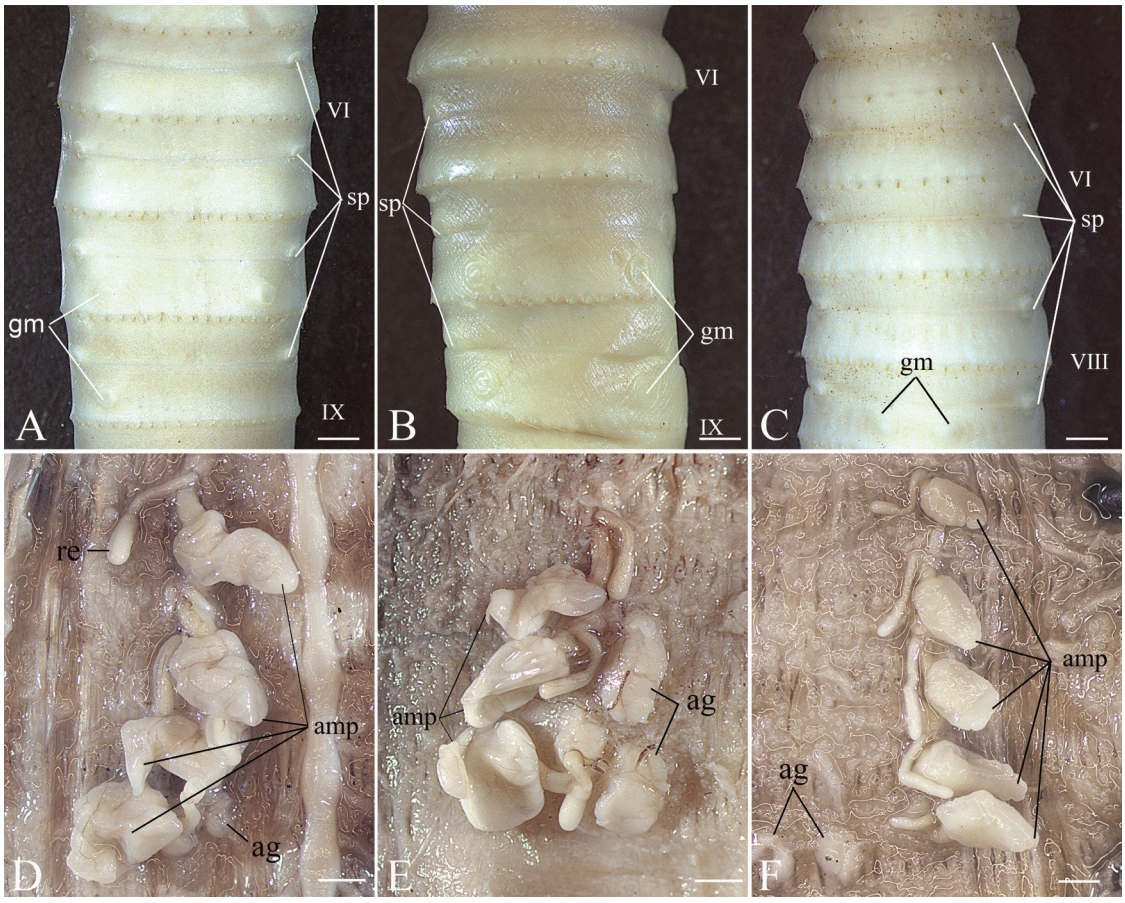

Figure 2. *Amynthas carnosus carnosus* variations on the number of spermathecal pores and spermathecae: four pairs (**A, D**) (specimen ID 362R1_06), three pairs (**B, E**) (specimen ID LFXHR7_05), five pairs (**C, F**) (specimen ID HNLNR2_05). Scale bars: 1 mm.

