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Two new Cambodian semi-aquatic earthworms in the genus *Glyphidrilus* Horst, 1889 (Oligochaeta, Almidae), based on morphological and molecular data

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Abstract

Combining morphological and molecular data is a powerful approach to support the discovery of new species. Here, two new species of the semi-aquatic earthworm genus *Glyphidrilus*, *G. jamiesoni* **sp. n.** and *G. kralanhensis* **sp. n.**, are described from the Mekong Basin in Cambodia. They are morphologically distinguished by the respective locations of wings and spermathecae; furthermore, *G. kralanhensis* **sp. n.** has three pairs of ovaries, probably an autapomorphic trait. In addition, two mitochondrial gene fragments (COI and 16s rRNA) were sequenced of types of the new species and of five further *Glyphidrilus* species described recently from the Mekong basin in Thailand and Laos. They revealed a high level of genetic divergence of the new species compared to the other earthworm taxa. The evolutionary relationships among the Mekong *Glyphidrilus* members is discussed with reference to the recent paleogeography of the Mekong River drainage.

Key words: new species, earthworms, *Glyphidrilus*, Cambodia, integrative taxonomy, phylogeny, paleogeography

Introduction

Semi-aquatic earthworms are an intriguing group of annelids that live specifically in riparian habitats or in the ecotone between freshwater and terrestrial ecosystems, along the muddy banks of rivers, streams, canals, ponds, lakes, waterfalls and in paddy fields (Chanabun *et al.* 2013). These earthworms belong to the family Almidae, whose distribution range covers wide areas from Central and South America, tropical Africa and the Nile Valley, to India and Southeast Asia. Members of this family are notable for their quadrangular-shaped posterior body section and extensions of the epidermis in the clitellar region, such as protuberances in *Drilocrius*, paddle-shaped claspers in *Alma* and keel-like structures called 'wings' or 'alae' in *Glyphidrilus* and *Progizzardus* (Jamieson 2006; Nair *et al.* 2010). The genus *Glyphidrilus* Horst, 1889 has a widespread occurrence in the Oriental region and East Africa (see Gates 1972 for taxonomic and biogeographic discussion of the African taxa). In recent years, several surveys and investigations have revealed a high species diversity of *Glyphidrilus* in Southeast Asia, which has led to the recognition of a total of 44 species and one subspecies (Chanabun *et al.* 2011, 2012a, b, 2013; Chanabun & Panha 2015, 2016).

Similar to other earthworm taxa, the taxonomy of *Glyphidrilus* has been based on a limited number of morphological characters, mainly the position of the clitellum, wings and gizzard, and the number and position of the genital markings and spermathecae (Chanabun *et al.* 2013). However, these key morphological features are highly variable in most aquatic and semi-aquatic groups (Brinkhurst & Jamieson 1971). Even though male pores have been used to distinguish between *Glyphidrilus weberi* Horst, 1889 and *G. quadrangulus* (Horst 1893), the

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positions of the genital pores (e.g. male, female and spermathecal pores) are of limited taxonomic use due to their minute size that leads to difficulties in their observation, and these characters were not recorded in other later descriptions of *Glyphidrilus*. Moreover, different *Glyphidrilus* species from adjacent river basins show overlapping ranges of some key characters (Chanabun *et al.* 2013), probably due to similar adaptations to soil habitats or high morphological plasticity (Novo *et al.* 2012a). This casts doubt on decisions regarding the range of intraspecific variation and interspecific diagnostic characters. However, a recent study using allozymes supports the species delimitation between *G. vangviengens*is Panha & Chanabun, 2011 and *G. mekongensis* Panha & Chanabun, 2012, and also revealed cryptic diversity in the Lower Mekong River basin (Jirapatrasilp *et al.* 2015).

Recently, the use of molecular markers, such as 'DNA barcoding', using (mostly) fragments of the cytochrome *c* oxidase I gene (COI) as a powerful tool to identify species, has become prevalent (Rougerie *et al.* 2009). The ongoing increase in DNA barcode sequences in the GenBank database has resulted in an upward trend of species discovery and delimitation based on this molecular data (Kekkonen *et al.* 2015; Prévot *et al.* 2013). Earthworm systematics strongly benefits from DNA barcoding, since this soil fauna lacks highly specialized organs, has few informative characters and is probably highly homoplasic, and exhibits a high intraspecific variability and overlapping ranges of characters among taxa (Novo *et al.* 2010; Pérez-Losada *et al.* 2009). To further establish a firm taxonomy of earthworms, DNA barcodes of 'hologenetype' or 'paragenetypes' are necessary to unambiguously link the molecular data to its respective morphological information, and so provide 'an objective standard of reference' for future specimen identification and comparison (Blakemore *et al.* 2010; Chakrabarty 2010). Recently, molecular barcoding has facilitated the discovery of several earthworm species new to science (Boyer *et al.* 2011a; Díaz Cosín *et al.* 2014; Fernández Marchán *et al.* 2014; Novo *et al.* 2012b), revealed cryptic diversity (King *et al.* 2008; Novo *et al.* 2010), and resolved some taxonomical disputes (James *et al.* 2010). The use of DNA barcoding in integrative taxonomy (Dayrat 2005), combining morphological, molecular and other data of earthworms, is gaining more popularity in systematic studies, both to delimit species and support the designation of new species (Chang & James 2011; Decaëns *et al.* 2013). In addition, DNA barcodes have also facilitated the identification of some species complexes of earthworms used in ecotoxicological tests (Römbke *et al.* 2016) and to detect invasive species (Dupont *et al.* 2012).

Cambodia is located in the Lower Mekong River basin and its terrain mostly consists of low-lying plains and the river delta. Knowledge of the country's biodiversity is currently scarce, but increasing due to the recent assistance of international organizations and researchers to conduct biodiversity surveys. The fauna inventories of Cambodia have mainly focused on vertebrates (e.g. Grismer *et al.* 2008; Hartmann *et al.* 2013) and some large groups of invertebrates (e.g. Monastyrskii *et al.* 2011), with far less or no attention on other taxonomic groups, including earthworms. Indeed, the diversity of earthworms in Cambodia has been totally neglected since the Colonial era, and has still received no attention from local researchers, in contrast to the neighboring Thailand (Bantaowong *et al.* 2014, 2015, 2016), Laos (Hong *et al.* 2014) and Vietnam (Nguyen & Nguyen 2015; Thai 2000). Until now, there has only been one publication regarding Cambodian earthworms, with *Glyphidrilus ceylonensis* Gates, 1945 reported in the checklist (Thai & Do 1989). Without access to the previously reported specimens, the presence of this species in Cambodia is questionable.

In this study, two new species of Cambodian semi-aquatic earthworms are described based on both their morphological and molecular data. In addition, the phylogenetic positions of the new taxa are discussed with respect to the molecular data from other related *Glyphidrilus* in the Mekong River Basin, in which their types were sequenced for the first time and the data are reported herein.

Material and methods

Earthworms were collected by digging up the topsoil from the banks of freshwater habitats where casts were apparent. The GPS coordinates of each locality were recorded and the habitat type photographed. All specimens were cleaned and then killed in 30% (v/v) ethanol, photographed and fixed in 95% (v/v) ethanol for morphological and molecular studies. Species identification and nomenclature for the taxonomic characters were based on Chanabun *et al.* (2013). Further comparative studies of *Glyphidrilus* type specimens were conducted at Chulalongkorn University, Museum of Zoology, Bangkok (CUMZ). The new species were described from observations under an OLYMPUS SZX16 stereomicroscope, and the holotype and paratypes were deposited in

CUMZ, The Natural History Museum, London, UK (NHMUK) and the Biozentrum Grindel und Zoologisches Museum, University of Hamburg, Germany (ZMH). The 14 collection localities of the specimens in this study are shown in Figure 1.

