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Polystoma luohetong n. sp. (Monogenea: Polystomatidae) from *Rana chaochiaoensis* Liu (Amphibia: Ranidae) in China

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Abstract *Polystoma chaochiaoensis* from the urinary bladder of the chaochiao frog *Rana chaochiaoensis* Liu was briefly described in a symposium abstract and presented at the Third Symposium on Parasitology of China in 1990. Types were not assigned and the original specimens collected are no longer available. The morphological description was incomplete and no illustrations were provided. We consider *Polystoma chaochiaoensis* a *nomen nudum* and provide a full description for the species and assign types. Based on morphological characteristics and molecular data of partial 18S rDNA sequences, we describe this species as *Polystoma luohetong* n. sp. Out of 578 frogs examined, 16 male and 38 female frogs were infected (prevalence 9.3%; mean intensity 1.02). *Polystoma luohetong* n. sp. is distinguished from all other *Polystoma* species by the presense of a prominent crest on the hamulus as well as by the shape and size of marginal hooklets and the intestinal arrangement. Furthermore, the phylogentic analysis based on the 18S rRNA gene shows *Polystoma luohetong* n. sp. well nested within the *Ploystoma* clade and as a sister taxon to *Polystoma integerrimum*.

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Introduction

Polystomatid flatworms (Monogenea: Polystomatidae Gamble, 1896) are globally distributed and currently there are 27 recognized genera with more than 180 species (Chaabane et al., 2019). The vast majority (19 genera) are known from amphibian hosts. Anuran polystomatids are represented in the Palaearctic realm by species of *Diplorchis* Ozaki, 1931, *Eupolystoma* Kaw, 1950, *Indopolystoma* Chaabane, Verneau & Du Preez, 2019, *Polystoma* Zeder, 1800, *Neoriojatrema* Imkongwapang & Tandon, 2010 and *Sundapolystoma* Lim & Du Preez, 2001. Of these, *Diplorchis* (6 species), *Indopolystoma* (6 species) and *Polystoma* (2 species) are known from China. While *Diplorchis* spp. and *Indopolystoma* spp. are restricted to the Palaearctic and Oriental realms, *Polystoma* spp. are known from all continents hospitable to anurans, with the exception of Australia. *Diplorchis* is represented by *D. grahami* Fan, Wang & Xu, 2007, *D. hangzhouensis* Zhang & Long, 1987, *D. latouchii* Zhang & Long, 1987, *D. lividae* Song, Xiao & Ding, 2008, *D. nigromaculatus* Lee, 1936 and *D. shilinensis* Fan, Pan & Wang, 2006. *Indopolystoma* is represented by *I. carvirostris* (Fan, Li & He, 2008), *I. leucomystax* (Zhang & Long, 1987), *I. mutus* (Meng, Song & Ding, 2010), *I. parvum* Chaabane, Verneau & Du Preez, 2019, *I. pingbianensis* (Fan, Wang & Li, 2004) and *I. zuoi* (Shen, Wang & Fan, 2013). *Polystoma* is represented by *P. xinpingensis* Gao, Chen & Fan, 2012.

Based on morphological characteristics alone, *Polystoma chaochiaoensis* from *Rana chaochiaoensis* was named and briefly described in an abstract presented at the 3th Symposium on Parasitology of China, held by Compilation of Parasitology Society of Chinese Society of Zoology in 1990. This description was incomplete, without illustrations, and with no type material assigned. According to the International code for Zoological Nomenclature (ICZN) Chapter 3, Section 8, Article 1, a symposium abstract does not qualify as a valid publication outlet and thus we consider *Polystoma chaochiaoensis* a *nomen nudum* and herein we provide a full description for this species.

Materials and methods

Sample collection and morphological study

Frogs were collected at Lufeng County, Yunnan Province, China, in the months of April, May and December during the period 2005–2009. The taxonomic status of frogs was confirmed according to Yang (1991). Frogs were transported to the laboratory to be euthanised and dissected to examine the parasites using an Olympus SZX7 dissecting microscope (Olympus, Japan). Monogeneans were removed from the urinary bladder and some (n = 3) were fixed in 100% ethanol for molecular stydies and the remainder were fixed in 5% formol-saline under coverslip pressure. After 12 h, the fixed specimens were transferred to a 5% formol-saline solution for preservation. Polystomes were stained overnight in a weak solution of alum carmine and washed thereafter in several changes of tap water. Specimens were dehydrated in an ethanol series, cleared in clove oil or xylene and mounted in Canada balsam. All specimens were examined and measured using a microscope Olympus CX41 and images captured using a Digi Retina 16 microscope camera. Digital image analysis system (TCapture Imaging Application 4.2) was used for morphometric analysis. Measurements are given in micrometres as the range with the mean in parentheses.