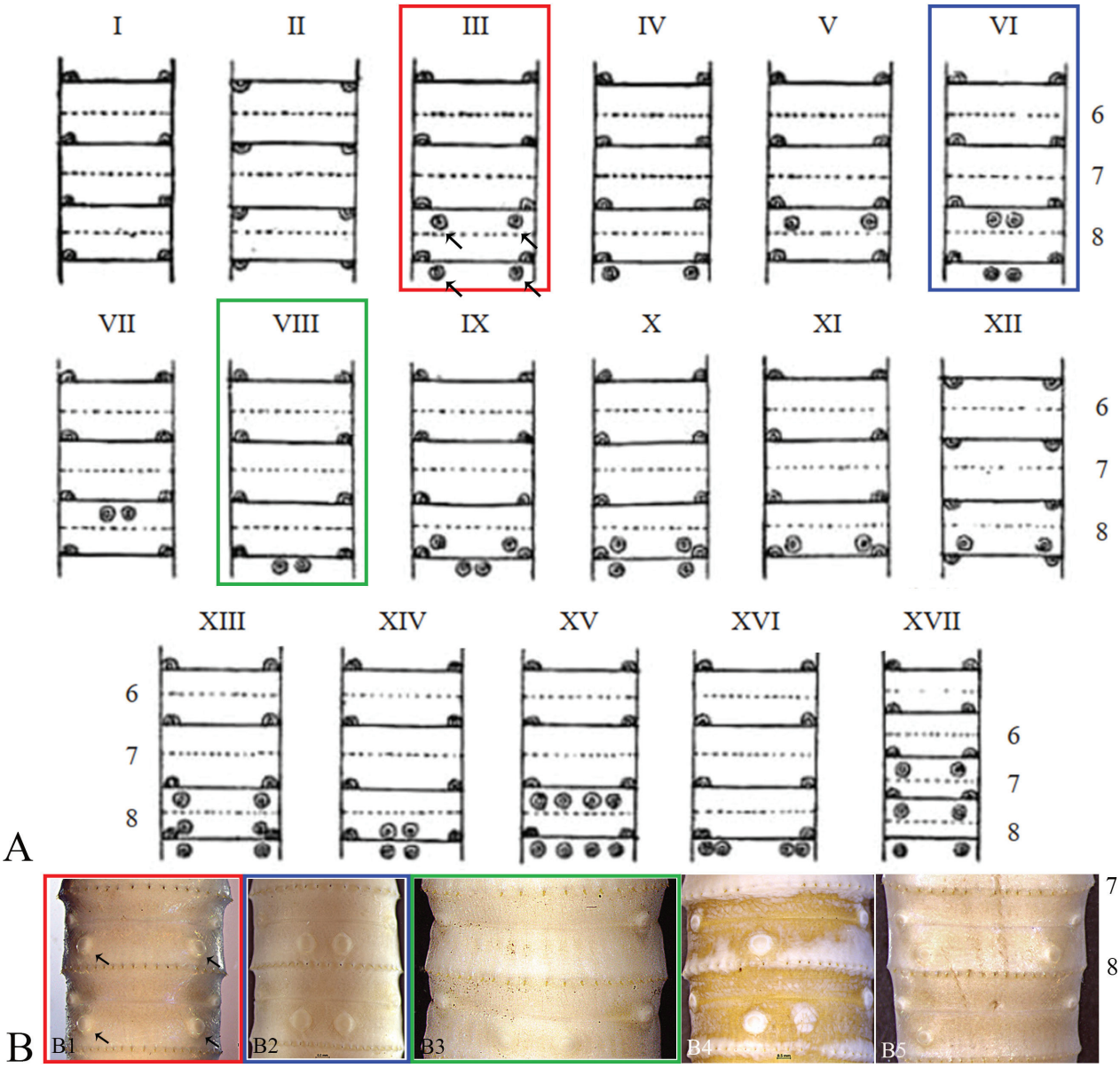

Figure 3. Pre-clitellar genital marking (arrows) variations of *Amynthas carnosus*. **A.** Modified fig. 1 of the variations of pre-clitellar genital markings from Kobayashi (1936); **B.** This study. **B1.** (specimen ID 362R1_01); **B2.** (specimen ID 533R70_09), and **B3.** (specimen ID HNLNR2_05) comply with the "permissible variations" [termed by Blakemore (2012)] of Kobayashi's (1936) Type III (red), VI (blue), and VIII (green).

Variations. For the *A. c. carnosus* from China, the number of spermathecal pores and spermathecae are variable: 14 out of 16 specimens typically have four pairs in 5/6/7/8/9, one specimen has three pairs in 6/7/8/9 (LFXHR7_05), and another one has five pairs in 4/5/6/7/8/9 (HNLNR2_05) (Fig. 2). However, despite these variations, molecular analyses have shown them to belong in the same clade with little genetic divergence within the clade (Table 2, Fig. 1). Two specimens from South Korea and one specimen from the USA have three pairs of spermathecal pores in 6/7/8/9 (Kobayashi 1936; Carrera-Martínez and Snyder 2016). However, prior to this study, no other specimen with five pairs was recorded elsewhere.

Distribution. China (Liaoning, Beijing, Tianjin, Hebei, Henan, and Shanghai), Japan (Kyushu, Honshu, and Hokkaido), and South Korea (Incheon, Jeju Island).

Remarks. Detailed descriptions of *A. carnosus* were reported by Kobayashi (1936), Ohfuchi (1937), and Blakemore (2012, 2013a, c). Rather than typical closely spaced mid-ventral pre-clitellar genital markings (Blakemore 2012), widely spaced ones are mostly observed with the *A. carnosus* specimen from China, which resembles those Hikone specimens from Japan (Tokyo An-460-DNA JET-112) [cf. fig. 3 by Blakemore (2013a)]. In contrast, the closely spaced pre-clitellar genital markings of the Liaoning specimens (Dandong, DNA 533R) match those of the South Korean specimen from Geoman

