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Cryptic Diversity within the Endemic Mekong Giant Earthworm, *Amynthas mekongianus* (Cognetti, 1922) (Clitellata: Megascolecidae) Across the Lower Mekong River Basin with Descriptions of a New Species

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ABSTRACT.– The Lower Mekong Basin is recognized as a region of remarkable biodiversity. However, it is currently experiencing severe degradation due to rapid economic development and the intensifying effects of climate change. Numerous species, including earthworms, are under increasing threat. In addition, *Amynthas mekongianus* (Cognetti, 1922) exhibits considerable intraspecific morphological variability, making its identification challenging. Therefore, the study aims to clarify species boundaries within the *A. mekongianus* complex in the Lower Mekong River Basin using both morphological and molecular approaches. Specimens were collected from multiple sites in the basin, and analyses revealed at least two putative species. Phylogenetic reconstructions (NJ, ML, and BI) together with species delimitation methods (ASAP, GMYC, and bPTP) consistently supported the recognition of two distinct lineages. These lineages differ primarily in body length, segment number, and spermathecae morphology. Accordingly, they are recognized as *A. mekongianus* sensu stricto and *A. sirindhornae* Nantarat & Bantaowong, sp. nov., which is formally described herein. This study enhances understanding of the evolutionary complexity and taxonomic status of the group, providing a foundation for future conservation and management strategies in the region.

KEYWORDS: conservation, earthworm, phylogeny, systematics, taxonomy

INTRODUCTION

Accurately understanding and measuring biological diversity is one of the priority issues that needs to be addressed to successfully apply conservation policies (Agapow et al., 2004; Sattler et al., 2007). The Mekong River region is one of the most important global biodiversity hotspots with a length of 5,400 km that passes through six countries. It's originating from China, through Myanmar, Laos, Thailand, Cambodia, and finally Vietnam (Narumon and Boonsoong, 2006; Lu et al., 2014). Moreover, the Mekong River basin is among the most diverse riverine systems in the world and contains various endemic species (Narumon and Boonsoong, 2006; Lu et al., 2014; Jeratthitikul et al., 2019). The basin comprises various iconic and endangered species such as tigers, snails, giant freshwater stingrays and giant earthworms (Strong et al., 2008; Lu et al., 2014; Jeratthitikul et al., 2019). Currently, the biodiversity of this region is experiencing severe degradation driven by economic development, human population expansion, deforestation, overexploitation, and natural resource pollution, further exacerbated by the impacts of climate change (Strong et al., 2008; Jeratthitikul et al., 2019). Climate change will shift ecosystem boundaries and significantly diminish the size of habitats. As remaining patches become more isolated and fragmented, this will lead to species extinctions and a loss of biodiversity (Camacho et al., 2017; Singh et al., 2019). Moreover, so far, the diversity of earthworms in this region has not been intensively studied along its course.

Earthworms belong to the phylum Annelida and are commonly found worldwide (Fragoso et al., 1999; Edwards and Arancon, 2022). As soil-dwelling animals with burrowing and feeding behaviors, earthworms act as living engineers. They play a vital role in shaping and enhancing soil structure, decomposers, fertility, and improving their habitat in ways that benefit the entire ecosystem (Edwards and Arancon, 2022). Their actions facilitate the breakdown of organic matter, aeration of the soil, and nutrient cycling, effectively creating a more hospitable environment for plants to thrive (Fragoso et al., 1999; Edwards and Arancon, 2022). There are approximately 5,406 described species of earthworms over times and possibly more than 8,000 species in the total global species diversity estimation (Misirlioğlu et al., 2023). Among described species, the terrestrial earthworm genus *Amynthas* Kinberg, 1867 is one of the most diverse groups and is dominant in mainland Asia and Southeast Asian archipelagos. It consists of more than 713 species

worldwide (Misirlioğlu et al., 2023). *Amynthas* are earthworms found on land, inhabiting diverse environments such as natural forests, agricultural fields, paddy fields, and areas near rivers. One of the iconic earthworms in the Mekong River basin is a Mekong giant earthworm, *A. mekongianus* (Cognetti, 1922). This is the key flagship species and is endemic to the vicinity of the Mekong River. The iconic earthworm is typically long-sized, measuring between 690 and 1920 mm in length and 4 to 10 mm in width (Cognetti, 1922; Blakemore et al., 2007). However, earthworm morphological traits exhibit considerable variation across different populations within species (Novo et al., 2010; Shekhovtsov et al., 2013). Traditional classification methods, which relied heavily on morphology, frequently reveal geographic variation. Identifying and differentiating species based on only morphological characteristics is challenging and often imprecise (Jirapatrasilp et al., 2016; Jeratthitikul et al., 2017). Additionally, the Mekong River basin's plentiful discharge and various landforms become a major base for large-scale construction projects that cause earthworm habitat destruction (He et al., 2014). So, the endemic earthworm of this region may be under threat and need to clarify its taxonomic status.

Molecular techniques have effectively identified various earthworm species, particularly in cases where morphological features were inadequate. The mitochondrial cytochrome oxidase subunit I (COI) gene has proven especially valuable for species identification (Jirapatrasilp et al., 2016; Jeratthitikul et al., 2017). In this study, we combine traditional taxonomic approaches with molecular phylogenetics using COI sequences to define species boundaries and explore the phylogenetic relationships within *A. mekongianus* from the Mekong River region. Identifying cryptic species enables their inclusion in conservation strategies and biodiversity assessments.

MATERIALS AND METHODS

Ethics

All animal experiments were conducted in accordance with the guidelines approved by the Institute of Animals for Scientific Purpose Development (IAD) and the National Research Council of Thailand (permit number U1-03304-2559, UI-09066-2563 issued to Nattawadee Nantarat and Ueangfa Bantaowong, respectively). This study adhered to all relevant national regulations and institutional policies concerning the humane care and use of animals. Animal procedures were approved by the Institutional Animal Care and Use Committee by Khon Khaen university (IACUC-KKU-32-65).