Molecular data

Specimens for molecular study were washed in double-distilled water and fixed in 96% ethanol. Genomic DNA was extracted from each individual specimen using phenol-chloroform method. DNA extraction, amplification and sequencing followed the methods of Bentz et al. (2001) and Sinnappah et al. (2001). The primers F18 (5’-ACC TGG TTG ATC CTG CCA GTA G-3’) and Rev18 (5’-TAC CGG TGT ACT ATT TAG CAG-3’) were used to amplify a fragment of the 18S rRNA gene (Bentz et al. 2001, 2003, 2006; Sinnappah et al. 2001). PCR amplification reaction was performed in a total of 50 µl, containing 5 µl of 10 × buffer, 4 µl of 2.5 mmol/l dNTP, 1 µl of 20 µmol/l of each primer, 2 µl of 25 mmol/l of $MgCl_2$, 2U *Taq* polymerase and 50 ng of DNA template, under the following conditions: 5 min at 95°C, 1 min at 45°C, 2 min at 72°C elongation, 1 min at 95°C, 1 min at 45°C and 35 cycles of 2 min at

72°, followed by a final extension step at 72° for 10 min. Samples without genomic DNA were included as a negative control in each run (Eppendorf Mastercycler gradient). PCR products were detected on a 1% agarose gel stained using ethidium bromide. The sequencing reactions were sequenced and analyzed using the ABI PRISMTM 377XL DNA Sequencer and ABI PRITM-3730XL DNA Analyzer (Takara Bio, Dalian, China). Sequences were analysed using DNAMAN 7.0 and Sequencher 5.0 (Gene Codes Corp., Ann Arbor, Michigan, USA) software. Species identity of the polystome from *Rana chaochiaoensis* described here was compared against sequences of other polystomes using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

The software package Geneious R11 (http://www.geneious.com (Kearse et al., 2012)) was used for sequence alignment and phylogenetic analysis. Representative sequences were downloaded from GenBank and aligned using the MUSCLE alignment tool (Edgar, 2004) to the sequence generated in the present study. *Pseudopolystoma dendriticum* was selected as the outgroup taxon with the final alignment made up of 17 sequences. A model test was performed to determine the most suitable nucleotide substitution model, according to the Akaike information criterion (AIC) using jModelTest 2.1.7 (Guindon & Gascuel, 2003; Darriba et al., 2012). The model with the best AIC score for the 18S rDNA sequence alignment was the General Time Reversible (Tavaré, 1986) model, with estimates of invariable sites, and a discrete gamma distribution ($GTR + I + \Gamma$). For the Maximum likelihood (ML) analysis, nodal support was assessed using 1000 rapid bootstrap inferences.

Class Monogenea Van Beneden, 1858
Order Polystomatidea Lebedev, 1988
Family Polystomatidae Gamble, 1896

Polystoma luohetong n. sp.

Type-host: *Rana chaochiaoensis* Liu (Amphibia: Ranidae).

Site in host: Urinary bladder.

Type-locality: Lufeng County (25.160930°N, 101.895574°E; altitude 1,640–1,800 m), the central area of Yunnan Province in southwest of China.

Type-material: Holotype (NMB P554) and five paratypes (NMB P555-P559) housed in the Parasitic Worm Collection, National Museum, Bloemfontein, South Africa and four paratypes 200505C015, 200505C018-2, 200505C023 and 200505C070 housed in the School of Life Science of Yunnan Normal University, Kunming, China.

Representative DNA sequence: 18S rRNA gene (GenBank: EU734834).

Levels of infection: Of the 578 *R. chaochiaoensis* collected, 16 male and 38 females were found to be infected with between one and three parasites (prevalence 9.3%; mean intensity 1.02).