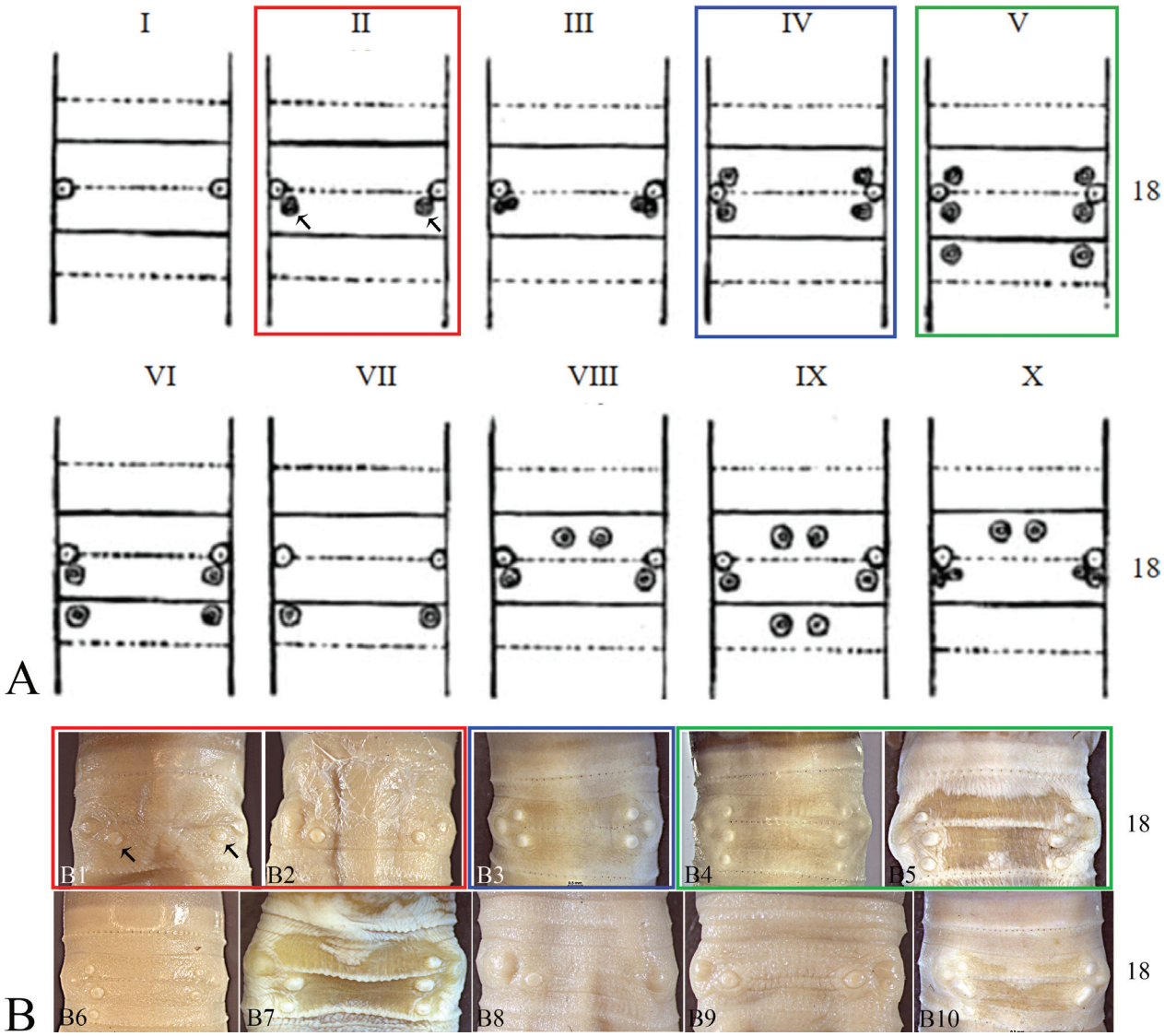

Figure 4. Post-clitellar genital marking (arrows) variations of *Amynthas carnosus*. **A.** Modified fig. 2 of the variations of post-clitellar genital markings from Kobayashi (1936); **B.** This study. **B1, B2. (**specimen IDs 362R1_07 and HNLNR2_04); **B3** (specimen ID 362R1_04); **B4, B5. (**specimen IDs 362R1_01 and 533R70_10) comply with the "permissible variations" [termed by Blakemore (2012)] of Kobayashi's (1936) Type II (red), IV (blue), and V (green).

(NIBR IV261234-DNA w37) and the Japanese neotype of *A. carnosus* (Tokyo An435) [cf. fig. 2 by Blakemore (2013a)].

A comparison of characters from the specimens of China, Japan (a neotype NSMT An435 from the Tokyo Museum) (Blakemore 2012), and the USA (Kansas) (Chang et al. 2016) is presented in Suppl. material 1: table S2. External characters such as the number of spermathecal pores and segment locations of pre-clitellar genital markings match among specimens from different countries. However, internal character variations are observed in the position of the intestinal caeca, which was reported to begin at XXVII and extend to XXIII or XXIV (Blakemore, 2012; 2013b; 2013c; Chang et al. 2016), while the intestinal caeca in the Chinese specimens extends up to XX, XXI (or XXIII) (Fig. 5), 2–3 segments more anterior than those from the two previous accounts. Moreover, some character measurements that were not presented in the other two accounts, such as the ventral distances between male pores (0.25–0.29 mm), spermathecal pores (0.28–0.30 mm), and genital markings (latero-ventrally with 0.21–0.29 mm distance apart or mid-ventrally with 0.08 mm distance apart), were added to further aid species identification.

Kobayashi (1936), in his thorough investigation of *A. carnosus*, presented "permissible" variations on the pre-clitellar and post-clitellar genital markings [text-figs. 1–2 in Kobayashi (1936)]. The pre-clitellar genital making variations in the *A. c. carnosus* from China comply with Kobayashi's Types IIII, VI, and VIII (see Fig. 3), while the post-clitellar genital marking variations comply with Kobayashi's Types II, IV, and V (see Fig. 4). It is important to take note that genital marking patterns can also be considered a distinctive character for species identification (e.g., Nguyen et al. 2020; Aspe et al. 2021).

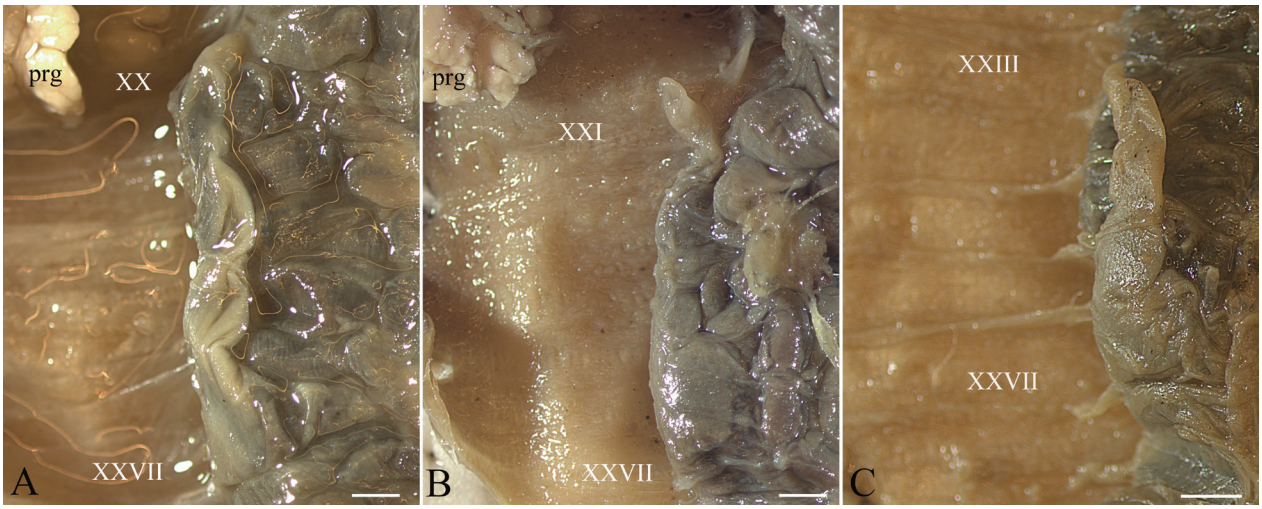

Figure 5. *Amynthas carnosus carnosus* intestinal caeca showing the segment length variations: **A.** (specimen ID 533R70_10); **B.** (specimen ID 362R1_05), and **C.** (specimen ID HNLNR2_05). Scale bars: 1 mm.

Amynthas carnosus naribunji Blakemore, 2013

Amynthas pingi: Blakemore 2013a: 60, figs 4, 5.