Collecting specimens, morphological investigation and identification

Adult earthworms of *A. mekongianus* s.s. were collected from the Mekong River region in Thailand and Laos (Fig. 1; Table 1). The specimens were anesthetized in 30% (v/v) ethanol, fixed in 10% (v/v) formalin, and preserved in 70% (v/v) ethanol for morphological study. Small muscle tissue samples from the area behind the clitellum were isolated post-anesthesia and preserved in 95% (v/v) ethanol for molecular analysis. Descriptions were based on observations from dorsal dissections, with dimensions and segment counts taken from clitellate adult specimens using an Olympus SZX7 stereoscopic light microscope. Illustrations were created to depict body segments, distinct external features, and internal organs. The number of segments and the body width and length were measured in both full adults and juveniles; measured data are presented as the range (min-max) and mean ± one standard deviation. Holotype and paratype specimens were deposited in the Chulalongkorn University Museum of Zoology, Bangkok, Thailand (CUMZ) and Natural History Museum of the National Science Museum, Thailand (THNHM)

Anatomical abbreviations

fp, female pore; gm, genital markings; gmg, genital marking glands; ic, intestinal caeca; mp, male pores; pg, prostate gland; sc, spermathecae; sp, spermathecal pores; sv, seminal vesicles.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from the integument tissue of the posterior region of the earthworms using Geneaid™ DNA extraction kits. The molecular markers targeted were regions of the mitochondrial cytochrome c oxidase subunit I (COI). Polymerase chain reaction (PCR) amplification was carried out in a 50 µl reaction mixture, which included 0.5–1 µL of DNA template, 2.5 µL each of forward and reverse primers (5 µM), 25 µL of Ultra-Pure Taq PCR Master Mix with emerald dye, and 19–19.4 µL of distilled water (ddH_2O). The COI region was amplified using the forward primer LCO1490 and the reverse primer HCO2198 (Folmer et al., 1994). The PCR thermal cycling conditions were as follows: an initial denaturation at 94 °C for 5 minutes, followed by 36 cycles of 94 °C for 45 seconds, 42 °C for 60 seconds, and 72 °C for 90 seconds, with a final extension at 72 °C for 10 minutes. The amplified products were analyzed by 1% (w/v) agarose gel electrophoresis in 1x TBE buffer, running the gels at 100 V for 20 minutes. Visualization was achieved using RedSafe® nucleic acid staining solution and UV transillumination. The PCR products were purified

TABLE 1. Details of collecting locations of earthworms across Mekong River in Thailand and Laos.

No. on map	Species	Localities and References	GPS	Accession No.
1	*Amynthas mekongianus* s.s.	Wiang Kaen, Chiang Rai, Thailand (This study)	N 20° 11' 45.2" E 100° 27' 32.0"	PV460046-PV460048
2	*Amynthas mekongianus* s.s.	Xanamkhan, Vientiane, Laos (This study)	N 17° 54' 37. 2" E 101° 41' 12.2''	PV460051-PV460053
3	*Amynthas mekongianus* s.s.	Sangkhom, Nong Khai, Thailand (This study)	N 18° 1' 24.36" E102° 22' 32.84"	PV460049-PV460050
4	*Amynthas mekongianus* s.s.	Champasak, Champasak, Laos (This study)	N 15° 6' 45.4" E 105° 48' 49.9"	PV460054-PV460055
-	*Amynthas mekongianus* s.s.	Wiang Kaen, Chiang Rai, Thailand (Jeratthitikul et al., 2017)	N 20°11'45.2" E 100°27'32.4"	KU565196.1
-	*Amynthas alexandri*	Hat Kham, Kui Buri, Prachuap Khiri Khan, Thailand (Jeratthitikul et al., 2017)	N 12°03'24.5" E 99°37'40.4"	KU565173.1
-	*Amynthas alexandri*	Pha Sing, Muang Nan, Nan, Thailand (Jeratthitikul et al., 2017)	N 12°03'24.5" E 99°37'40.4"	KU565176.1
-	*Amynthas phatubensis*	Srisaket, Nanoi, Nan, Thailand (Jeratthitikul et al., 2017)	N 18°21'55.8" E 100°50'24.0"	KU565206.1 and KU565208.1
-	*Amynthas papulosus*	B. Mor, Houaphanh, Laos (Jeratthitikul et al., 2017)	N20°03'23.4" E103°38'13.2"	KU565200.1
	Amynthas papulosus	Khlong Sok, Phanom, Surat Thani, Thailand (Jeratthitikul et al., 2017)	N8°54'55.7" E98°31'39.4"	KU565201.1
	Amynthas comptus	Ban Huat, Ngao, Lampang, Thailand (Jeratthitikul et al., 2017)	N18°36'22.0" E99°54'12.2"	KU565185.1
-	*Amynthas comptus*	Huai Rong, Rong Kwang, Phrae, Thailand (Jeratthitikul et al., 2017)	N18°26'32.3" E100°27'00.0"	KU565186.1-
-	*Metaphire birmanica*	Phu Laen Chang, Nakhu, Kalasin, Thailand (Jeratthitikul et al., 2017)	N16°41'01.7" E103°57'21.6"	KU565261.1
-	*Metaphire birmanica*	Huai Yang, Muang Sakon Nakhon, Thailand (Jeratthitikul et al., 2017)	N17°07'22.1" E104°01'08.4"	KU565262.1
-	*Metaphire birmanica*	Sai Yok, Sai Yok, Kanchanaburi, Thailand (Jeratthitikul et al., 2017)	N14°29'48.5" E98°50'19.0"	KU565264.1
-	*Metaphire baii*	Southwestern Vietnam (Unpublished)	-	OP787166.1-OP787167.1

using the BigDye® Terminator v3.1 cycle sequencing kit. Sequencing was performed with the original amplification primers, and the sequencing reaction products were processed by 1st BASE DNA Sequencing Services (Applied Biosystems). The sampling localities and GenBank accession numbers for the specimens and outgroups analyzed are provided in Table 1.