ZooBank registration: To comply with the regulations set out in Article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Polystoma luohetong* n. sp. is urn:lsid:zoobank.org:act:0C176439-B965-42B2-8C44-B050A0DAC6DC.

Etymology: The new species is named in recognition of Professors Y. D. Luo, H. M. He and J. R. Tong, who first collected this species.

Description

[Based on 22 whole-mounted adult specimens; see Table 1 and Figs. 1–3.] General characteristics typical of *Polystoma*. Body elongate, 1,876–7,610 (4,215) long, 788–2,980 (1,960) in maximum width, 509–2,034 (1,033) wide at level of vaginal ducts (Fig. 1). Pharynx 119–279 (164) long, 103–188 (148) wide. Intestine bifurcate; with multiple anastomoses to form reticulated network extending well into haptor (Fig. 1). Haptor 465–2,560 (1,126) long, 800–2,610 (1,734) wide, mean haptor length/body length ratio 0.22–0.36 (0.27). Haptoral sucker 206–474 (340) in diameter; hamulus with deep cut between roots (Fig. 2A), hook to tip of handle ("X" in Fig. 2B) 221–513 (344), hook to tip of guard ("Y" in Fig. 2B) 188–396 (282), recurved hook 38–64 (51), prominent crest with grooves for muscle attachment, X:Y ratio 1.04–1.40 (1.22). Marginal hooklets positioned as for other polystomes, hooklets 1 and 2 posteriormost between posteriormost suckers, hooklets 3-5 at base of suckers, hooklets 6-8 anterior in haptor between anteriormost suckers; marginal hooklet 1 largest, 26.9–32.2 (29.2) long (Fig. 2C); marginal hooklets 2-8 of equal length 18.8–24.5 (22.1) (Fig. 2D).

Testis follicular, prominent, occupies most of the region posterior to the ovary, masses of sperm observed between testicular follicles. Vas deferens median, coalescing anteriorly to form a seminal vesicle.

Ovary prominent, elongate-oval (Fig. 3), 99–778 (495) long, 54–549 (338) wide. Uterus short, tubular, anterior to the ovary, holding up to 13 eggs. Intrauterine eggs 118–261 × 90–151 (220 × 115), no intrauterine development observed. Genital atrium ventral, posterior to intestinal bifurcation. Genital bulb with 8–9 genital spines 15.0–49.8 (33.3) long. Vitellarium indistinct, with follicles distributed throughout most of the body extending into the haptor. A pair of vaginae, prominent at level of uterus anterior to ovary, 11% from anterior extremity. Measurements and hosts of related and recorded Chinese polystomes are given in Table 1.

Remarks

Phylogenetic analysis supports *P. luohetong* n. sp. as a valid taxon of genus *Polystoma* (see below). *Polystoma luohetong* n. sp. differs from all other species of *Polystoma* known from Asia in a combination of morphometric characteristics. The prominent crest opposing the hook of the hamuli is unique and distinguish *P. luohetong* n. sp. from all other known polystomes. The reticulated network intestine separates it from *P. xinpingensis*, the only other polystome from China, which has no more than two prehaptoral anastomoses. Whereas species of *Polystoma* as a rule possess a pear-shaped or comma-shaped ovary, the ovary of *P. luohetong* n. sp. is elongate-oval, a characteristic that further separates it from *P. xinpingensis*.

Molecular analyses

The ML phylogenetic analysis (Fig. 4) based on 18S rDNA sequences showed with high confidence values the phylogenetic relationship among species of *Polystoma*. *Polystoma luohetong* n. sp. *(*GenBank: EU734834), *P. gallieni* Price, 1939 (GenBank: AM051070), *P. integerrimum* (Frölich, 1791) Rudolphi, 1819 (GenBank: AM051071) and *P. xinpingensis* (GenBank: EU979386) forms a Palaearctic Realm cluster. Polystoma *gallieni* is sister to *P. xinpingensis* both species infecting tree frogs of the genus *Hyla* while *P. luohetong* n. sp. is sister to *P. integerrimum*, both infecting river frogs of the genus *Rana*.

