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First report of *Schistosoma sinensium* infecting *Tupaia belangeri* and *Tricula* sp. LF

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ABSTRACT

Schistosoma sinensium belongs to the Asian *Schistosoma* and is transmitted by freshwater snails of the genus *Tricula*. Rodents are known definitive hosts of *S. sinensium*. In 2016, suspected schistosome eggs were found in the feces of the northern tree shrew (*Tupaia belangeri*) in a field in Lufeng County (latitude, 25°04′50″ N; longitude, 102°19′30″ E; altitude 1820 m), Yunnan Province, China. Morphological analysis suggested that the schistosome was *S. sinensium*. 18S, 12S and CO1 genes sequencing and phylogenetic analysis showed that this species had the highest similarity to and occupied the same evolutionary branch as *S. sinensium* from Mianzhu, Sichuan, China. Meanwhile, based on 16S and 28S rDNA sequencing and morphological identification, the snail intermediate host was identified as a species of *Tricula*, and was found in irrigation channels. Phylogeny indicated that *Tricula* sp. LF was a sister taxon to *T. bambooensis, T. ludongbini*. The *S. sinensium* was able to experimentally infect the captive-bred *Tupaia belangeri,* and *Schistosoma* eggs were recovered from all *Tupaia belangeri* exposed. In this study, we report the infection of *Tupaia belangeri* and *Tricula* sp. LF with *S. sinensium* in Lufeng, Yunnan, southwest China. These findings may improve our understanding of the host range, evolution, distribution, and phylogenetic position of *S. sinensium*.

1. Introduction

Schistosoma Weinland, 1858, is a genus of trematodes (blood flukes) that causes schistosomiasis in mammals, including humans, and is prevalent in tropical and subtropical areas. Schistosomiasis is a serious and debilitating disease affecting humans and animals and is considered by the World Health Organization as the second most socioeconomically important parasitic disease after malaria. Over 20 schistosome species associated with human or animal diseases have been identified to date (Attwood et al., 2007). The host of an individual schistosome is restricted, although some schistosomes have a wide range of hosts including humans and some livestock (Attwood et al., 2002a).

Schistosoma sinensium Bao, 1958, was first isolated from an unidentified snail in Mianzhu County, Sichuan Province, China. This schistosome species was described on the basis of the morphological analysis of adult worms and eggs (Pao, 1959), and the snail species was identified as *Tricula hortensis* by Attwood (Attwood et al., 2003). *Schistosoma sinensium* is found throughout southern China, southeast Asia, and northern India. Molecular analysis showed that *S. sinensium* belongs to the Asian *Schistosoma* and is a sister clade to *S. japonicum,* although egg morphology was similar to that of *Schistosoma mansoni*, with a lateral spine (Agatsuma et al., 2001; Lawton et al., 2011). *Schistosoma sinensium* has been located in Weishan, Yunnan Province, and Napo, Guangxi Province of China (Hu et al., 2003; Yang et al., 1995). Kruatrachue et al. (1983) isolated *S. sinensium* from the snail *Tricula bollingi* in northwest Thailand. Field rodents and laboratory rabbits are definitive hosts of *S. sinensium*.

Triculine snails (Pomatiopsidae Stimpson, 1865: Triculinae Annandale, 1924) are found in freshwater habitats in southern China and southeast Asia. The Triculinae show high biodiversity and major adaptive radiation in Yunnan/Sichuan Province, southwest China, the lower Mekong Basin, and Hunan Province, China (Attwood et al., 2002a).

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Table 1
Sequence analyses of *Schistosoma sinensium* and *Tricula* sp. LF.

Species	Locus	Primer sequence	Authors	length(bp)
Schistosoma sinensium	18S	5′- ACAGGTGTCGATGGGTTAATGA -3′ 5′- GTAAAAACTGGCACCGCACC -3′		798
	12S	5′-TTTGTCCACAGTTATAACTGAAAGG -3′ 5′- GATTCTTCAAGCACTACCATGTTACGAC -3′	Bowles et al. (1993)	331
	CO1	5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ 5′-TAAAGAAAGAACATAATGAAAATG-3	Bowles et al. (1993)	402
Tricula sp. LF	16S	5′- CGCCTGTTTATCAAAAACAT -3′ 5′- CCGGTCTGAACTCAGATCACGT -3′	Palumbi. (1996)	516
	28S	5′-GTTAGACTCCTTGGTCCGTG-3′ 5′- ACCTCAGATCGGACGAGATTAC-3′	Wade and Mordan. (2000)	764