Phylogenetic analysis

Sequence alignment was performed with Clustal W (Thompson et al., 1994), implemented in MEGA X (Kumar et al., 2018). The alignments were improved manually where necessary. All base frequencies and molecular character statistics were calculated using MEGA X program (Kumar et al., 2018). Sequences from both directions were assembled and checked for stop codons, which might indicate nuclear pseudogenes, using the MEGA X program (Kumar et al., 2018). The nucleotide sequences obtained in this study have been deposited in the GenBank (NCBI) nucleotide sequence databases under accession numbers PV460046–PV460055 (Table 1).

Phylogenetic trees were constructed using neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) methods. The best-fit evolutionary substitution model, determined using the Akaike Information Criterion (AIC) (Akaike, 1974) and implemented in jModelTest 2 (Darriba et al., 2012), was GTR + I + G for COI. NJ trees were generated using MEGA X (Kumar et al., 2018) with 1000 bootstrap replicates, while ML trees were constructed using PhyML v3 (Guindon et al., 2009) with 1000 bootstrap replicates. Bootstrap values greater than 70% were considered to indicate strong support (Felsenstein, 2008). Bayesian inference was performed with MrBayes v3.2.1 (Ronquist et al., 2012), running the Markov Chain Monte Carlo (MCMC) search with four chains for 10,000,000 generations, a heating parameter of 0.70, tree sampling every 100 generations, and a burn-in of 25%. Posterior probabilities ≥0.95 were considered significant. Tree topologies were visualized using FigTree v1.4.3 (Rambaut, 2010). Genetic divergences between and within taxa were assessed using Kimura 2-parameter (K2P) distances, calculated in MEGA X (Kumar et al., 2018).

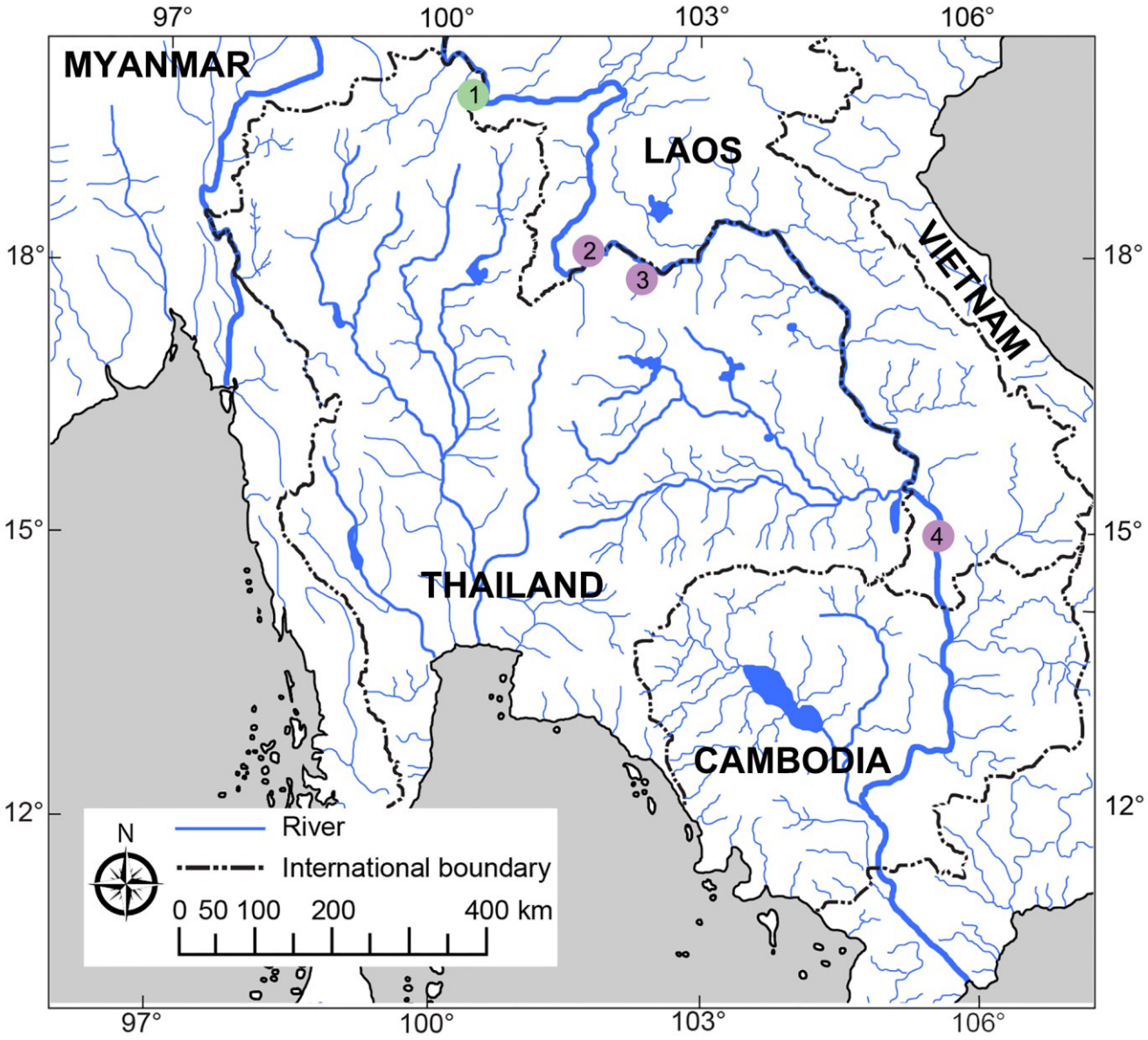

FIGURE 1. Map showing the collecting locations where earthworms were collected from across Thailand and adjacent Laos, respectively.