Material examined. Specimen IDs: 551R3 (01–10), 10 adults from Longwan Park, Huludao Pref., Liaoning Prov. One specimen from Beijing is a juvenile and was poorly preserved. Thus, its morphological examination was not performed. However, the molecular data is presented (DNA ID: BJCY_42, Fig. 1).

Diagnosis. Spermathecal pores four pairs in 5/6/7/8/9, located latero-ventrally (0.29–0.30 mm), each with pre-intersegmental hemispherical arc (spermathecal papillae) anterior to intersegments (Fig. 6). Pre-clitellar genital markings absent (complying with Kobayashi Type I in Blakemore 2013a); if present, one to three pairs; pre-setal in VIII and IX when one or two pairs and having a post-setal pair in VIII when three pairs (complying with Kobayashi's Types III, IV, and VIII); asymmetrical patterns also present (Fig. 7). Male pores superficial in XVIII having "disc-like" genital markings paired posterio-median to male pores (Fig. 8).

Description. Length 185–228 mm. Color of preserved specimens may have varying shades of brown but dorsum generally dark brown in pre-clitellar region to brown in post-clitellar region, fading to lighter brown towards posterior end with darker clitellum, while ventrum part is paler/fleshy color. Clitellum width 5.8–8.3 mm. Segments 115–137. Prostomium epilobous. First dorsal pore on 12/13. Clitellum annular at XIV–XVI without setae or dorsal pores. Setal arrangement perichaetine, setae between male pore 18–19. Female pore single and circular, midventral at XIV.

Spermathecal pores large, having four pairs in 5/6/7/8/9 and widely-spaced, latero-ventral (0.29–0.30 mm) in pre-intersegmental hemispherical arc (spermathecal papillae). Pre-clitellar genital markings circular in shape, latero-ventral (0.25–0.29 mm), randomly located in pairs (three pairs/two pairs/one pair, a total of 2–6 genital markings), or asymmetrically located on one side (1–2 genital markings), about 0.38–0.59 mm in diameter.

Male pores superficially paired in XVIII close to lateral margin (with ventral distance 0.26–0.29 mm) on large circular porophores. Post-clitellar genital markings distinguishably paired, post-setally in XVIII, mid-ventral to male pore, 0.42–0.69 mm in diameter.

Septa 4/5–7/8 and 10/11–14/15 thickened, 8/9/10 absent. Esophageal gizzard within VIII−X. Intestinal origin at XV (or XIV). Intestinal caeca simple, paired in XXVII, extending anteriorly to XXII. Last hearts in XIII.

Four pairs of spermathecae in VI−IX. Ampulla ovate, wrinkled; ducts short and stout. Diverticula reaching one-third to half of ampulla with a slender stalk and a wider seminal chamber; seminal chamber elongated or botuliform. Accessory glands sessile and round.

Seminal vesicles paired in XI and XII, large, smooth, yellowish, posterior pair larger but not as obvious compared to *A. c. carnosus*, each with a dorsal lobe. Ovaries present. Prostate glands paired in XVIII, large, lobulated, covering XVI−XX; ducts thick and large, U-shaped. Accessory glands round, sessile, or slightly lobed, corresponding to each genital marking around male pore area.

Distribution. Northern China (Liaoning, Beijing) and South Korea (Ulleung Island).

Remarks. There is not much of a thorough morphological description of *A. carnosus naribunji* in the original account of Blakemore (2013a) (see Suppl. material 1: table S3) aside from its single illustration of paired post-clitellar genital markings in the male pore area and a spermathecal pore with corresponding spermathecae shown in fig. 4 by Blakemore (2013c).

Notable features of *A. c. naribunji* in comparison with *A. c. carnosus* were its slightly larger size with lengths of 185–228 mm, typically wide-spaced pre-clitellar genital markings with a maximum number of six (three pairs) to at least three genital markings; pre-setal/post-setal in VIII and pre-setal in IX. In contrast, *A. c. carnosus* is