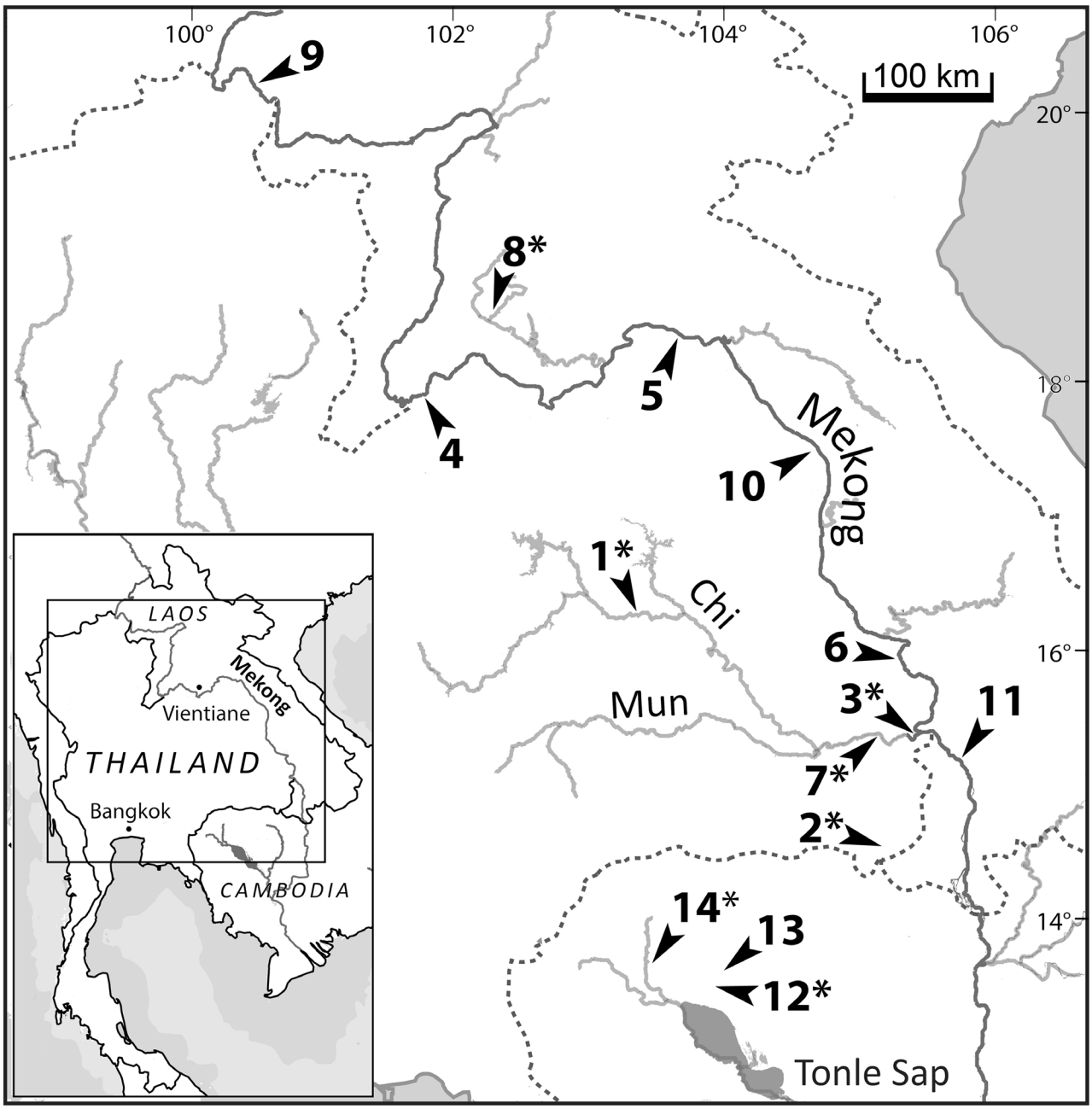

FIGURE 1. Map of sampling localities of *Glyphidrilus* spp. in the Mekong River Basin in Thailand, Laos and Cambodia. Locality numbers (1–14) correspond to those in Table 1 and 2. Numbers with an asterisk designate type localities. Loc. 1: Rice field at Ban Thatoom, Mueang, Mahasarakham, Thailand. Loc. 2: Huailuang waterfall, Najahlauy, Ubon Ratchathani, Thailand. Loc. 3: Mekong River, Khong Chiam, Ubon Ratchathani, Thailand. Loc. 4: Khut Khu Islets, Chiang Khan, Loei, Thailand. Loc. 5: Bueng Kan Mekong River pathway, Mueang, Bueng Kan, Thailand. Loc. 6: Salung Beach, Pho Sai, Ubon Ratchathani, Thailand. Loc. 7: Kang Saphue, Mun River, Phibunmangsahan, Ubon Ratchathani, Thailand. Loc. 8: Song River, Vangvieng, Vientiane, Laos. Loc. 9: Phonevichith Guesthouse, Houayxay, Bokeo, Laos. Loc. 10: Ban Arjsamart, Muang, Nakon Panom, Thailand. Loc. 11: Pakse Mekong River pathway, Pakse, Champasak, Laos. Loc. 12: Stream near Praduk Temple, Banteay Srei, Siem Riep, Cambodia. Loc. 13: Banteay Srei Temple, Banteay Srei, Siem Riep, Cambodia. Loc. 14: Tapan River, Kralanh, Siem Riep, Cambodia.

Genomic DNA was extracted from the integument tissue of the posterior part of the earthworms using Geneaid™ DNA extraction kits. DNA extraction was performed on type specimens and some additional materials