Species delimitation

The COI data were used to delimited by the following methods: (1) Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021) with the K2P (Kimura, 1980) substitution model and the default settings on the web server (https://bioinfo.mnhn.fr/abi/public/asap/), (2) generalized mixed Yule-Coalescent (GMYC) (Fujisawa and Barraclough, 2013) was performed on the GMYC web server (https://species.h-its.org/gmyc/) with a single threshold and (3) Bayesian Poisson tree processes (bPTP) (Zhang et al., 2013) methods was performed on the bPTP web server (https://species.h-its.org/ptp/). A 660 bp alignment of COI dataset was used as input and pairwise uncorrected p-distances were calculated. ASAP partitions were ranked by ASAP-score; the lowest-score partition was selected as the preferred delimitation. If two partitions had near-identical ASAP-scores (<0.05 difference) the partition with the higher score and concordance with morphological and other molecular delimitation methods was chosen. Both GMYC and bPTP methods were done after re-constructed an ultrametric tree from the BEAST v1.10.4 package (Suchard et al., 2018). BEAUti v1.10.4 generated an XML file based on the relaxed log-normal clock algorithm and the GTR+I+G model. The tree was reconstructed for 1×10^7 generations with sampling every 1,000 steps. The output tree was analysed using

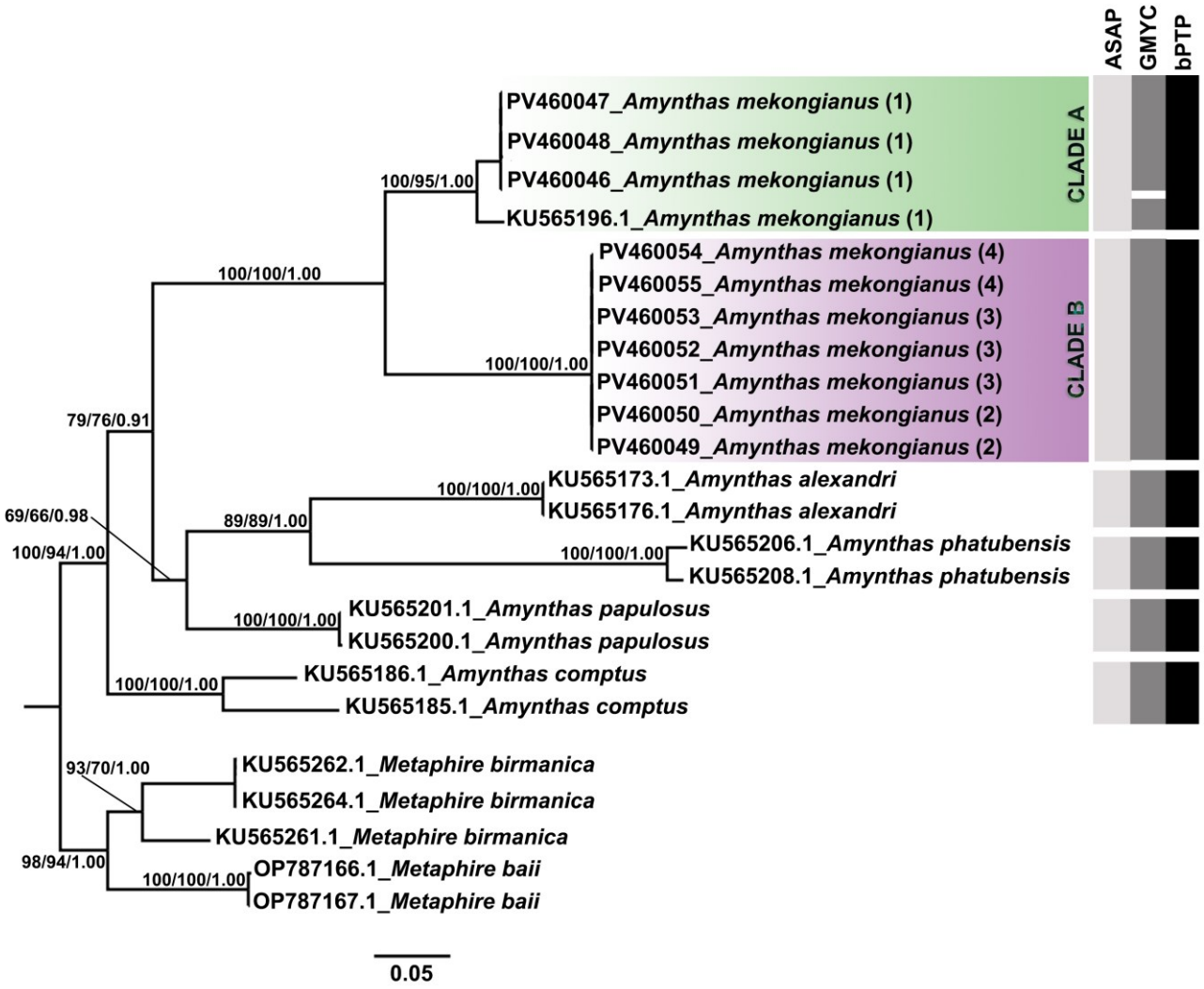

FIGURE 2. The ML tree of *Amynthas mekongianus* s.s. and related species based on 660 bp of COI. Bootstrap support values (BS; >65) and posterior probabilities (PP; >0.70) for individual are shown on the tree. Bars (grey to black) indicate delineated OTUs by different methods: ASAP, GMYC, and bPTP.

TreeAnnotator v1.10.4. The tree file was displayed using FigTree v1.4.3 (Rambaut, 2010). Results from the ASAP, GMYC, and bPTP analyses were then compared with the prior morphological-based taxonomic classifications.

RESULTS

Molecular analysis

Phylogeny and species delimitation

The COI alignment consisted of 660 bp across 29 specimens. Of these, 248 sites (37.6%) were variable, and 240 sites (36.4%) were parsimony informative. The overall base composition was A: 28.9%, T: 30.7%, C: 21.9%, and G: 18.5%, with a mean GC content of 40.4%

The results showed that all trees estimated by NJ, ML and BI gave similar topologies and small differences in the unsupported basal clades were detected (Fig. 2). Both BI and ML trees recovered a well-supported clade for all taxa. Outgroup was genus *Metaphire* that is the close related species (Jeratthitikul et al., 2017). Monophyly of *Amynthas* and *Metaphire* was supported in this case. *Amynthas mekongianus* s.s. was sister with *A. alexandri*, *A. phatubensis, and A. papulosus* with moderate supports. The earthworm species *A. mekongianus* s.s. were represented by two lineages with strong support (clade A and B; Fig. 2). Clade A contains specimens of *A. mekongianus* s.s. from Wiang Kaen, Chiang Rai, Thailand from our study and a previous report by Jeratthitikul et al. (2017) (Table 1), whereas clade B contains *A. mekongianus* s.s. from Vientiane, Laos PDR, which is thought to be the type locality of *A. mekongianus*, as well as nearby Thai locations (Blakemore et al., 2007). In addition, the morphology of the earthworms from clade B was more

comparable to the original description (Cognetti, 1922; Blakemore et al., 2007) than the earthworm from clade A.