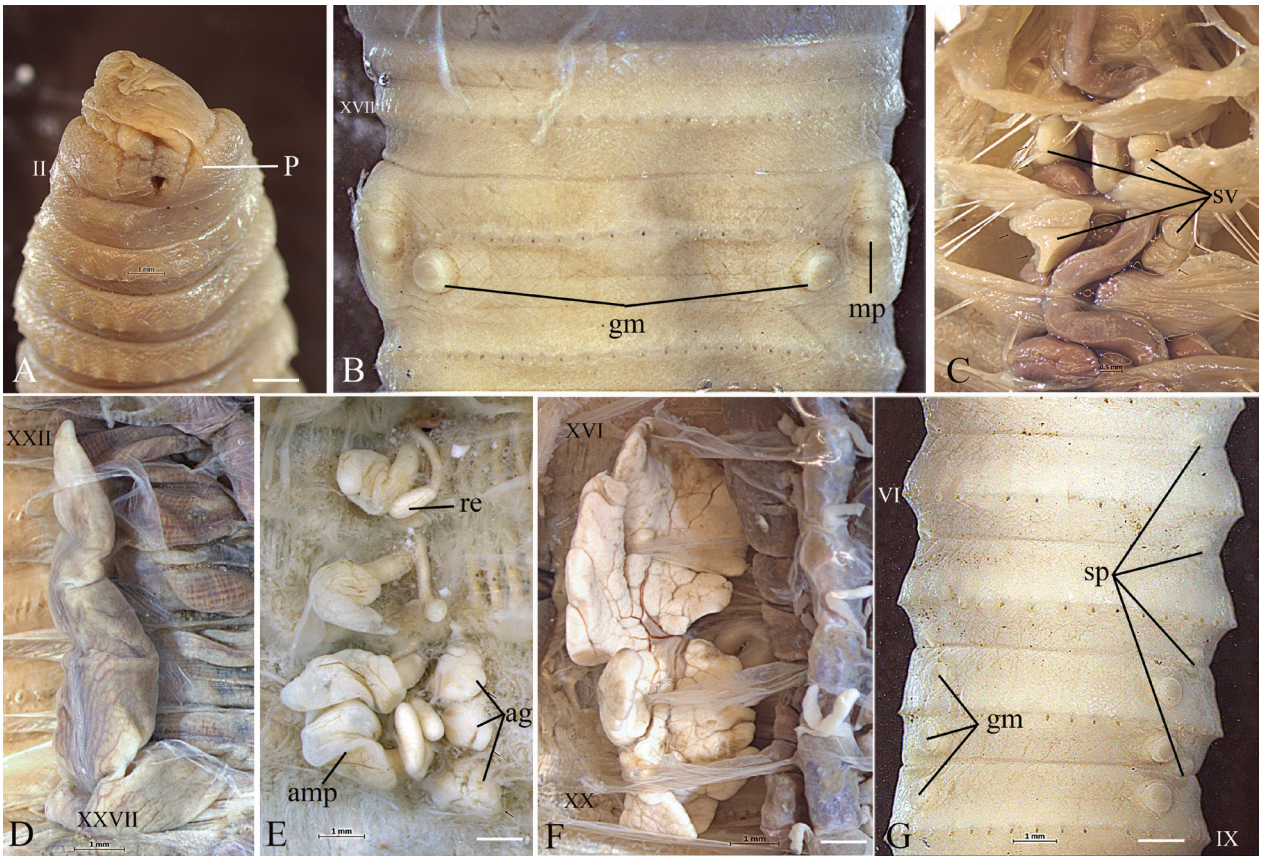

Figure 6. *A. carnosus naribunji* (specimen ID 551R3_01): Prostomium (**A**); Male pore with posterio-median paired genital marking (**B**); Seminal vesicles (**C**); Intestinal caeca (**D**); spermathecae (**E**); Prostate gland with U-shaped think duct (**F**); and Spermathecal pores with three pairs of pre-clitellar genital markings (**G**). Scale bars: 1 mm.

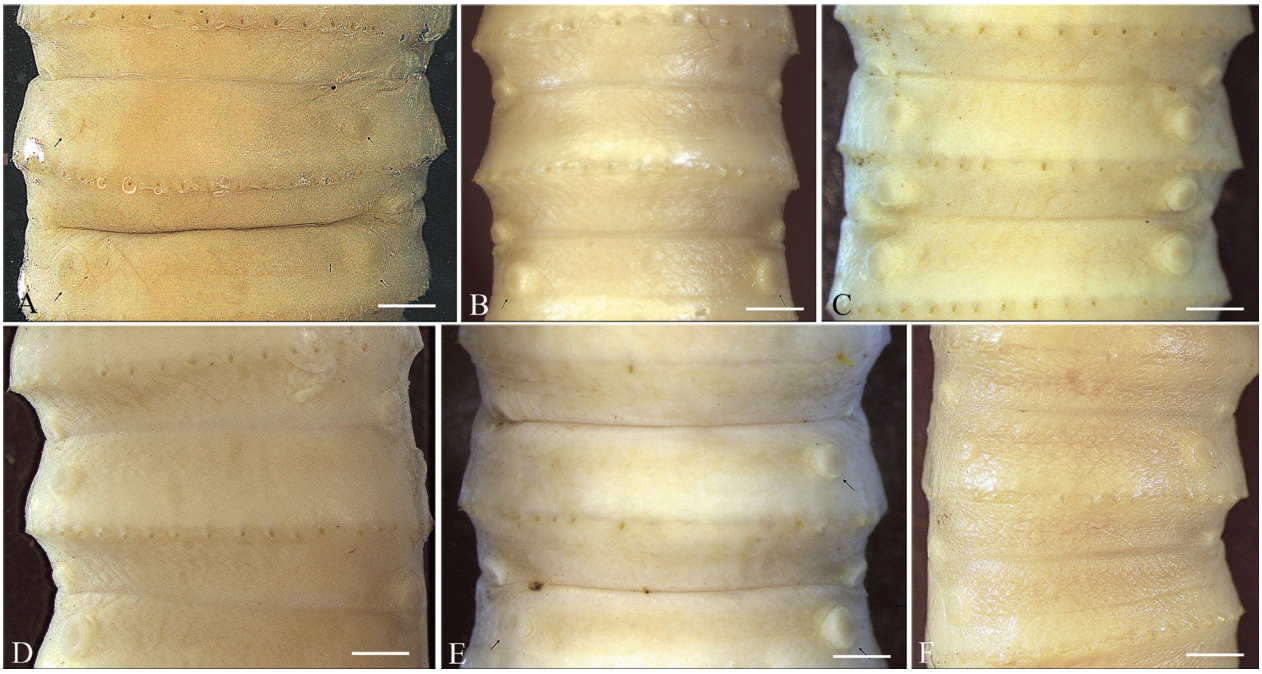

Figure 7. *A. carnosus naribunji* pre-clitellar genital marking variations; **A–C.** Complying with Kobayashi's (1936) Types III, IV, and VIII, respectively; **D–F.** Displays asymmetrical patterns. Scale bars: 1 mm.

typically medium to smaller size, with mostly only four genital markings (two pairs) or less, either wide or closely-spaced pre-clitellar (Table 3).

The distinctive character of having "a pair of genital markings posterio-median to male pores" in *A. c. naribunji* may distinguish it from those of *A. c. carnosus*