of *Glyphidrilus chiensis*, *G. quadratus*, *G. huailuangensis*, *G. mekongensis* and *G. vangviengensis* from CUMZ, in order to establish the species boundary compared to other Mekong taxa. In addition, DNA was extracted from *Pontoscolex corethrurus* (Glossoscolecidae) for use as an outgroup. *Glyphidrilus chiensis* and *G. quadratus sensu* Chanabun were preliminarily retrieved as polyphyletic (Prasankok, unpublished data) so only specimens from the type localities were used in this analysis. The molecular markers used were regions of the mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S ribosomal DNA (16S rRNA) genes. Their polymerase chain reaction (PCR) amplification was performed in a 50 µl reaction comprised of 0.6–1 µL of DNA template, 2.5 µL each of the forward and reverse primers (5 µM), 25 µL of Ultra-Pure *Taq* PCR Master Mix with emerald dye and 19–19.4 µL of ddH_2O. The COI region was amplified using the universal forward primer LCO1490 (Folmer *et al.* 1994) and the earthworm reverse primer Meta-1R (Minamiya *et al.* 2009), which resulted in ~900 bp fragments and included the 658 bp 'DNA barcode.' Templates that gave an unsuccessful amplification were then amplified using the designed internal primer COI_R_Gly (5' GAAATTGAGCCAAAYCCTGG 3'). The PCR thermal cycling was performed as: 94 °C for 15 min, followed by 36 cycles of 94 °C for 45 s, 42 °C for 60 s and 72 °C for 90 s, and then followed by a final 72 °C for 10 min. The 16S rRNA fragment was amplified using primers designed from the conserved regions of the mitochondrial genomes of five earthworm species: *Lumbricus terrestris* (U24570.1; Boore & Brown 1995), *Perionyx excavatus* (EF494507.1; Kim *et al.* 2007), *Amynthas aspergillus* (KJ830749.1; Zhang *et al.* 2014), *Metaphire vulgaris* (KJ137279.1; Zhang *et al.* 2016) and *Tonoscolex birmanicus* (KF425518.1; Wang *et al.* 2015). The forward primer 16SF_EW (5' TATTTCGACTGTTTAACAAAAACATTG 3') covered the same region as the universal 16Sar primer (Palumbi *et al.* 1991), while the reverse primer 16SR1_EW (5' GATAGAAGCTAACCTGGCTTAC 3') was slightly further 3' than the 16Sbr primer region (Palumbi *et al.* 1991). The PCR thermal cycling was performed as above except using an annealing temperature of 50 ºC rather than 42 ºC. The PCR products were resolved using 1% (w/v) agarose gel electrophoresis in 0.5x TBE buffer and detected with SYBR® Safe DNA gel staining under UV transillumination. DNA products were purified using PEG precipitation and then sent to be commercially direct cycle-sequenced at Bioneer Co., Korea. The relevant sequences from *Lumbricus terrestris* (Lumbricidae) and *Hormogaster sylvestris* (Hormogastridae) were obtained from GenBank and included as additional outgroups. Sampling localities and GenBank accession numbers of both mitochondrial markers for the hologenetype, paragenetypes (Chakrabarty 2010), additional specimens and outgroups analyzed herein are shown in Table 1.

Sequence alignment and editing were performed using the MEGA 6.13 software (Tamura *et al.* 2013). Both mitochondrial sequences were checked for substitution saturation and phylogenetic signal using the DAMBE v. 5.6.21 software (Xia 2013). For COI, saturation tests were performed using all codon positions and only the third codon position. However, no saturation was detected in the sequences and so all the codon positions were used in the subsequent analysis. The program Kakusan4 (Tanabe 2011) was used to find the best-fit models of nucleotide substitution, as judged by the Akaike information criterion (AIC: Akaike 1974). The models were then implemented into the concatenated dataset to construct phylogenetic trees based on maximum likelihood (ML) and Bayesian inference (BI) methods. The ML analysis was performed with RAxML-HPC2 on XSEDE v.8.2.4 (Stamatakis 2014) with default settings and 1000 bootstrap replicates in the CIPRES Science Gateway (Miller *et al.* 2010). MrBayes v3.2.2 (Ronquist *et al.* 2012) in the CIPRES Science Gateway was used to construct the BI tree, with two runs in parallel for 2 million generations (with default heating values). The analysis started with a random tree, and trees were sampled every 100 generations. Fifty percent of the sampled trees were discarded as burn-in and the remaining trees were used to calculate the posterior probability. In addition, MEGA 6.13 was used to construct phylogenetic trees based on the Neighbor Joining (NJ) distance method, using Kimura 2-Parameter model (Kimura 1980) with 1,000 bootstrap replicates. Tree topologies with bootstrap values of 70% or greater for ML/NJ, and a posterior probability of 0.95 or greater for the BI were regarded as highly supported (Huelsenbeck & Hillis 1993; Larget & Simon 1999). Uncorrected pairwise sequence distances and intraspecific divergence of both mitochondrial markers among the Mekong *Glyphidrilus* were also calculated using the MEGA 6.13 program.

Anatomical abbreviations and explanations. The following abbreviations used in the figures are as appeared in Chanabun *et al.* (2013): **wi**, wings; **gm**, genital markings; **gi**, gizzard; **he**, hearts; **sv**, seminal vesicles; **sc**, spermathecae; **ov**, ovaries; **np**, nephridia.

The terms 'pre-wing' and 'post-wing' used in the descriptions mean the position 'in front of wings' and 'behind wings', respectively.

TABLE 1. Sampling localities and GenBank accession numbers of the type and non-type specimens of *Glyphidrilus* from the Mekong river basin analyzed in this study, including outgroups. Locality numbers correspond to those in Figure 1.

Species	Country, Locality	Type	Specimen Code	COI	16S rRNA
G. chiensis Chanabun & Panha, 2013	Thailand, Loc. 1	Holotype	C*	KU885471	KU885506
G. chiensis	Thailand, Loc. 1	Paratype 1	C1	KU885472	KU885507
G. chiensis	Thailand, Loc. 1	Paratype 2	C2	KU885473	KU885508
G. chiensis	Thailand, Loc. 1	Paratype 3	C3	KU885474	KU885509
G. chiensis	Thailand, Loc. 1	Paratype 4	C4	KU885475	KU885510
G. huailuangensis Chanabun & Panha, 2013	Thailand, Loc. 2	Holotype	H*	KU885476	KU885511
G. huailuangensis	Thailand, Loc. 2	Paratype 1	H1	KU885477	KU885512
G. huailuangensis	Thailand, Loc. 2	Paratype 2	H2	KU885478	KU885513
G. huailuangensis	Thailand, Loc. 2	Paratype 3	H3	KU885479	KU885514
G. mekongensis Panha & Chanabun, 2012	Thailand, Loc. 3	Holotype	M*	KU885480	KU885515
G. mekongensis	Thailand, Loc .3	Paratype	M1	KU885481	KU885516
G. mekongensis	Thailand, Loc. 4	Non-type	M2	KU885482	KU885517
G. mekongensis	Thailand, Loc. 5	Non-type	M3	KU885483	KU885518
G. mekongensis	Thailand, Loc. 6	Non-type	M4	KU885484	KU885519
G. quadratus Chanabun & Panha, 2013	Thailand, Loc. 7	Holotype	Q*	KU885485	KU885520
G. quadratus	Thailand, Loc. 7	Paratype 1	Q1	KU885486	KU885521
G. quadratus	Thailand, Loc. 7	Paratype 2	Q2	KU885487	KU885522
G. quadratus	Thailand, Loc. 7	Paratype 3	Q3	KU885488	KU885523
G. quadratus	Thailand, Loc. 7	Paratype 4	Q4	KU885489	KU885524
G. vangviengensis Panha & Chanabun, 2011	Laos, Loc. 8	Holotype	V*	KU885490	KU885525
G. vangviengensis	Laos, Loc. 8	Paratype	V1	KU885491	KU885526
G. vangviengensis	Laos, Loc. 9	Non-type	V2	KU885492	KU885527
G. vangviengensis	Thailand, Loc. 10	Non-type	V3	KU885493	KU885528
G. vangviengensis	Laos, Loc. 11	Non-type	V4	KU885494	KU885529
G. jamiesoni **sp. n.**	Cambodia, Loc. 12	Holotype	J*	KU885495	KU885530
G. jamiesoni	Cambodia, Loc. 12	Paratype 1	J1	KU885496	KU885531
G. jamiesoni	Cambodia, Loc. 12	Paratype 2	J2	KU885497	KU885532
G. jamiesoni	Cambodia, Loc. 13	Non-type	J3	KU885498	KU885533
G. jamiesoni	Cambodia, Loc. 13	Non-type	J4	KU885499	KU885534
G. kralanhensis **sp. n.**	Cambodia, Loc. 14	Holotype	K*	KU885500	KU885535
G. kralanhensis	Cambodia, Loc. 14	Paratype 1	K1	KU885501	KU885536
G. kralanhensis	Cambodia, Loc. 14	Paratype 2	K2	KU885502	KU885537
G. kralanhensis	Cambodia, Loc. 14	Paratype 3	K3	KU885503	KU885538
G. kralanhensis	Cambodia, Loc. 14	Paratype 4	K4	KU885504	KU885539
Pontoscolex corethrurus (Glossoscolecidae)	Outgroup specimen		*P. corethrurus*	KU885505	KU885540
Lumbricus terrestris (Lumbricidae)	Outgroup specimen		*L. terrestris*	U24570.1[a]	U24570.1[a]
Hormogaster sylvestris (Hormogastridae)	Outgroup specimen		*H. sylvestris*	HQ621981.1[b]	HQ621874.1[b]