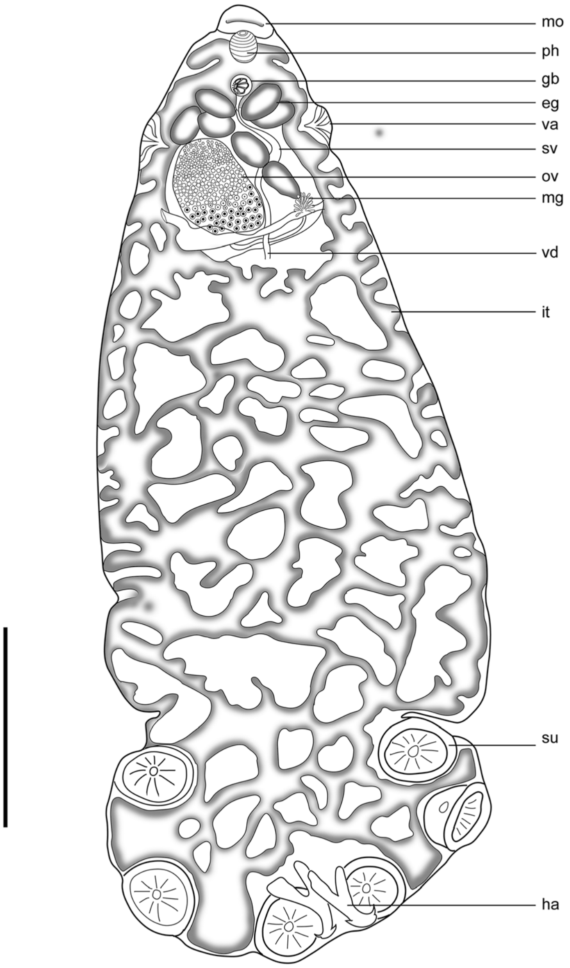

Fig. 1 *Polystoma luohetong* n. sp. Ventral view of the holotype. *Abbreviations*: eg, egg; gb, genital bulb; ha, hamuli; it, intestinal caecum; mg, Mehlis'gland; mo, mouth; ov, ovary; ph, pharynx; su, sucker; sv, seminal vesicle, va, vagina, vd, vas deferens. *Scale-bar*: 1,000 μm

Discussion

Polystomatid flatworms have two main structures to secure a firm attachment in the host, haptoral suckers and hamuli. Larval forms at first have neither suckers nor hamuli, but posess a set of 16 marginal hooklets that ensure a firm attachment to the host tissue. These marginal hooklets do not develop further and are

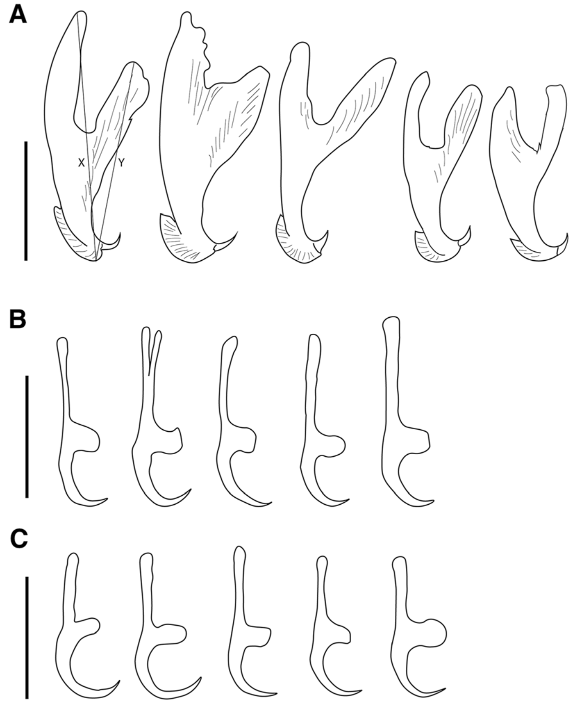

Fig. 2 *Polystoma luohetong* n. sp.. A, Hamuli of the holotype and paratypes; B, Marginal hooklets 1; C, Marginal hooklets 2–7. *Scale-bars*: A, 200 µm; B, C, 20 µm