Species delimitation analysis (ASAP, GMYC and bPTP) based on COI sequences almost the same number of operational taxonomic units (OTUs). The ASAP and bPTP methods suggested the existence of the following 2 OTUs of *A. mekongianus* s.s., except for GMYC method produced a higher count at 3 OTUs (Fig. 2). The ASAP analysis yielded partitions of 2 groups. The partition graph showed a major inflexion point at two groups (ASAP-score = 1.03), consistent with species-level divergences. This lowest-scoring partition was selected because it was congruent with morphological differences and supported by GMYC and bPTP results. The K2P distance within species was around 0.8% on average, whereas the divergence between species was about 20.0%. The maximum K2P distance was 28.1% between *A. mekongianus* s.s. clade B and *A. phatubensis*, while the minimum was 14.19% between *A. mekongianus* s.s. clade A and *A. mekongianus* s.s. clade B (Table 2). The previous study suggested that genetic divergence among *Amynthas* species at around 13% (Jeratthitikul et al., 2017). As such, the two OTUs of *A. mekongianus* s.s. as defined by (1) length of earthworm body, (2) number of segments, are interpreted as two different species under the morphology and phylogenetic species concepts. So, *A. mekongianus* s.s. clade A would be a new species and described herein.

Taxonomy

Description of new species

Family Megascolecidae Rosa, 1891

Genus *Amynthas* Kinberg, 1867

Type species.– *Amynthas aeruginosus* Kinberg, 1867, by monotypy.

Amynthas sirindhornae Nantarat & Bantaowong, sp. nov.

http://zoobank.org/urn:lsid:zoobank.org:act: ABA70CD9-48F3-4003-95BF-116271746CB7

(Figs 3, 4, Table 3)

Type materials.– Holotype: CUMZ 3829 (Fig. 3), Wiang Kaen, Chiang Rai, Thailand (N 20°11' 45.2" E 100° 27' 32.0", elev. 359 m. 15 March 2014), coll. S. Panha, R. Chanabun, P. Tongkerd, W. Siriwut & U. Bantaowong. Paratypes: four adults (CUMZ 3830), one adult (THNHM); same collection data as holotype.

Etymology.– The specific epithet '*sirindhornae*' is a patronym for Princess Maha Chakri Sirindhorn.

Diagnosis.– Elongate and cylindrical body with 419-494 mm length, width 65–70 mm. Four pairs of spermathecal pores at 5/6–8/9, slit-like. Female pores mid-ventral on xiv. Male pores paired, on round placed disc, protuberant, alate on xviii. Spermathecae ellipsoidal ampulla, diverticulum long zig zag loops in the same plane. Testis sacs paired in x and xi. Seminal vesicles paired in xi, xii. Prostate glands paired in xvii-xix. Intestinal caecum simple in xxvii–xxiv

Description of holotype.– Length 415 mm, width 7 mm at segment vii, cylindrical body with 187 segments. The body color dark gray in newly collected specimens after placement in 30 % (v/v) ethanol. Setae regularly distributed around segmental equators, numbering 88 at segment vii, 92 at segment xx, and 5 between male pores at segment xviii. Setal formula AA:AB:ZZ:ZY= 2:1:1:1 at segment xii. Prostomium tanylobous. First dorsal pore in 12/13. Clitellum annular in xiv–xvi with no dorsal pores or setae.

Single female pore on the ventral side at segment xiv.

Male pores are superficial on the setal line of segment xviii; each situated on a large, markedly protuberant, light-colored, round placed disc, each disc

TABLE 2. Range of genetic divergence (COI; K2P-distance). Average intraspecific distances within each taxon are shown in bold.

Species	1)	2)	3)	4)	5)	6)
1) *A. mekongianus* clade A	1.31±0.28					
2) *A. mekongianus* clade B	14.19±1.58	0.00±0.00				
3) *A. alexandri*	22.53±2.18	25.47±2.29	0.00±0.00			
4) *A. comptus*	20.19±1.97	22.28±2.06	21.97±2.08	0.65±0.40		
5) *A. papulosus*	18.83±2.03	21.55±2.17	19.30±1.96	17.65±1.63	0.17±0.17	
6) *A. phatubensis*	24.94±2.34	28.12±2.56	21.12±2.10	24.66±2.18	20.59±2.05	2.58±0.69