[a] Boore & Brown (1995), [b] Novo *et al.* (2011)

Results

Systematics

Family ALMIDAE Duboscq, 1902

Genus *Glyphidrilus* Horst, 1889

Type species. *Glyphidrilus weberi* Horst, 1889, by monotypy

1. *Glyphidrilus jamiesoni* Jirapatrasilp, Chanabun & Panha, sp. n.
(Figure 2)

Type specimens. Holotype CUMZ 3397 (adult; Fig. 2), paratypes CUMZ 3398 (23 adults and 57 juveniles), NHMUK (two adults), and ZMH 14585 (two adults), leg. P. Jirapatrasilp, C. Sutcharit, W. Siriwut, and R. Srisonchai, 8 Feb 2015.

Type locality. Stream banks near Praduk Temple, Banteay Srei District, Siem Riep Province, Cambodia, 13º28'46.4"N, 103º56'18.1"E, 15 m amsl. (Fig. 1 Loc. 12, Fig. 4A).

Other material examined. Three adults and 20 juveniles (CUMZ 3399), Banteay Srei Temple, Banteay Srei District, Siem Riep Province, Cambodia, 13º35'56.1"N, 103º57'47.8"E, 15 m amsl (Fig. 1 Loc. 13), 7 Feb 2015.

Diagnosis. Wings between segment 23, 24–31, 32, 33; clitellum in 17, 18–35, 36; genital markings paired or unpaired on **aa** in pre-wing 12, 13, 14, and post-wing 31, 33, paired or asymmetrical on **bc** in pre-wing 15, 16, 17, 18–22, 23, and post-wing 31, 32; intestine enlarged in 16; ovaries in 13–14; spermathecae sessile, elongated oval or globular in 13/14–18/19 (Table 2).

Description of holotype. Body length 114 mm, diameter 3.82 mm in segment 8, 4.70 mm in front of wings in segment 22, 4.35 mm behind wings in segment 43; body cylindrical in anterior part, quadrangular in cross-section behind clitellum. 307 segments. Body color pale brown with reddish tint from the first segment to clitellar portion. At posterior end dorsal surface broader than ventral. Clitellar wings on ventro-lateral part of clitellum, left wing in 24–32, right wing in ½24–32, 4.75–5.37 mm in height, 0.6 mm wide. Prostomium zygolobous. Dorsal pores absent. Clitellum annular but not totally fused in 17–35. Four pairs of setae per segment from 2, setal formula **aa: ab: bc: cd: dd** = 1.59: 0.80: 1.53: 0.74: 1.86 in segment 6 and 1.79: 0.73: 1.75: 0.59: 2.19 in postclitellar segments. Female, male and spermathecal pores not visible. Genital markings unpaired on **aa** in 12 and 13, and paired on **bc** in 17–22.

Septa 5/6–7/8 thickest, 8/9–11/12 thick and 12/13 to the last segment thin. Gizzard globular within 7–8. Intestine enlarged from 16. Dorsal blood vessel anterior to 7. Hearts in 7–11. A pair of nephridia in each segment, rudimentary in 13, small in 14, and normal in 15 onwards. Seminal vesicles in 9–12. Ovaries in 13–14. Testes in 10–11. Prostate and accessory glands absent. Spermathecae sessile, elongated oval or globular in 13/14–18/19, about 0.2 mm in diameter, one to seven on each side per segment.

Variation. Body lengths of adult paratypes (27) and non-types (3) ranged from 82–114 mm (mean ± S.D. = 101.7 ± 8.8), with 165–307 segments. Wings begin in 23 or 24 and end in 31, 32, or 33; the most frequent position is 24–32. Clitellum begins in 17 or 18 and ends in 35 or 36. Most specimens have genital markings paired or unpaired on **aa** in pre-wing 12, some occur in 13, 14, and post-wing 31, 33. Genital markings paired or asymmetrical on **bc** start in pre-wing 15, 16, 17, or 18 and ends in 22, 23, and some occur in post-wing 31, 32; the most frequent position is 18–22 (Table 2).

Distribution. The new species is known from the type locality, Praduk Temple, and also from Banteay Srei, Siem Riep, Cambodia.

Habitat. The species was found on stream banks and in paddy fields where the worm casts covered the surface (Fig. 4B). Earthworms occurred in the muddy loam topsoil at about 10–20 cm depth.

Etymology. The specific name was dedicated to Barrie G. M. Jamieson, an Australian oligochaetologist, who extensively reviewed the taxonomy and systematics of semi-aquatic earthworms, including the family Almidae.

Remarks. *Glyphidrilus jamiesoni* **sp. n.** is similar to *G. vangviengensis* in the wing locations, but has spermathecae in 13/14–18/19. For further comparison of Mekong *Glyphidrilus* species, see Table 2.