gradually replaced by suckers and hamuli (Tinsley, 2017). As a rule, suckers and hamuli start developing after the parasite larvae have become attached in the target microhabitat in the host; however, in species of *Madapolystoma* Du Preez, Raharivolololniaina, Verneau & Vences, 2010 suckers and hamuli start developing while the larvae are still *in utero* (Landman et al., 2018). Marginal hooklets are always retained in mature parasites but do not contribute further to attachment and may often be difficult to locate as they are small and obscured by other tissues. Species of all of the 27 known polystome genera have three pairs of haptoral suckers except for *Sphyranura* that have one pair. On the other hand, hamuli are not always present. Of the anuran-infecting polystomes, species of *Eupolystoma*, *Madapolystoma*, *Neoriojatrema* Imkongwapang & Tandon, 2010, *Parapseudopolystoma* Nasir & Fuentes Zambrano, 1983, *Pseudodiplorchis* Yamaguti, 1963, *Pseudopolystoma* Yamaguti, 1963 and *Riojatrema* Lamothe-Argumedo, 1964 do not possess hamuli. The hippopotamus-infecting polystome *Oculotrema* Stunkard, 1924 and the chelonian polystome *Neopolystoma* Price, 1939 also lack hamuli. *Polystomoidella* Price, 1939, *Uropolystomoides* Tinsley & Tinsley, 2016 and *Uteropolystomoides nelsoni* (Du Preez & Van Rooyen, 2015) as well as one anuran-infecting polystome *Protopolystoma* Bychowsky, 1957 possess two pairs of hamuli.

The surface of the hamuli is not smooth but with multiple grooves, especially on the two roots thus allowing for muscle attachment. Although a crest opposite to the hook on the handle has been indicated for other *Polystoma* spp. including *Polystoma mazurmovici* Batchvarov, 1980, *Polystoma pricei* Vercammen-Grandjean, 1960 and *Polystoma gabonensis*

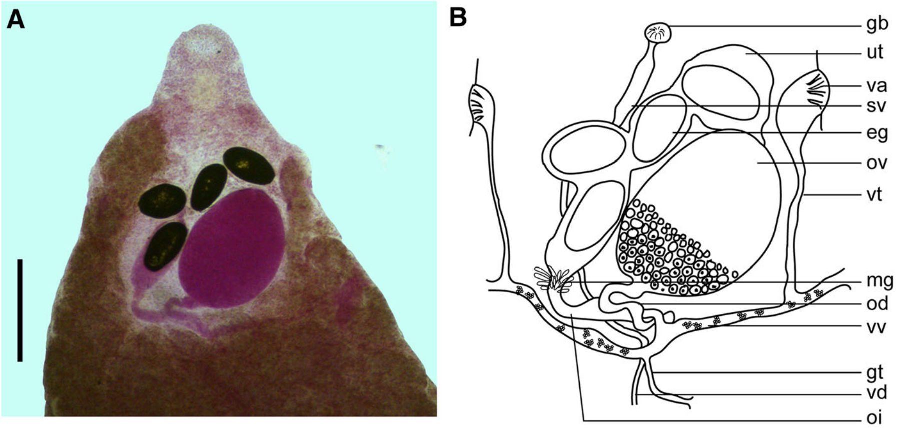

Fig. 3 *Polystoma luohetong* n. sp.. Reproductive system in ventral view. A, Micrograph of reproductive system; B, Annotated drawing of reproductive system. *Abbreviations*: eg, egg; gb, genital bulb; gt, genito-intestinal duct; mg, Mehlis'gland; od, oviduct; oi, oö-vitelline duct; ov, ovary; sv, seminal vesicle; ut, uterus; va, vagina; vd, vas deferens; vt,vaginal duct; vv, vitello-vaginal duct. *Scale-bar*: A, 500 µm

Euzet, Combes & Knoepffler, 1966, *P. luohetong* n. sp. is the first species of *Polystoma* with this characteristic present in all specimens examined. At the roots of the hamuli, the crest has prominent grooves indicating that the crest allows for muscle attachment.

Polystomatid flatworms infecting anurans are generally regarded as strictly host-specific (Du Preez & Kok, 1997; Aisien & Du Preez, 2009; Berthier et al., 2014) and host identity is often used as an additional character for parasite identification (Combes & Channing, 1979; Combes & Gueorgui, 1995; Héritier et al., 2017), and proves to be quite valuable in identifying members of the genus *Polystoma* (see Santos & Amato, 2012). All specimens collected in the present study from the type-locality displayed the same morphological features and the new species appears to display a high level of host specificity.