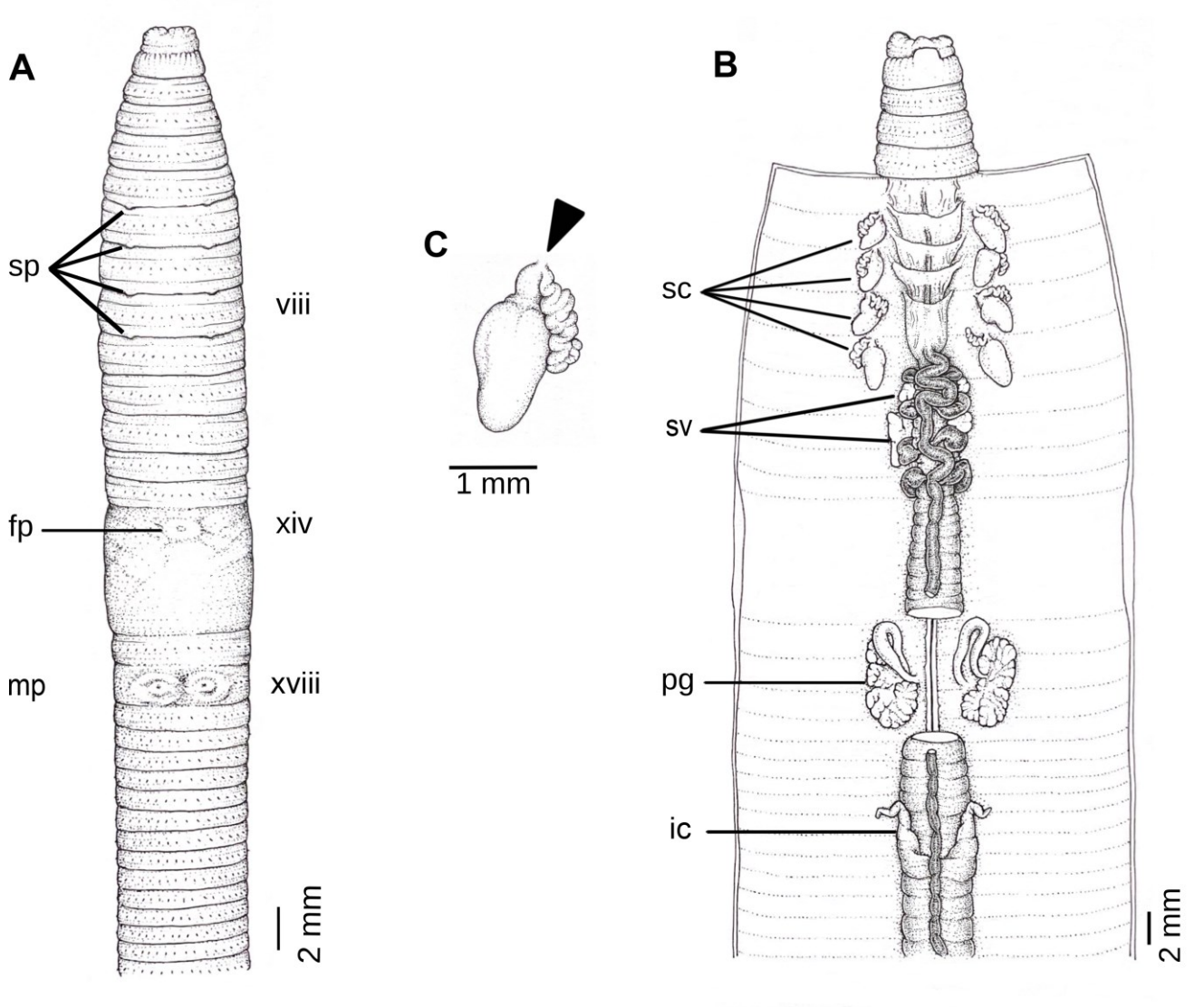

FIGURE 3. External and internal morphology of hohlotype (CUMZ 3829) of *Amynthas sirindhornae* sp.nov. **A.** external ventral view, **B.** internal dorsal view and, **C.** spermatheca; black arrow indicates the connection of the spermatheca and spermathecal pore.

extending from 17/18 to 18/19, distance between male pore 3 mm, 0.25 body circumference apart. Tiny four pairs of spermathecal pores in 5/6–8/9, distance between each pair is 0.16 body circumference ventrally apart. There are no genital markings elsewhere.

The septa at 5/6–7/8 very thick, absent in 8/9, thin in 9/10–11/12, and very thin behind 12/13. Intestine begins at segment xv. Gizzard muscular weak behind 7/8. Long and simple intestinal caeca in xxvii–xxiv. Esophageal hearts four pairs in x–xiii. Holandric; testes and funnels in segments x and xi. Seminal vesicles are paired in x–xi. Prostate glands compact, located in segments xvii–xix, and divided into four lobules. Prostate duct large folded into the hairpin shape. Ovaries paired in xiii. Spermathecae 4 pairs are situated in segments vi, vii, vii, ix. Each ampulla ellipsoidal in shape, duct moderately sharply marked off from the ampullar, stout, slightly shorter than the later. Its diverticulum slightly shorter than main pouch, usually in a fairly regular approximation to zigzag, with many or most of the loops in the same plane, the limbs of the loops in contact.

Variation.– Holotype measures 415 mm body length. Five paratypes range in body length from 438–494 mm (446.40 ± 28.09 mm), with 138–223 segments.

Biology.– The cocoon is cream color, elongate oval, approximately 7 mm in high, and surface casting appear heap-like structure (Fig. 4).

Distribution.– Currently only known from one locality in the Wiang Kaen, Chiang Rai.