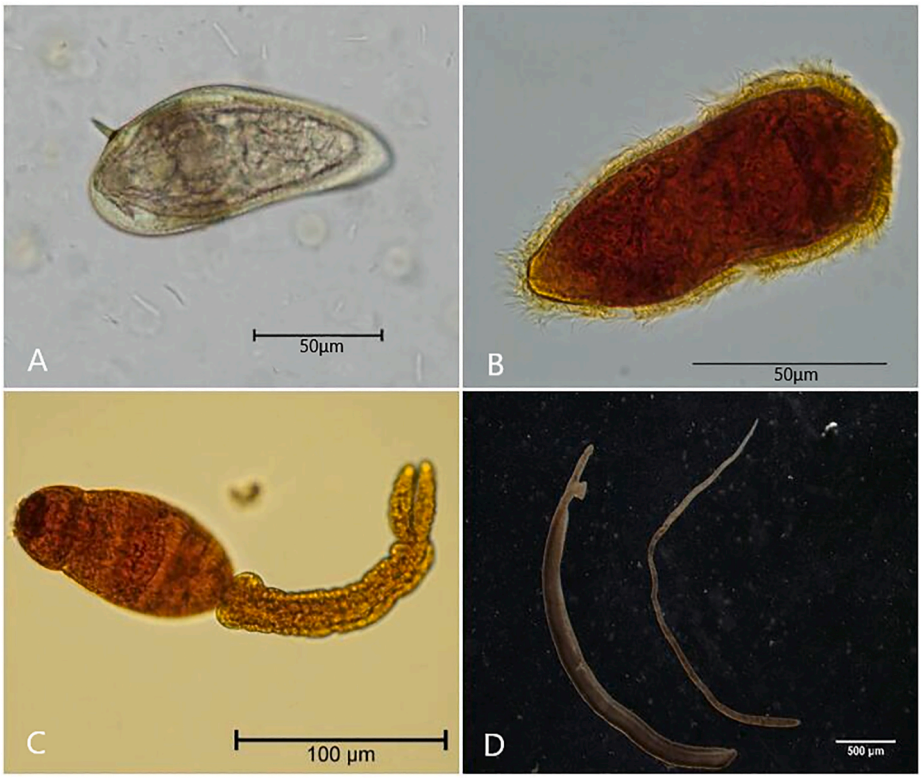

Fig. 1. Morphology of *S. sinensium* at different life cycle stages. Miracidia and cercariae were stained with iodine. **A.** Egg. **B.** Miracidium. **C.** Cercaria. **D.** Male and female adult worms.

Tricula bollingi (Kruatrachue et al., 1983), and *T. hortensis* (Attwood et al., 2003) were intermediate hosts of *S. sinensium*.

The northern tree shrew (*Tupaia belangeri*) is a small mammals closely related to primates and highly similar to humans in terms of anatomy and physiology, neural development, and responses to viral infection and psychological stress (Xu et al., 2013). These animals live in tropical and subtropical jungles and are widely distributed in South and Southeast Asia and Southwest China. Tree shrews have been proposed as experimental models for biomedical research (Xu et al., 2012).

In August 2016, schistosome eggs were found in the feces of a female *Tupaia belangeri* in Lufeng County, Yunnan Province, China. Egg morphology was similar to that of *Schistosoma mansoni*, with a long lateral spine. Subsequently, two other cases were observed. Adult worms were recovered from the veins of a deceased *Tupaia belangeri* that had shed eggs. The worms were identified as *S. sinensium* based on morphology and 18S, 12S and CO1 genes sequencing. The snail intermediate host, *Tricula* sp., was found in the same geographic location, and named *Tricula* sp. LF (LF is the abbreviation of Lufeng). Subsequently, infection experiments were performed to verify that the snail and *Tupaia belangeri* can act as hosts of *S. sinensium*. This study reports that *Tupaia belangeri* and a novel *Tricula* sp. LF were infected with *S. sinensium* in nature. These findings help understand the evolution and distribution pattern of *S. sinensium*.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Animal Care and Welfare Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College. All procedures were performed according to ethical standards and practices.

2.2. Geographic location and ecological environment

Tupaia belangeri specimens were collected at the outskirts of Qinfengying Town, Lufeng County, Yunnan Province, China (latitude, 25°04′50″ N; longitude, 102°19′30″ E; altitude, 1820 m). Freshwater snails resembling Pomatiopsidae were collected from flowing streams in irrigation canals in the same area where the animals were trapped. The