Polystomatids display a wide diversity of life-cycle patterns, amongst the most varied in the Platyhelminthes (see Tinsley, 1983). At one extreme they infect primarily aquatic frogs (e.g. *Protopolystoma* spp. in *Xenopus laevis* Daudin) which allows for continual reinfection. At the other extreme they infect arid adapted frogs (e.g. *Pseudodiplorchis americanus* (Rodgers & Kuntz, 1940) Yamaguti, 1963 infecting *Scaphiopus couchi* Baird) where annual parasite transmission takes place one day a year following the first major rainfall event of the season. *Polystoma* spp. are often found in anuran hosts with explosive breeding behaviour. Large numbers of eggs therefor have to be produced in a matter of a few days. A prominent ovary packed with oöcytes to meet this requirement is a characteristic of the genus. The reproductive strategy of *P. luohetong* n. sp. in its

Table 1 Measurements (in micrometres) for *Polystoma* spp. from China

Species **Host**	***P. luohetong* n. sp.** ***Rana chaochiaoensis* Liu**	***P. xinpingensis* Gao, Chen & Fan, 2012** ***Hyla annectans* Jerdon**
Prevalence	9.34% (54/578)[a]	14.5% (18/124)[a]
No. of specimens	22	12
Body length	1,876–7,610 (4,215; n = 22)	2,082–3,940 (2,967)
Body maximum width	788–2,980 (1,960; n = 22)	– (1,187)
Width at vagina	509–2,034 (1,033; n = 21)	– (904)
Haptor length	465–2,560 (1,126; n = 22)	532–1,501 (801)
Haptor width	800–2,610 (1,734; n = 22)	867–1,451 (1,211)
Haptor to body length ratio	0.22–0.36 (0.27; n = 22)	–
Pharynx length	119–279 (164; n = 19)	– (200)
Pharynx width	103–188 (148; n = 19)	– (154)
Egg length	118–261 (220; n = 47)	– (251)
Egg width	90–151 (115; n = 47)	– (91)
No. of intrauterine eggs	1–13	1
Ovary length	99–778 (495; n = 21)	– (414)
Ovary width	54–549 (338; n = 21)	185–298 (242)
Haptoral sucker diameter	206–474 (340; n = 119)	229–323 (275)
X	221–513 (344; n = 24)	250–368 (304)
Y	188–396 (282; n = 24)	222–261 (239)
X/Y	1.22 (n = 24)	1.27
No. of spines	8–9	7–9
Genital spine length	15.0–49.8 (33.3; n = 79)	24.5–37.4 (29.8)
No. of anastromoses	Network	2
Marginal hooklet 1	26.9–32.2 (29.2; n = 12)	
Marginal hooklets 2–8	18.8–24.5 (22.06; n = 17)	