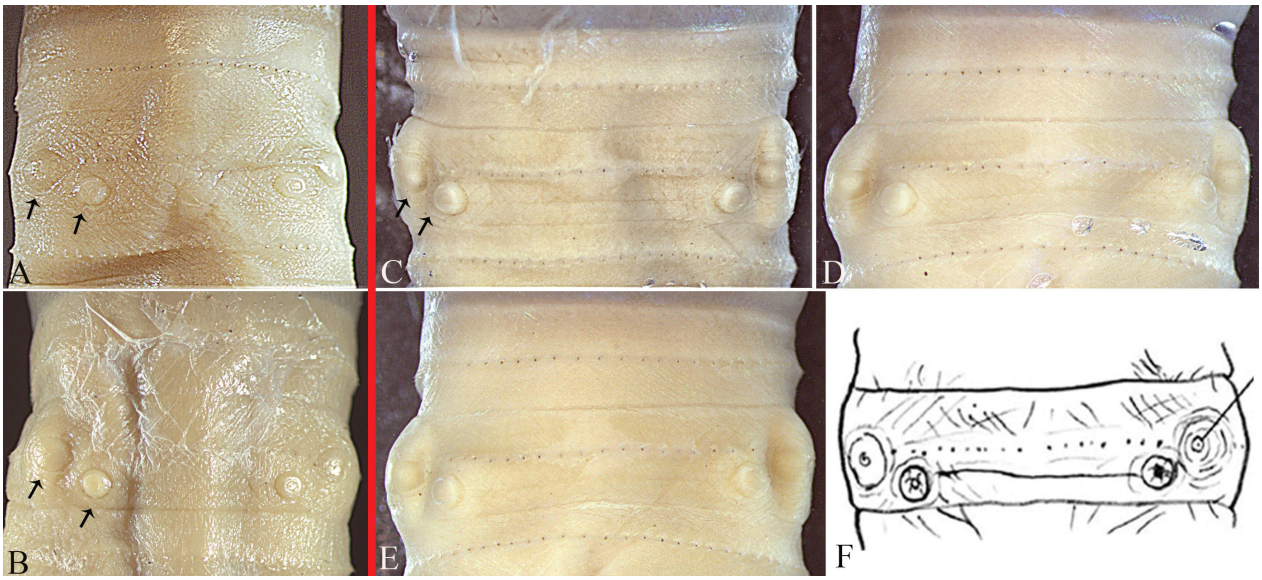

Figure 8. Image comparison of male pore porophores and paired posterio-median genital markings between *A. carnosus carnosus*. **A, B.** (specimen IDs 362R1_07 and HNLNR2_04) and *A. carnosus naribunji* from Liaoning; **C–E.** (specimen IDs 551R3_01, 551R3_09, and 551R3_06) and Ulleungdo, **F.** (specimen ID w54).

Table 3. Character comparison among *A. c. carnosus*, *A. c. naribunji*, and *A. c. roki*. The asterisk stands for the figure in Blakemore and Lee 2013a, p. 131, yet there is no definite description.

Characters	*A. carnosus carnosus*	*A. carnosus naribunji*	*A. carnosus roki*
Length	105–340 mm	185–228 mm	175–300 mm
No. of segments	96–179	121–137	136
No. of setae between mp	16–20	17–20	14
Male pore	round or elliptical porophores	on large circular porophores,	superficial on small mounds within concentric rings
Distance between Male pores (circumference apart)	0.25–0.29	0.26–0.29	0.30
Spermathecal pore	6/7/8/9 (three pairs, rarely); 5/6/7/8/9 (four pairs); 4/5/6/7/8/9 (five pairs, rarely); with pre-intersegmental spemathecal papillae	5/6/7/8/9 (four pairs) with pre-intersegmental spermathecal papillae	post-intersegmental pores 5/6/7/8/9 (four pairs) with post-intersegmental spermathecal papillae
Distance between spermathecal pores (circumference apart)	0.28–0.30	0.29–0.30	NA
Pre-clitellar gm (circumference apart)	closely paired or widely spaced pre-setal in VIII and IX, mid-ventral (mostly two pairs, total of 1–4 GMs)	widely spaced, randomly located in pairs (three pairs or two pairs or one pair, a total of 2–6 GMs)	absent
Post-clitellar gm (circumference apart)	up to three pairs of genital markings near male pores, pre-/post-setal in VXIII and pre-setal in XIX. Second pair, post-setal more medial than the first	distinguishably paired, post-setal in XVIII, mid-ventral to male pore	absent
Spermathecae (circumference apart)	typically four pairs in VI–IX, first pair often slightly smaller; or three pairs in VII–IX or five in IV–IX	four pairs in VI–IX	four pairs in VI–IX
Ampulla and Duct (circumference apart)	Wrinkled; Ovate to narrowly ovate	Wrinkled; Ovate to narrowly ovate	Narrowly ovate*
Prostate gland (circumference apart)	racemose at XVIII, covering XV (or XVI)–XX	racemose at XVIII, covering XVI–XX	racemose in XVIII
Intestinal caeca (circumference apart)	paired in XXVII, simple, extending to XX, or XI, or XIII	paired in XXVII, simple, extending to XXII	Simple from XXVII
Gizzard (circumference apart)	VIII–X	VIII–X	NA
Intestine	XV (or XIV)	XV	NA

(Blakemore, 2013a). However, it is now undeniable that this seemingly "distinctive character" was likewise observed in the *A. c. carnosus* specimens from Liaoning and Henan (Fig. 8). Here, we dismiss the former notion of Blakemore (2013a) "to consider for exclusion Kobayashi's Types I and II markings" as a distinctive character for the *A. c. carnosus* subspecies, *A. c. naribunji*. Although this might be the case, one cannot ignore the certain degree of dissimilarity between the two subspecies' morphological characters (e.g., the shape of male pore porophore and the distinct shape of the genital markings in *A. c. naribunji*, described as "disc-like" by Blakemore). As such, applications of state-of-the-art methodologies such as high-throughput sequencing or geometric morphometric (Marchán et al. 2020) may be adopted that go beyond traditional methods of taxonomic diagnosis

(which in this case is rather insufficient to "quantify" the degree of variations). Nevertheless, the incorporation of genetic data such as DNA barcoding and calculating K2P intraspecific distances (as conducted in this study) may aid in suggesting subspecies delineation.

***Amynthas carnosus roki* Blakemore & Lee, 2013**

Material examined. In China, only molecular data is available in the Genbank (Accession No. KT252956).

Description. See Blakemore and Lee 2013: 129–132.

Distribution. South Korea, China.

Morphological comparison among the subspecies of *A. carnosus*

A list of character comparisons between *A. c. carnosus*, *A. c. naribunji*, and *A. c. roki* is summarized in Table 3. Most of the distinctive characters for *A. c. carnosus* and *A. c. naribunji* are discussed above. As for *A. c. roki*, "distinctive characters are the tendency to have large size (175–300 mm) post-intersegmental spermathecal pores with U-shaped spermathecal papillae. Obvious distinctive character accounts for the lack of genital markings, thereby complying with Kobayashi's Types II and I" (Blakemore and Lee 2013). Blakemore and Lee (2013) added further: "On these characters, the present subspecies appears to differ from the nominal taxon's neotype and from other synonyms in Blakemore." Aside from the external morphological differences, slight internal character differences were also observed (Table 3).