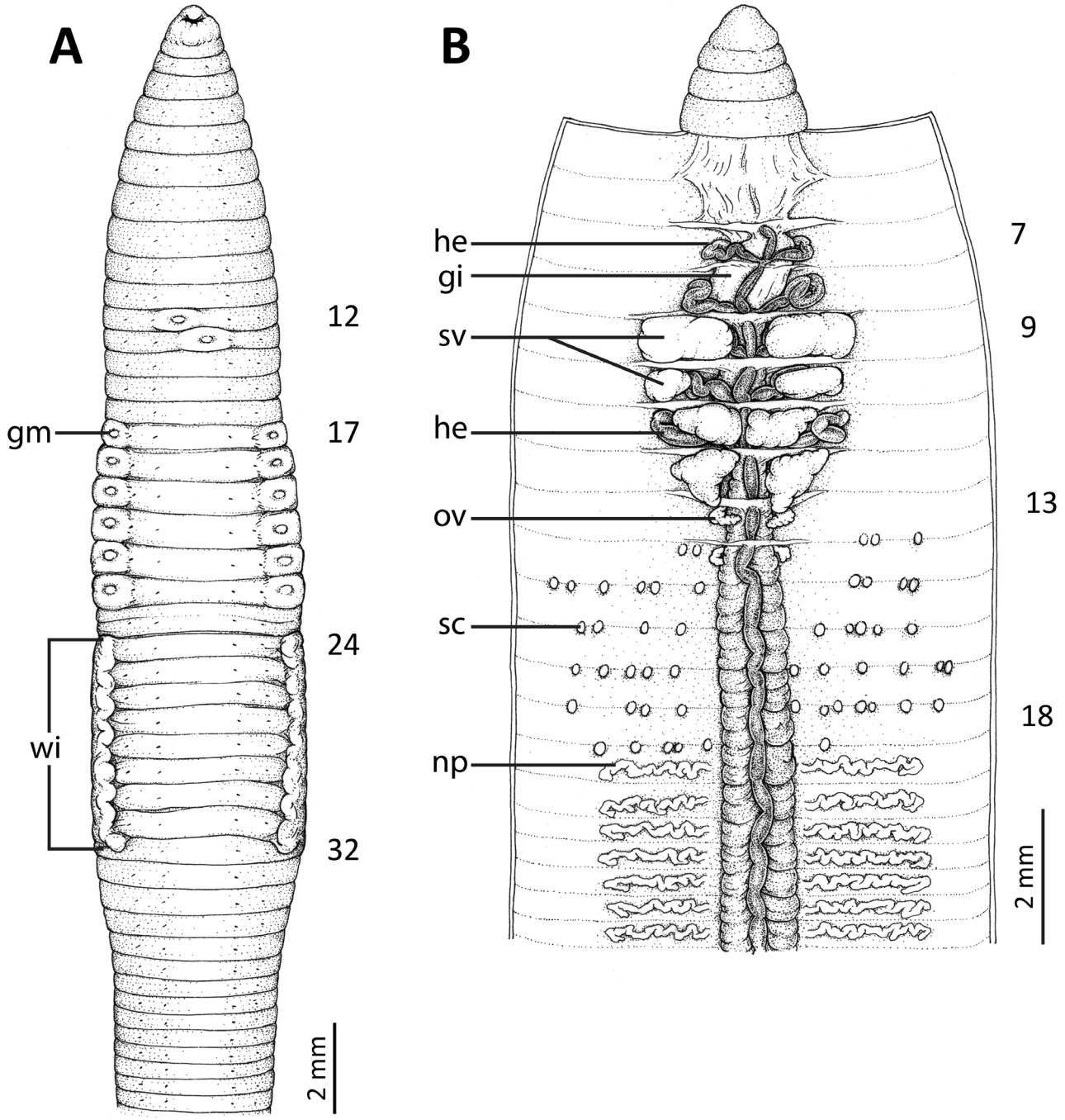

FIGURE 2. Morphology of the holotype (CUMZ 3397) of *G. jamiesoni* **sp. n. A.** External ventral view. **B.** Internal dorsal view after dissection; some anterior pairs of nephridia are not shown.

2. *Glyphidrilus kralanhensis* Jirapatrasilp, Chanabun & Panha, sp. n.
(Figure 3)

Type specimens. Holotype CUMZ 3400 (adult; Fig. 3), paratypes CUMZ 3401 (three adults and six juveniles), and ZMH 14586 (one adult), leg. P. Jirapatrasilp, C. Sutcharit, W. Siriwut and R. Srisonchai, 6 Feb 2015.

Type locality. Tapan River, Kralanh District, Siem Riep Province, Cambodia, 13º35'24.5"N, 103º24'27.8" E, 8 m amsl. (Fig. 1 Loc. 14, Fig. 4C).

Diagnosis. Wings between segment 23, 24, ½25, 25–26, 27, ½28, 29, 30, 31; clitellum in 19, 20–35, 37; genital markings paired or asymmetrical on **bc** in pre-wing 20, 22–24, and post-wing 26–29, 30; intestine enlarged in 16; ovaries in 13–15; spermathecae sessile, elongated oval or globular between 13/14–21/22 (Table 2).

Description of holotype. Body length 107 mm, diameter 3.63 mm in segment 8, 4.06 mm in front of wings in segment 22, 2.71 mm behind wings in segment 43; body cylindrical in anterior part, quadrangular in cross-section behind clitellum. 314 segments. Body color pale brown with reddish tint from the first segment to clitellar portion. At posterior end dorsal surface broader than ventral. Clitellar wings on ventro-lateral part of clitellum, left wing in 25–30, right wing in 24–29, both 3.90 mm in height and 0.71 mm wide. Prostomium zygolobous. Dorsal pores absent. Clitellum annular but not totally fused in 20–37. Four pairs of setae per segment from 2, setal formula **aa: ab: bc: cd: dd** = 1.27: 0.59: 1.25: 0.74: 1.26 in segment 8 and 1.25: 0.46: 1.05: 0.52: 1.65 in postclitellar segments. Female, male and spermathecal pores not visible. Genital markings paired on **bc** in 23.