[a]No. of infected/Total no. examined frogs

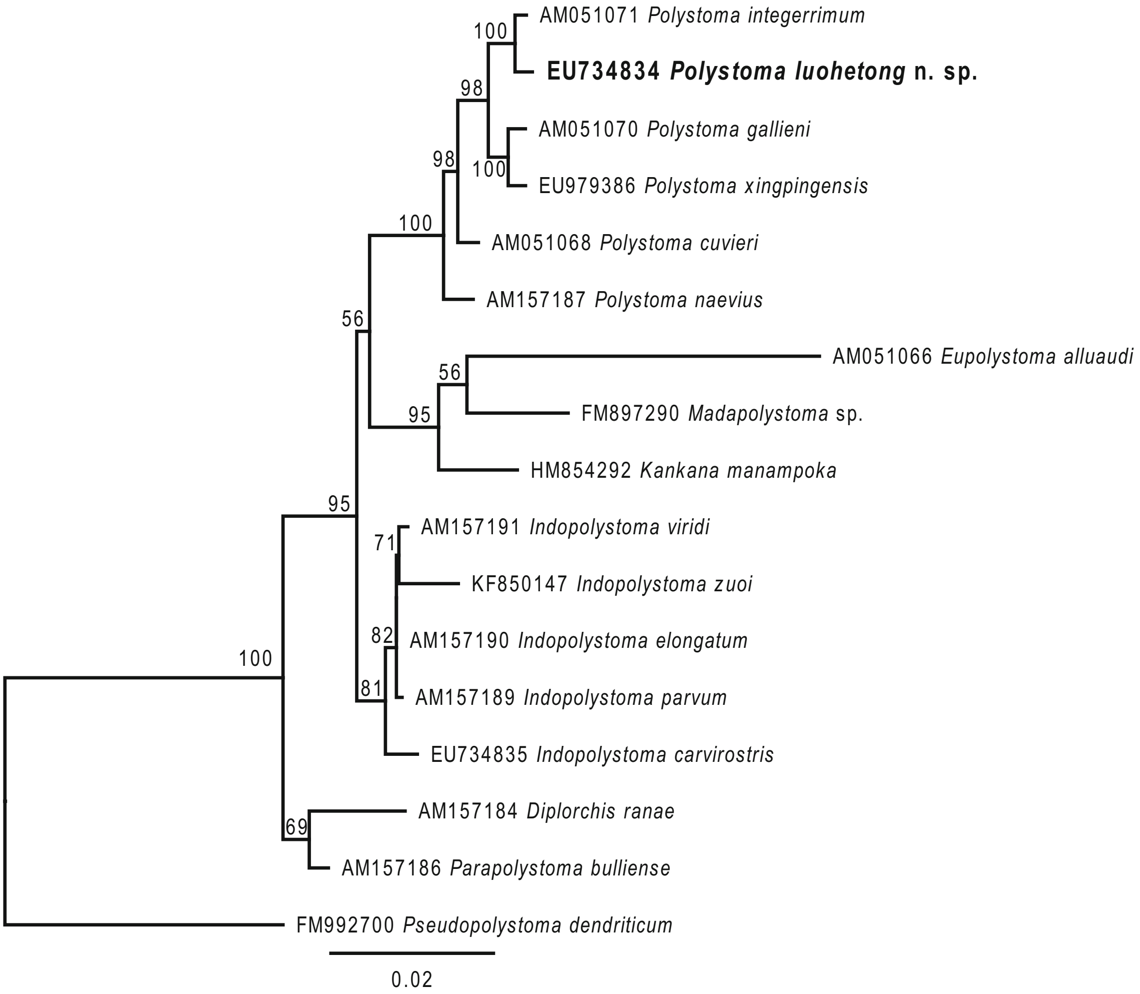

Fig. 4 Phylogenetic tree inferred by the Maximum Likelihood method (1,522 nt). *Pseudopolystoma dendriticum* was selected as the outgroup taxon

anuran host *R. chaochiaoensi* fits the above profile. This frog is found is a moderately arid environment and is also an explosive breeder. Although the elongate-oval shape of the ovary of *P. luohetong* n. sp. is somewhat atypical for the genus, it is packed with oöcytes and the vitellarium is extensive, providing the materials needed for rapid egg production.

In comparisons, the sequence data for *P. luohetong* n. sp. (AM051071) matched the sequence KR856125 (Héritier et al., 2015) on GenBank with 99.8% simiarity and a difference of three nucleotide positions. We confirm that the *Polystoma* sp. listed as *P. dianxiensis* (KR856125) is a synonym to *P. luohetong* n. sp. When a specimen of *R. chaochiaoensis* was sent to Professor Verneau and Dr Héritier to include in the Héritier et al. (2015) paper, the specimen was labelled *P. dianxiensis*. However, this was a provisional name at the stage and never formally published.

The Yunnan Province and in particular the Hengduan Mountains in southwestern China, is regarded a biodiversity hot spot (Linnemann et al., 2018) and currently seven of the 14 anuran polystomes known to occur in China have been recorded in this geographical area. With more than 120 species of amphibians recorded in this area (Yang, 1991) and with the high degree of host specificity within the genus *Polystoma*, we can expect that that the rich anuran diversity might be mirrored by a similarly rich polystome diversity.

No chelonian polystomes have been reported from China in spite of the fact that China has a rich diversity of freshwater chelonians. One can assume that many polystomes await discovery and description.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed. Ethical clearance were obtained from the Yunnan Normal University (Ethics no: ynnuethic2020-8).

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