According to Blakemore (2012), "the genital marking variation in *A. carnosus* allowed for by Kobayashi in his detailed and most thorough account is excessive, rather representing a congery of morphs, if not separate species'', this can be said out from the preliminary DNA results forming new taxa as *A. c. naribunji* and *A. c. roki* (Blakemore 2013a; Blakemore and Lee 2013). Genital marking variations as observed in our specimens here did appear to have variations exceeding that of Kobayashi's permissible" genital marking variations (with asymmetrical GMs also observed).

Conclusion

Our results agree with Blakemore's subspecies assignment of *A. c. naribunji* and *A. c. roki* (additional molecular data only). Furthermore, the attempt to justify the presumed "distinctive character" of having a pair of genital markings posterio-median to male pores in *A. c. naribunji*, which is distinct from the *A. c. carnosus* as noted by Blakemore (2013a, c), has now been invalidated, given that both *A. c. naribunji* and *A. c. carnosus* specimens in China possess this same character. Here, a pairwise distance for the *A. carnosus* subspecies was shown to be 10% and below. With new occurrences of *A. carnosus* in China, patterns of morphological as well as genetic variations in different geographical occurrences of this species can be further elucidated. Still, a more thorough investigation should be carried out by conducting a broader sampling collection in the country, which may contribute to new distribution records. Moreover, with the updated and detailed morphological descriptions of *A. c. naribunji* provided here, elaborate data can now be used to clarify morphological distinctions. Thus, similar specimens from any region may now be compared genetically to an increasingly refined formulation of *A. carnosus*, its subspecies, and even synonymous species.

Acknowledgments

We thank the reviewer, Parin Jirapatrasilp, for his valuable suggestions. This study was supported by the National Natural Sciences Foundation of China (42071059); the Northeast Asia Biodiversity Research Center (2572022DS09); by the China National Tobacco Corporation of Science and Technology Major Projects (110202201018 [LS-02]); by the "One Belt, One Road" international scholarship from the Ministry of Science and Technology of China (DL2023130001L); and by the President's International Fellowship Initiative from the Chinese Academy of Sciences (2024VCA0009); the National Science and Technology Fundamental Resources Investigation Program of China (2018FY100301).

References

Admassu B, Juen A, Traugott M (2006) Earthworm primers for DNA-based gut content analysis and their cross-reactivity in a multi-species system. Soil Biology & Biochemistry 38(6): 1308–1315. https://doi.org/10.1016/j.soilbio.2005.08.019

Aspe NM (2016) The geographic distribution of the genera in the *Pheretima* complex (Megascolecidae) in eastern Asia and the Pacific region. Kaiyo Monthly 48: 39–45.

Aspe NM, Manasan RE, Manlavi AB, Ma. Patiluna LE, Sebido MA, Obusan MCM, Simbahan JF, James SW (2021) The earthworm fauna of Palawan, Philippines with description of nineteen new pheretimoid species (Clitellata, Megascolecidae). Journal of Natural History 55(11–12): 11–12, 733–779. https://doi.org/10.1080/00222933.2021.1923849

Bely AE, Wray GA (2004) Molecular phylogeny of naidid worms (Annelida, Clitellata) based on cytochrome oxidase I. Molecular Phylogenetics and Evolution 30(1): 50–63. https://doi.org/10.1016/S1055-7903(03)00180-5

Blakemore R (2009) Cosmopolitan Earthworms–A Global and Historical Perspective. 257–283. https://doi.org/10.1002/9780470455203.ch14

Blakemore R (2012) *Amynthas carnosus* (Goto & Hatai, 1899) re-described on its neotype (Oligochaeta, Megadrilacea, Megascolecidae). Journal of Species Research 1(1): 35–46. https://doi.org/10.12651/JSR.2012.1.1.035

Blakemore R (2013a) *Megascolex* (*Perichaeta*) *diffringens* Baird, 1869 and *Pheretima pingi* Stephenson, 1925 types compared to the *Amynthas corticis* (Kinberg, 1867) and *A. carnosus* (Goto & Hatai, 1899) species-groups (Oligochaeta, Megadrilacea, Megascolecidae).

Journal of Species Research 2(2): 99–126. https://doi.org/10.12651/JSR.2013.2.2.099

Blakemore R (2013b) Jeju-do earthworms (Oligochaeta, Megadrilacea)-Quelpart Island revisited. Journal of Species Research 2(1): 15–54. https://doi.org/10.12651/JSR.2013.2.1.015

Blakemore R (2013c) Ulleung-do earthworms - Dagelet Island revisited. Journal of Species Research 2(1): 55–68. https://doi.org/10.12651/JSR.2013.2.1.055

Blakemore R, Lee W (2013) Survey of Busan Oligochaeta earthworms supported by DNA barcodes. Journal of Species Research 2(2): 127–144. https://doi.org/10.12651/JSR.2013.2.2.127

Carrera-Martínez R, Snyder B (2016) First report of *Amynthas carnosus* (Goto & Hatai, 1899) (Oligochaeta, Megascolecidae) in the Western Hemisphere. Zootaxa 4111(3): 297–300. https://doi.org/10.11646/zootaxa.4111.3.7

Chang C-H, Lin S-M, Chen J-H (2008) Molecular systematics and phylogeography of the gigantic earthworms of the Metaphire formosae species group (Clitellata, Megascolecidae). Molecular Phylogenetics and Evolution 49(3): 958–968. https://doi.org/10.1016/j.ympev.2008.08.025

Chang C-H, Snyder B, Szlávecz K (2016) Asian pheretimoid earthworms in North America north of Mexico: An illustrated key to the genera *Amynthas*, *Metaphire*, *Pithemera*, and *Polypheretima* (Clitellata, Megascolecidae). Zootaxa 4179(3): 495–529. https://doi.org/10.11646/zootaxa.4179.3.7

Chang C-H, Bartz M, Brown G, Callaham M, Cameron E, Davalos A, Dobson A, Gorres J, Herrick B, Ikeda H, James S, Johnston M, McCay T, McHugh D, Minamiya Y, Nouri Aiin M, Novo M, Ortiz-Pachar J, Pinder R, Szlávecz K (2021) The second wave of earthworm invasions in North America: Biology, environmental impacts, management and control of invasive jumping worms. Biological Invasions 23(11): 1–32. https://doi.org/10.1007/s10530-021-02598-1

Chen Y (1933) A preliminary survey of the earthworms of the Lower Yangtze Valley. Contributions from the Biological Laboratory of the Science Society of China 9: 177–296.