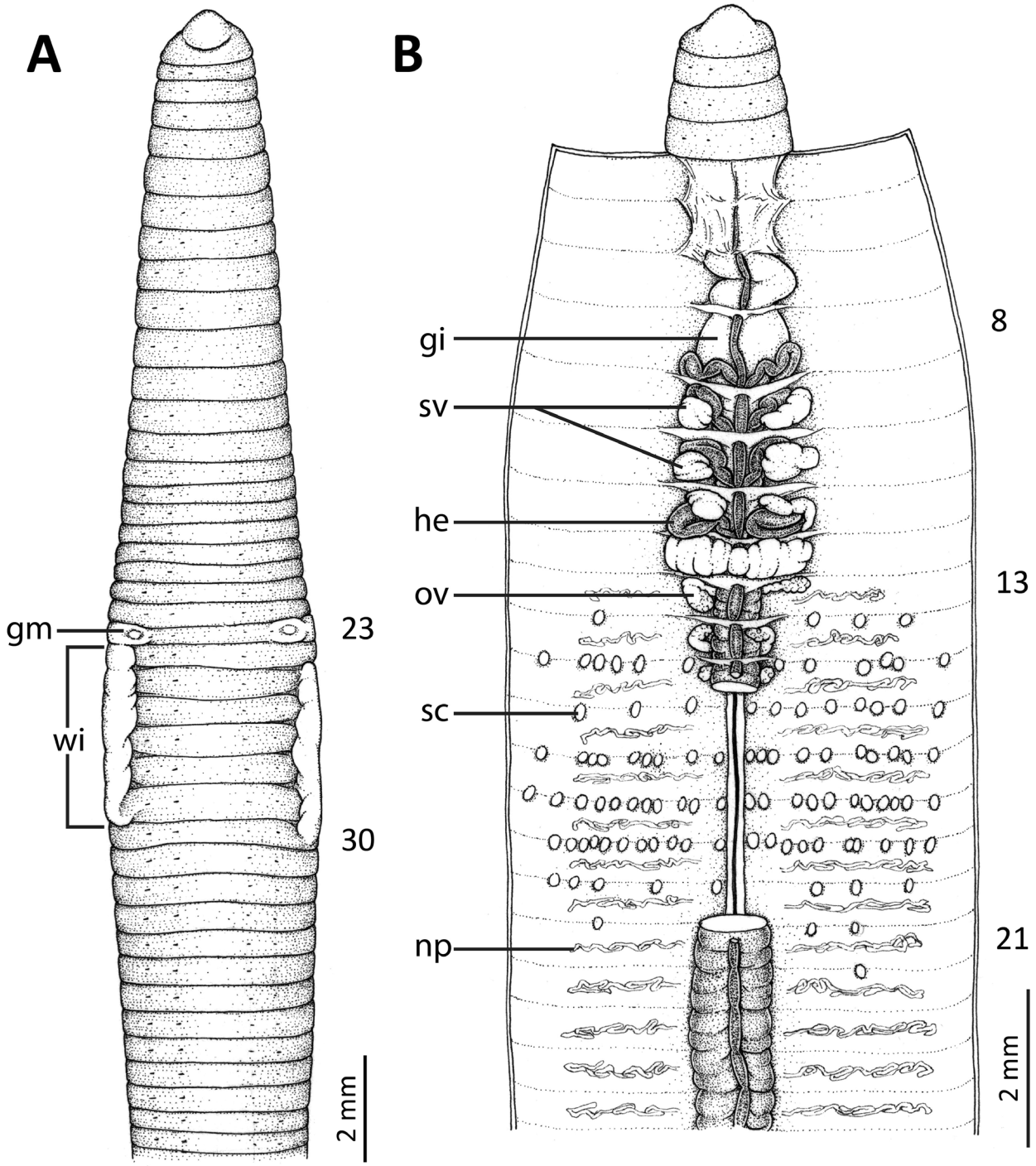

FIGURE 3. Morphology of the holotype (CUMZ 3400) of *G. kralanhensis* **sp. n. A.** External ventral view. **B.** Internal dorsal view after dissection; some anterior pairs of nephridia are not shown.

Septa 5/6–7/8 thickest, 8/9–11/12 thick and 12/13 to the last segment thin. Gizzard globular within 8. Intestine enlarged from 16. Dorsal blood vessel anterior to 7. Hearts in 7–11. A pair of nephridia in each segment, rudimentary in 13, small in 14, and normal in 15 onwards. Seminal vesicles in 9–12. Ovaries in 13–15. Testis in 10–11. Prostate and accessory glands absent. Spermathecae sessile, elongated oval or globular in 13/14–21/22, about 0.2 mm in diameter, one to eleven on each side per segment.

Variation. Body lengths of adult paratypes (4) ranged from 85–116 mm (mean ± S.D. = 100.5 ± 13.7), with 205–314 segments. Wings begin in 23, 24, ½25, or 25 and end in 26, 27, ½28, 29, 30, or 31; the most frequent position is 24–30, 31. Clitellum begins in 19 or 20 and ends in 35 or 37. All specimens have genital markings paired or asymmetrical on **bc** in pre-wing 23, some in 20–22, 24, and post-wing 26–29, 30. The most frequent position of spermathecae is 13/14–20/21; some occur in 21/22 (Table 2).

Distribution. The new species is known only from the type locality.

Habitat. The species was found on river banks where the worm casts covered the surface. The earthworms occurred in the loamy sand topsoil at about 10 cm depth.

Etymology. The species was named after Kralanh district, the type locality.

Remarks. *Glyphidrilus kralanhensis* **sp. n.** is the only species in the genus to contain three pairs of ovaries in 13–15 (hypergyny), and also has spermathecae in more posterior segments compared to the other taxa. For further comparison of Mekong *Glyphidrilus* species, see Table 2.

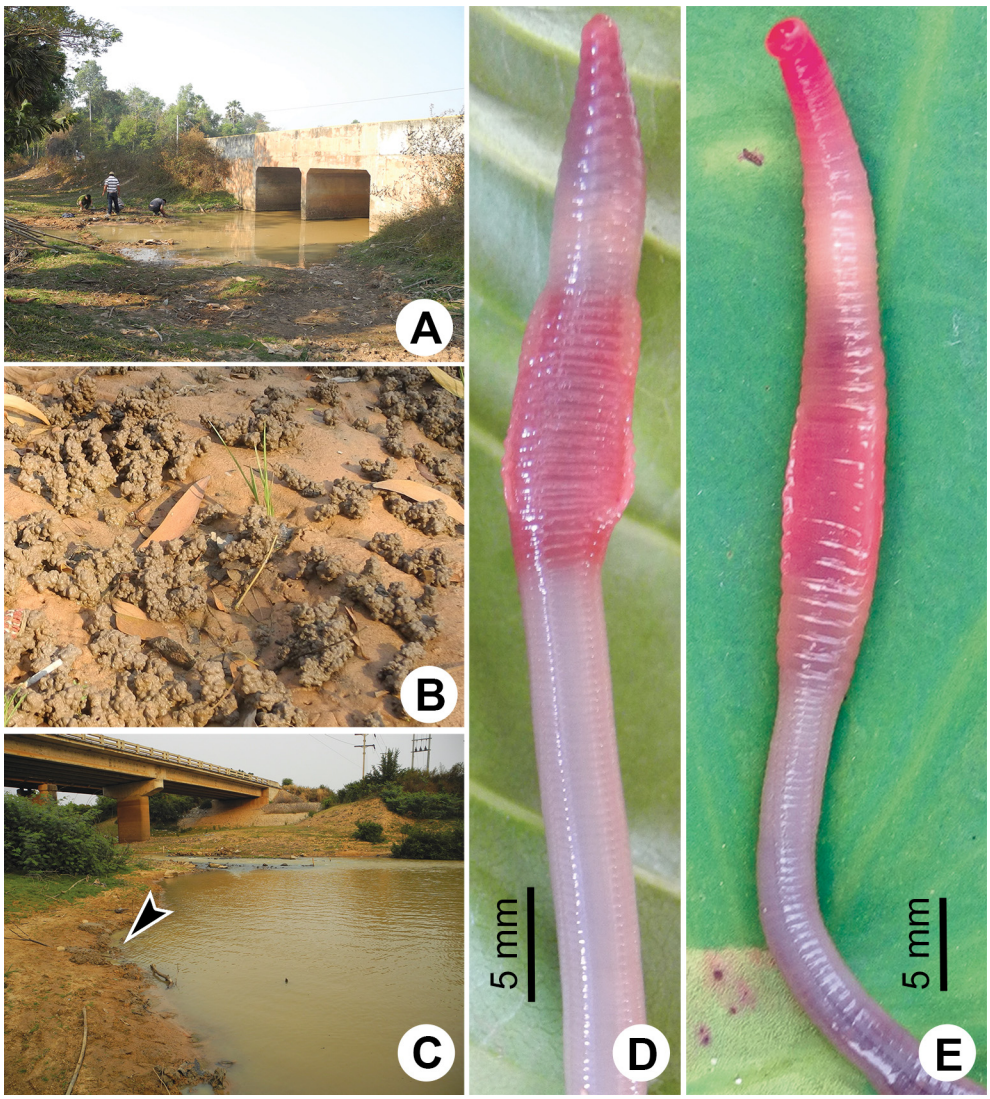

FIGURE 4. A. Type locality of *G. jamiesoni* **sp. n.** on the stream banks near Praduk Temple, Banteay Srei, Siem Riep. **B.** Same as A, casts. **C.** Type locality of *G. kralanhensis* **sp. n.** on the bank of Tapan River, Kralanh, Siem Riep. **D.** Paratype specimen of *G. jamiesoni* **sp. n.** (CUMZ 3398). **E.** Paratype specimen of *G. kralanhensis* **sp. n.** (CUMZ 3401). Both specimens photographed immediately after the first preservation in 30% (v/v) ethanol.