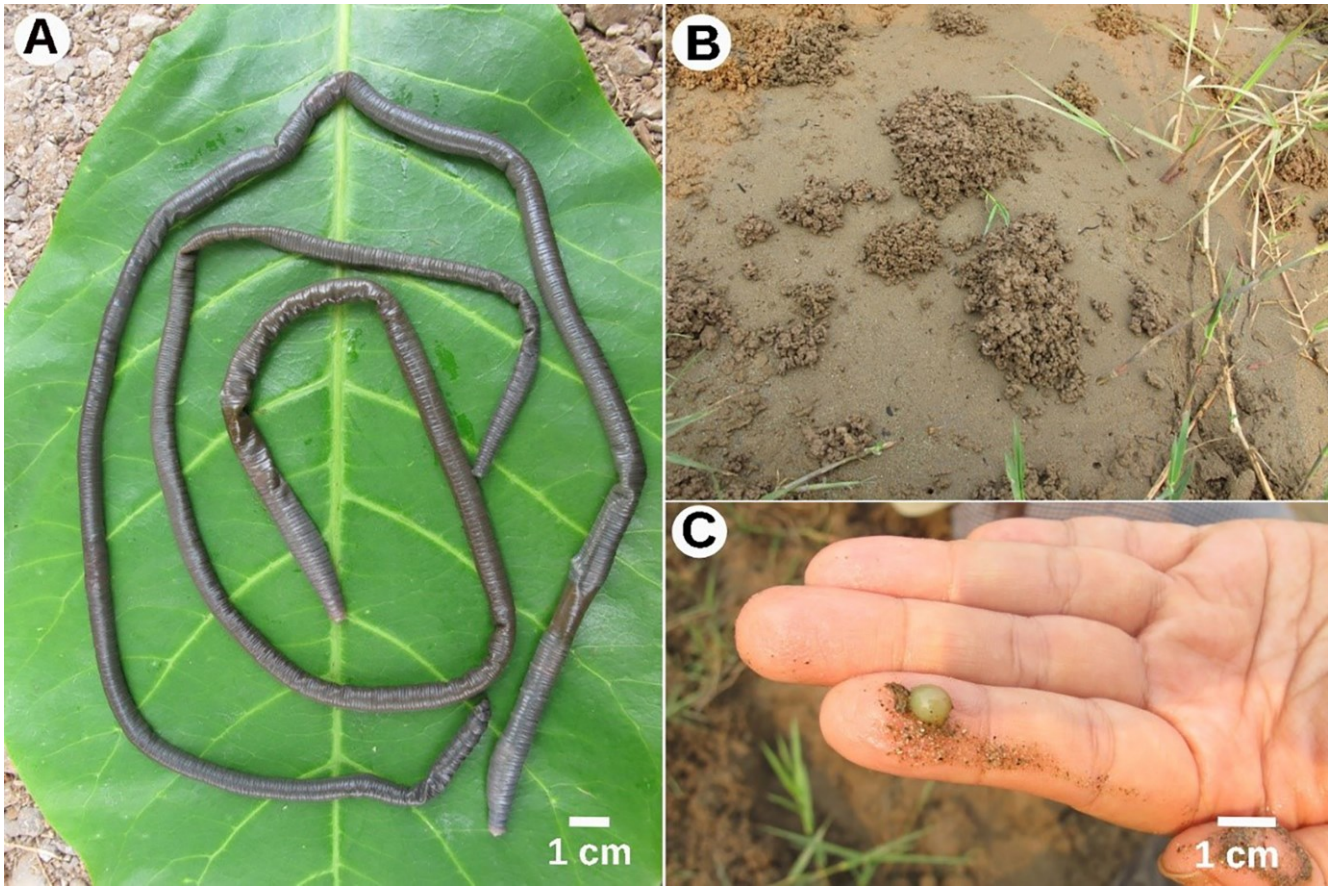

FIGURE 4. Photographs showing, **A.** coloration of living *Amynthas sirindhornae* sp. nov. **B.** surface casting and **C.** cocoon.

Remarks.– A new species, *A. sirindhornae* Nantarat & Bantaowong, sp. nov. from clade A (Fig. 2) from Wiang Kaen, Chiang Rai, Thailand showed the significant difference from the earthworms from clade B that contains *A. mekongianus* s.s. from Vientiane, Laos PDR, which is thought to be the type locality of *A. mekongianus* (Blakemore et al., 2007) and also supported by species delimitation analysis (ASAP, GMYC and bPTP) based on COI sequences. In addition, *A. sirindhornae* sp. nov. is assigned to the *corticis* species group which comprise more than one hundred species (Hong and James, 2013; Nguyen et al., 2020a, 2020b; Bantaowong et al., 2023; Jin et al., 2024) based on the presence of four pairs of spermathecal pores in segments 5/6–8/9. The new species is readily distinguished from *A. feae* (Rosa, 1888) by the markedly protuberant morphology of the male pores, and from *A. oyamai* (Ohfuchi, 1937) and *A. popi* Shen and Chang, 2025 by the complete absence of genital markings in both pre- and post-clitellum regions. Among Thai representatives of the *corticis* group, *A. sirindhornae* sp. nov. differs from *A. alexandri* Beddard, 1900 in possessing a larger body size (>300 mm vs. <300 mm), protuberant rather than depressed male pores, and a weakly developed gizzard. Morphologically, the species most closely resembles *A. mekongianus* (Cognetti, 1922) from Laos (Blakemore et al., 2007); however, it can be clearly separated by its shorter body length, larger prostate glands extending through segments xvii–xix, and a longer diverticulum with a more regular zigzag configuration. These features provide clear diagnostic distinctions supporting the recognition of *A. sirindhornae* sp. nov. as a new species (Table 3).

DISCUSSION

Combining DNA analysis with morphological classification, the phylogeny and species delimitation methods suggest that the accurate count of earthworm species in the genus *Amynthas* is probably at least 51 (Sims and Easton, 1972; Jeratthitikul et al., 2017; Bantaowong et al., 2023; Chanabun et al., 2023). Our studied complex of morphologically similar *A. mekongianus* s.s. is separated into clades A and B with good support from NJ, ML, and BI phylogenetic analyses (bootstrap values of 100 and 100 and posterior probability values of 1) (Fig. 2). The earthworms in clade B include *A. mekongianus* s.s. from Vientiane, Laos PDR, which is thought to be the type locality for *A. mekongianus* (Cognetti, 1922; Blakemore et al.,

TABLE 3. Comparison of characters among *Amynthas sirindhornae* new species and other *corticis* species group with a body size more than 270 mm; *A. feae* (Rosa, 1888), *A. alexandri* Beddard, 1900, *A. mekongianus* (Cognetti, 1922), *A. oyamai* (Ohfuchi, 1937), and *A. popi* Shen & Chang, 2025. Missing data are shown with a question mark (?) and data from Gates (1972) show with an asterisk (*).