Chen Y (1936) On the terrestrial Oligochaeta from Szechuan II with the notes on Gates' types. Contributions of Biological Laboratory of Science Society of China. Zoology 11: 269–306.

Dong Y, Law MMS, Jiang J, Qiu J (2019) Three new species and one subspecies of the *Amynthas corticis*-group from Guangxi Zhuang Autonomous Region, China (Oligochaeta, Megascolecidae). ZooKeys 884: 23–42. https://doi.org/10.3897/zookeys.884.30988

Easton EG (1981) Japanese earthworms: A synopsis of the Megadrile species (Oligochaeta). Bulletin of the British Museum (Natural History). Zoology : Analysis of Complex Systems, ZACS 40: 33–65.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Gates GE (1939) On some species of Chinese earthworms with special reference to specimens collected in Szechuan by Dr. D.C. Graham. Proceedings of the United States National Museum 85: 405–507. https://doi.org/10.5479/si.00963801.3040.405

Goto S, Hatai S (1899) New or imperfectly known species of earthworms. No. 2. Annotationes Zoologicae Japonenses 3: 13–24.

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98. https://doi.org/10.14601/phytopathol_mediterr-14998u1.29

Huang J, Xu Q, Sun Z, Tang G, Su Z (2007) Identifying earthworms through DNA barcodes. Pedobiologia 51(4): 301–309. https://doi.org/10.1016/j.pedobi.2007.05.003

Jiang J, Qiu J (2018) Origin and evolution of earthworms belonging to the family Megascolecidae in China. Shengwu Duoyangxing 26(10): 1074–1082. https://doi.org/10.17520/biods.2018105

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16(2): 111–120. https://doi.org/10.1007/BF01731581

Kobayashi S (1936) Distribution and some external characteristics of *Pheretima* (*Ph.*) *carnosa* (Goto et Hatai) from Korea. Science Report of the Tohoku Imperial University 11: 115–138.

Kobayashi S (1938) Earthworms of Korea I. Science Report of the Tohoku Imperial University 13: 89–170.

Marchán DF, Novo M, Sánchez N, Domínguez J, Díaz Cosín DJ, Fernández R (2020) Local adaptation fuels cryptic speciation in terrestrial annelids. Molecular Phylogenetics and Evolution 146: 106767. https://doi.org/10.1016/j.ympev.2020.106767

McCay TS, Brown G, Callaham Jr MA, Chang C-H, Dávalos A, Dobson A, Görres JH, Herrick BM, James SW, Johnston MR, McHugh D, Minteer T, Moore J-D, Nouri-Aiin M, Novo M, Ortiz-Pachar J, Pinder RA, Richardson JB, Snyder BA, Szlavecz K (2020) Tools for monitoring and study of peregrine pheretimoid earthworms (Megascolecidae). Pedobiologia 83: 150669. https://doi.org/10.1016/j.pedobi.2020.150669

Nguyen TT, Nguyen NQ, Lam DH, Nguyen AD (2020) Six new species of the genus *Metaphire* Sims & Easton, 1972 (Annelida, Oligochaeta, Megascolecidae) from southeastern Vietnam. The Raffles Bulletin of Zoology 68: 220–236. https://doi.org/10.26107/RBZ-2020-0019

Novo M, Almodóvar A, Diaz Cosin DJ (2009) High genetic divergence of hormogastrid earthworms (Annelida, Oligochaeta) in the central Iberian Peninsula: Evolutionary and demographic implications. Zoologica Scripta 38(5): 537–552. https://doi.org/10.1111/j.1463-6409.2009.00389.x

Ohfuchi S (1937) On the species possessing four pairs of spermathecae in the genus *Pheretima*, together with the variability of some external and internal characteristics. Saito Ho-On Kai Museum Research Bulletin: 31–136.

Sims RW, Easton EG (1972) A numerical revision of the earthworm genus *Pheretima* auct. (Megascolecidae, Oligochaeta) with the recognition of new genera and an appendix on the earthworms collected by the Royal Society North Borneo Expedition. Biological Journal of the Linnean Society, Linnean Society of London 4(3): 169–268. https://doi.org/10.1111/j.1095-8312.1972.tb00694.x

Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Stephenson J (1925) Oligochaeta from various regions, including those collected by the Mount Everest Expedition 1924. Proceedings of the Zoological Society of London 95: 879–907. https://doi.org/10.1111/j.1469-7998.1925.tb07109.x

Stephenson J (1931) Oligochaeta from Burma, Kenya and other parts of the world. Proceedings of the Zoological Society of London 101: 33–92. https://doi.org/10.1111/j.1469-7998.1931.tb06185.x

Sun J (2013) Taxonomy and Molecular Phylogeny of *Amynthas* Earthworms from China. Shanghai: Shanghai Jiao Tong University.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Sudhir K (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28(10): 2731–2739. https://doi.org/10.1093/molbev/msr121

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22(22): 4673–4680. https://doi.org/10.1093/nar/22.22.4673

Xiao N (2019) Terrestrial Earthworms (Oligochaeta, Opisthopora) of China. Elsevier Inc, China, 395 pp. https://doi.org/10.1016/B978-0-12-815587-5.09986-8

Zhang L, Sechi P, Yuan M-L, Jiang J, Dong Y, Qiu J (2016) Fifteen new earthworm mitogenomes shed new light on phylogeny within the *Pheretima* complex. Scientific Reports 6(1): 20096. https://doi.org/10.1038/srep20096

Supplementary material 1

Taxa list with the accession numbers and comparison of morphological characters

Authors: Anne Charis N. Han, Yufeng Zhang, Pu Miao, Shaolong Wu, Nengwen Xiao, Mingyan Qin, Donghui Wu, Huifeng Zhao, Nonillon M. Aspe

Data type: docx

Link: https://doi.org/10.3897/zse.100.119292.suppl1