Molecular analyses

The mitochondrial sequences of the Mekong *Glyphidrilus* species analyzed herein were composed of 658 base pairs (bp) of partial COI gene (DNA barcode region) and 483–486 bp of partial 16S rRNA gene. The variable and parsimony informative sites of the aligned COI sequences were 246 and 236 positions respectively, and for 16S were both 106 positions. The mean intraspecific distances among the Mekong *Glyphidrilus* ranged from 0–5.18% for COI and from 0–1.51% for 16S, whereas the interspecific divergences ranged from 13.45–20.99% for COI and from 0.79–13.18% for 16S (Table 3). Compared to the outgroups, the interspecific divergences ranged from 19.45–23.47% for COI and from 19.04–22.39% for 16S.

The monophyly of *Glyphidrilus* and of each analyzed Mekong species was strongly supported by all phylogenetic analyses (NJ/ML/BI), yielding identical topologies with high bootstrap supports (93–100%) and a posterior probability of 1 for all major clades (Fig. 5). The exception was the relationship among *G. mekongensis*, *G. huailuangensis* and *G. kralanhensis* **sp. n.**, where both ML and BI trees depicted *G. mekongensis* and *G. kralanhensis* **sp. n.** as sister clades with a high bootstrap value (91%) but only a moderate posterior probability (0.65; Fig. 5), while the NJ tree retrieved *G. mekongensis* to be more closely related to *G. huailuangensis* (not shown). However, these three species are always recovered as monophyletic in all analyses, as well as the relationship between *G. chiensis* and *G. quadratus* (Fig. 5).

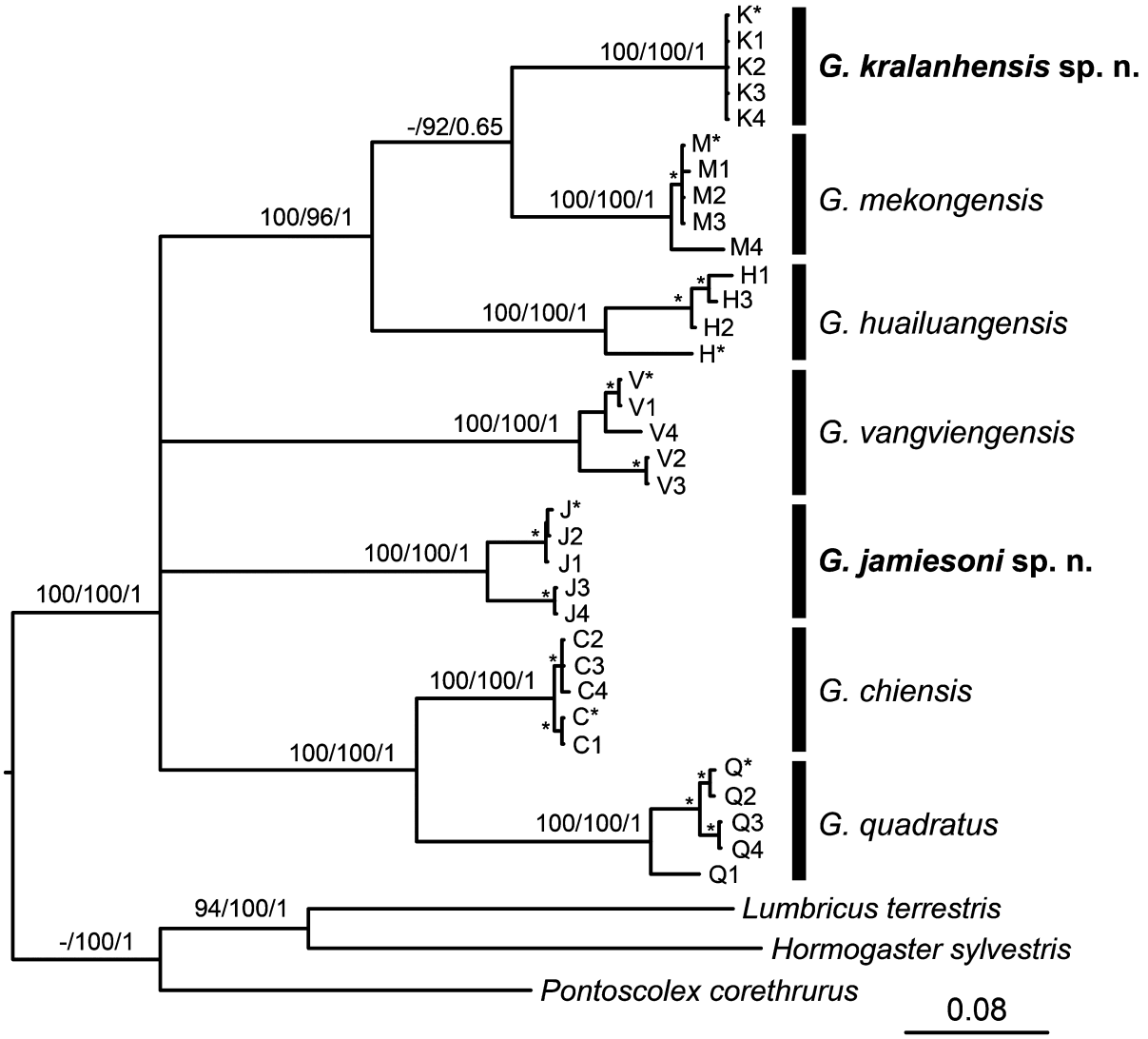

FIGURE 5. Bayesian inference tree based on the concatenated dataset of the partial COI and 16S rRNA gene sequences of the Mekong *Glyphidrilus* and outgroups. Specimen names correspond to those in Table 1. Bootstrap values of the corresponding NJ and ML trees (not shown), and posterior probabilities of this BI tree for the major branches are shown on the tree as NJ/ML/BI values, with asterisks indicating highly supported clades within each species.

TABLE 2. Comparison of the taxonomic characters and type localities of Mekong *Glyphidrilus* species analyzed in this study, based on the type specimens and the original descriptions. Locality numbers correspond to those in Figure 1. Wings, location: Numbers in bold indicate the most frequent position. Wings, segmental length: Only the most frequent extension is shown; '?' indicates uncertainty of data due to low number of specimens. Gm = genital markings. Spermathecae: Numbers in parentheses indicate infrequent locations.

Species	Wings, location	Wings, segmental length	Clitellum	Gm on aa pre-wing	Gm on aa post-wing	Gm on bc pre-wing	Gm on bc post-wing	Hearts	Intestinal origin	Ovaries	Spermathecae	Type locality
G. chiensis	**24**, 25–**30**	7	17, 18, 19–35, 36, 37	12–14	-	14, 15, 16, 17–23, 24	31	7–11	15	13–14	12/13–17/18 (18/19)	Loc. 1
G. huailuangensis	25, **26**–**30**, 31	5	12, 13, 16–32, 33	-	31	16–24	-	8–11	13	13–14	absent	Loc. 2
G. mekongensis	**24**–½33, 33, **34**, ½35	11	19–37, 38	-	-	23	-	7–11	15	13–14	absent	Loc. 3
G. quadratus	**23**, 24–**29**, 30	7	14, 15, 16–30, 31, 32, 33	13–14	-	15–23	-	7–11	15	13–14	12/13–17/18	Loc. 7
G. vangviengensis	**23**, **24**, 25–**31**, **32**	9	19, 20–35, 36, 37	12–14, 15	-	18, 19, 20, 21–24	33, 34	7–11	16	13–14	absent	Loc. 8
***G. jamiesoni* sp. n.**	23, **24**–31, **32**, 33	9	17, 18–35, 36	12, 13, 14	31, 33	15, 16, 17, 18–22, 23	31, 32	7–11	16	13–14	13/14–18/19	Loc. 12
***G. kralanhensis* sp. n.**	23, **24**, ½25, 25–26, 27, ½28, 29, **30, 31**	7–8?	19, 20–35, 37	-	-	20, 22–24	26–29, 30	7–11	16	13–15	13/14, 14/15–20/21 (21/22)	Loc. 14

TABLE 3. Percentage of uncorrected pairwise interspecific distances for the partial COI (above the diagonal) and 16S rRNA (below the diagonal) gene fragments among the Mekong *Glyphidrilus*. Intraspecific distances for COI/16S are shown in the diagonal.