Characters	*A. sirindhornae* sp. nov.	*A. mekongianus*	*A. feae**	*A. alexandri**	*A. oyamai*	*A. popi*
Body length (mm)	438–494	690–1,920	180–380	105–290	217–278	210–316
Body width (mm)	7.0	5.5–10.0	7.0–12.0	4.0–9.0	6.0–7.0	7.25–8.6
Segment numbers	138–223	580	130–160	90–141	116–123	138–150
First dorsal pore	12/13	12/13	12/13	12/13	12/13	12/13
Spermathecal pores	5/6–8/9	5/6–8/9	5/6–8/9	5/6–8/9	5/6–8/9	5/6–8/9
Male pores	xviii, markedly protuberant	xviii, circular or hour-glass shaped low	xvii, transversely elliptical disc	xviii, slightly depressed circular	xviii, slightly elevated oblong area	xviii, round porophore
Pre-clitellum genital markings	absent	absent	absent	absent	absent	paired in viii
Post-clitellum genital markings	absent	absent	absent	absent	paired in xviii	one medial to each male pore in xviii; widely paired in xix
Prostate gland	xvii–xix	xviii	xvi-xx	xvi-xxii	xv-xx	xviii
Spermathecae	ellipsoidal	sacciform	?	small sac	heart shaped	elongated oval-shaped
Intestinal caecum	simple, xvii–xix	simple, xxvii-xxiii	?	simple, xxvii-xx	simple, xxvii-xxv	simple, xxvii, -xxiv

2007). Cognetti identified the type location as "Ban Leum on Mekong River, Annam" and Sims and Easton as "from Vietnam" because Annam refers to Central Vietnam (1972: 223). Yet, the true Mekong River type location is unknown. At the time Cognetti's paper was published, the traditional territory of central Vietnam known as Annam had been incorporated into the French "Union of Indochina" since 1887, and Laos had been designated as a protectorate in 1893. The Truong Son range (Annamese Cordillera) separates north and central Annam from Laos and the Mekong to the west; the ridge then swings southeastward and stretches down the southern Annam coast, including plateaus that reach Cambodia and Cochin China's (South Vietnam) border. Thus, the type locality is unlikely to be in the current People's Republic of Vietnam, where the Mekong only exists in the south; rather, the site is more likely to be in Vientiane, Laos PDR (Blakemore et al., 2007). Earthworms from clades A and B exhibit very similar morphological traits. They share high similarities in certain features, such as the placement of the spermathecal pore in 5/6–8/9 and features of the male pores. The distinguishing characteristics between them are the number of segments, body length, and appearance of spermathecae. However, it can be identified by the following characteristics: the body is smaller, measuring 419–450 mm, with fewer segments, elongated spermathecae, a long zigzag diverticulum, and a compact prostate gland.

The previous study reported the genetic distance based on the COI gene fragment sequence between *Amynthas* species should be above 13%, which is comparable to the 14.2% in this study (Jeratthitikul et al., 2017). Our results also revealed a relationship in other species, such as *A. alexandri*, *A. phatubensis*, *A. papulosus*, and *A. comptus*. *Amynthas* and *Metaphire* are well separated in this study (Fig. 2). The classification of *Amynthas* and *Metaphire* is controversial because almost all anatomical and morphological structures of these genera show high similarity, except for the male pore area, which is represented as a distinct copulatory pouch in *Metaphire* but is absent in *Amynthas*. For the ASAP, GMYC, and bPTP analyses appeared to give confirmation of the new species. The numbers of K2P distances between the annelids are comparatively high when compared to the ~2% of most other animal taxa (Jeratthitikul et al., 2017). The earthworms' extensive genetic variation may result from their long evolutionary past (Chang and James, 2011), and selection forces that affect them directly. Being soil-dwelling invertebrates, the earthworms have adapted to survive in specific environments, and stabilizing selection may favor variations that are not in line with the ideal morphology (Bickford et al., 2007; Dyer et al., 2011). Normally, the dispersal capability is

one of the factors that is demonstrated by an isolation-by-distance pattern. The genetic variation may align closely with the distance between locations. It is not unexpected to find cryptic speciation in earthworms, but to find the cryptic endemic species that are under threatened environments like *A. mekongianus* s.s., is very important. Currently, the Mekong River basin is becoming a major base for large-scale construction projects and climate change that cause of earthworm habitat destruction and fragmentation (He et al., 2014). Additionally, it is unclear whether this worm species is commonly used or endangered locally. The Mekong basin's sedimentary embankments present few natural barriers to worm migration. However, the construction of 50 or more hydroelectric dams, such as the Nam-Ngum and Ban-Koum dams in Laos, may impact flow and sedimentation (Blakemore et al., 2007). Identifying and defining such cryptic species is a significant difficulty in evolutionary biology (Jeratthitikul et al., 2017; Ramesh et al., 2020). Lately, numerous studies utilizing analysis of mitochondrial DNA sequences have revealed proof of a significant number of hidden speciation occurrences in multiple earthworm species (Huyse and Volckaert, 2002; King et al., 2008; Novo et al., 2010; Shekhovtsov et al., 2013; Jeratthitikul et al., 2017; Misirlioğlu et al., 2023). In general, cryptic speciation is often found in allopatric populations (Finston et al., 2007). However, for earthworms, some cryptic species live together and hence share ecological niches like, in this case (Edwards and Arancon, 2022; Jeratthitikul et al., 2017). Cryptic species, or lineages that are genetically distinct yet morphologically identical, present a taxonomic challenge to biodiversity and conservation (Ramesh et al., 2020). As the rate of species discovery continues to rise, there is a growing need for a cohesive framework to identify taxa. This framework should account for the evolutionary processes that lead to cryptic species and also estimate their presence across different environments to guide conservation efforts (Shekhovtsov et al., 2013; Jeratthitikul et al., 2017; Ramesh et al., 2020). Our results indicate that the new species are not only subspecies glorified by the use of the phylogenetic species concept. Their high conservation value of a giant earthworm, *A. mekongianus* s.s., contributed mainly through endemism, but also demonstrates that describing new cryptic species is necessary to bridge the deep gap between the molecular phylogeny and the taxonomic knowledge.

CONCLUSIONS

The discovery of cryptic species by molecular taxonomy often serves as a reminder that our knowledge of taxonomic diversity is still far from complete. Due to their unrecognized status, cryptic species are rarely included in conservation efforts, even though some of them may be critically endangered or otherwise significant (Delić et al., 2017). We explored the presence of cryptic lineages within the endemic Mekong giant earthworm, *A. mekongianus* sensu lato, which we describe using molecular and morphological diagnoses. This study also emphasizes the significance of habitat destruction with increasing threats to earthworm habitats. Cryptic lineages must be recognized so that suitable conservation actions can be implemented.

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