	1. *G. chiensis*	2. *G. huailuangensis*	3. *G. mekongensis*	4. *G. quadratus*	5. *G. vangviengensis*	6. ***G. jamiesoni* sp. n.**	7. ***G. kralanhensis* sp. n.**
1	0.39/0	18.92	17.82	13.45	19.10	18.64	18.18
2	13.18	5.18/0	17.33	20.99	18.40	18.80	17.46
3	13.01	0.79	1.45/0.42	19.59	18.72	18.61	14.52
4	6.28	11.59	11.42	2.61/0.59	19.29	19.32	19.61
5	13.10	10.92	10.69	12.95	3.64/1.42	17.44	18.70
6	11.13	8.54	8.34	11.80	10.42	3.61/1.51	18.39
7	12.13	5.02	4.90	11.38	9.41	9.54	0/0

Discussion

The Cambodian earthworm fauna has received very little attention in the past, and the two new species reported in this study, *Glyphidrilus jamiesoni* **sp. n.** and *G. kralanhensis* **sp. n.,** are the first to be newly described from Cambodia in this century. The major external morphological characters used in identification (e.g. wings and genital markings) are known to be highly variable among *Glyphidrilus* taxa (Brinkhurst & Jamieson 1971; Chanabun *et al.* 2013). This was comprehensively shown previously by Gates (1958), who reported the level of variation in the wings and genital markings from within the same colony of *G. gangeticus* Gates, 1958. The location and segmental length of the clitellum are of limited use due to their variability and overlap among taxa, and also their changes in color and texture after preservation. Moreover, male, female and spermathecal pores are minute in size and can only be discerned in serial sections, whilst currently there are data on male pores from only two *Glyphidrilus* species (Horst 1893). However, the location and segmental length of the wings could be used to some extent, as some locations occur more frequently and the segmental length is consistently conserved (see Gates 1958 and Table 2). The internal anatomy proved to be highly valuable for species identification and is commonly used in earthworm taxonomy (Edwards & Bohlen 1996), especially in *Glyphidrilus* (Chanabun *et al.* 2013). Indeed, the internal organs are more conserved compared to the external morphology, but they may be similar or overlapping in shape, quantity and position among different taxa (Novo *et al.* 2012b). Thus, most of the internal characters (e.g. gizzard, hearts, intestinal origin and ovaries) are limited in their ability to resolve different species, except for those species with an exceptional set of characters (see *G. huailuangensis* and *G. kralanhensis* **sp. n.** in Table 2). It is worth noting that *G. kralanhensis* **sp. n.** is the only species in the genus to contain three pairs of ovaries (hypergyny), which could be an autapomorphic trait. The two new species also possess spermathecae in more posterior segments compared to the other Mekong taxa (Table 2). Despite the modest level of variation, the location of spermathecae proved to be crucial in the diagnosis of *Glyphidrilus* species. Thus, using both external and internal characters will likely yield a well-established identification although not a definitive one.

Molecular data, especially mitochondrial markers, have helped to support species delineations, including in defining which species are new to science (Díaz Cosín *et al.* 2014; Fernández Marchán *et al.* 2014; Novo *et al.* 2012b). All holotypes and most paratypes of the Mekong *Glyphidrilus* were sequenced, as encouraged by previous studies, to stabilize the taxonomy of earthworms (Blakemore *et al.* 2010; Boyer *et al.* 2011a) and to facilitate identification of specimens in the future, for example juveniles or environmental samples from soil and gut contents (Bienert *et al.* 2012; Boyer *et al.* 2011b; Richard *et al.* 2010; Šerić Jelaska *et al.* 2014). In accordance with the morphological data, the DNA sequence data of the two new species in this study were highly divergent from those of the other Mekong taxa (Fig. 5). The high interspecific genetic distances among the Mekong *Glyphidrilus* species (Table 3) are comparable to results in other earthworm studies (Chang *et al.* 2008; Fernández Marchán *et al.* 2014; Novo *et al.* 2010), where a COI genetic distance of 15% (Kimura-corrected) has been proposed to be a cut-off for different earthworm species (Chang & James 2011). Although the COI uncorrected divergences of two species pairs—*G. chiensis* / *G. quadratus* and *G. mekongensis* / *G. kralanhensis* **sp. n.**—were within the ambiguity range (9–15%), their different morphological characters and low intraspecific distances allow for their unambiguous species discrimination.

Even though the deeper nodes were not all resolved in the phylogenetic tree shown in Figure 5, some evolutionary relationships among the Mekong *Glyphidrilus* could still be inferred. All species in this study were retrieved as monophyletic groups, thus supporting the validity of the previously described morphospecies. The athecal species (without spermathecae) of *G. vangviengensis*, *G. huailuangensis* and *G. mekongensis* did not group together, which indicated that the absence of spermathecae may have occurred multiple times in different lineages. Furthermore, the presence/absence of spermathecae as a diagnostic character is now being questioned, as several earthworm species contain both thecal and athecal morphs (Bantaowong *et al.* 2011; Gates 1972; Shen *et al.* 2012). This notion is also supported by the observation that the thecal species *G. kralanhensis* **sp. n.** clustered together with the athecal *G. mekongensis* and *G. huailuangensis*, given that the secondary gain of spermathecae is unlikely. Dissimilar to *G. mekongensis,* which has a wide distribution along the Lower Mekong River, *G. huailuangensis* and *G. kralanhensis* **sp. n.** are so far known only from their respective type localities (Fig. 1). It is possible that speciation took place in those isolated areas, but whether this is due to a founder population that dispersed from the main stem river or caused by lineage separation due to a river course change is yet to be explored.

Glyphidrilus chiensis and *G. quadratus* both occupy habitats in the tributaries of the Mekong in Northeastern

Thailand, the Chi and the Mun, respectively (Fig. 1), and are phylogenetically closely related (Fig. 5). From a morphological point of view, these two species share the same segmental length, and have slightly different spermathecae and wings location. It is probable that the *G. chiensis* / *G. quadratus* clade diverged from the Mekong *Glyphidrilus* ancestor when the paleo-Mun River separated from the Mekong, and the river capture between the Mun and the Mekong might have occurred later. In the study site by the Mun, Claude *et al.* (2011) discovered aquatic reptile fossils that were not affiliated to the present Mekong taxa, indicating an ancient disconnection between the Mun and the Mekong. The authors proposed that the flow direction of the Mun was also different in the past from that of the present, flowing westwards into the Chao Phraya Basin in the Pleistocene at the latest. In any case, a larger sampling effort is needed to depict the actual distribution of these semi-aquatic earthworms, and the analysis of more specimens and appropriate molecular markers (e.g. nuclear genes) will yield a more complete picture of the evolutionary relationships and the biogeography of the Mekong *Glyphidrilus.*

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