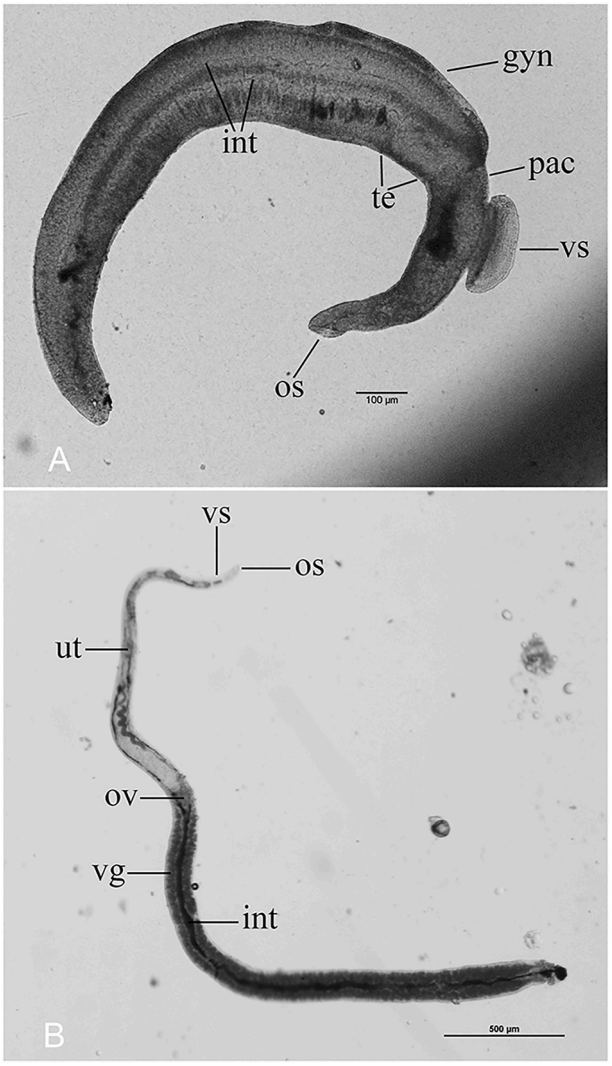

Fig. 2. Enlarged worm picture. **A.** Male, showing oral sucker (os), ventral sucker (vs), constriction (pac), testes (te), gut caeca (int) and gynecophoral canal (gyn). **B.** Female, showing oral sucker (os), ventral sucker (vs), utero (ut), ovary(ov), vitellarium (vg) and gut caeca (int).

water was clear, and the bottom sediment consisted of silt. This snail species lives mainly on stones, sediments, and floating leaves and branches of decaying plants. Other snail species of genera *Gyraulus* and *Radix* were also found in the same ecological environment.

2.3. Morphological and molecular identification of schistosomes

Infected animals were euthanized with excess sodium pentobarbital and adult worms recovered by perfusion. Eggs, miracidia, and cercariae were observed under a light microscope. A total of 100 mature eggs were measured (Nikon 50i, NIKON, Tokyo, Japan). Data are presented as ranges. Measurements of miracidia, cercariae, and worms were made using a stereoscope (Nikon SMZ 1500, NIKON, Tokyo, Japan).

Fresh fecal samples were sieved and washed with saline, and schistosome eggs were allowed to hatch in freshwater. Miracidia were collected using a pipette. Total DNA was extracted from 70 miracidia using a PureLink® Genomic DNA Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Primers were designed using Primer Premier version 6.0 (http://www.premierbiosoft.com/primerdesign/index.html) based on the *S. sinensium* 18S small-subunit ribosomal RNA gene (GenBank Accession No. AY157225.1), 12S and CO1 primers quoted in Bowles (Bowles et al., 1993), (Table 1), and PCR was carried out using a thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification products were sequenced and compared with GenBank sequences using BLAST. Phylogenetic trees were constructed to analyze evolutionary relationships. Highly similar sequences were selected for multiple sequence alignment using ClustalW (Thompson et al., 1994). Phylogenetic analysis was performed using the maximum likelihood method (ML) based on the Kimura 2-parameter model (Kimura, 1980), in addition, the evolutionary history was inferred using the maximum parsimony method (MP) in MEGA version 7.0.14 (Kumar et al., 2016). The sequences used in the present study were all from Genbank and selected after sequence alignment.

2.4. Morphological and molecular identification of snails

The morphology of 100 *Tricula* sp. were analyzed using a stereoscope (Nikon SMZ 1500, NIKON, Tokyo, Japan). Radular preparations were done. Counts of radular cusps were determined from scanning electron microscopy (SEM) images (HITACHI SU8100, Japan). These morphological characteristics were compared with those of *Tricula bambooensis* and *T. ludongbini*.

The snails were gently crushed, and the body were separated from the shell. Total genomic DNA was extracted using a Genomic DNA Mini Kit following the manufacturer's instructions (Thermo Fisher Scientific, Carlsbad, CA, USA). The PCR primers were designed by Palumbi (1996) and Wade and Mordan (2000) (Table 1), and PCR was carried out using a thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification products were sequenced, and the results were analyzed by SnapGene Viewer software and aligned by BLAST. Sequences with high similarity were selected for multiple sequence alignment using ClustalW (Thompson et al., 1994). Phylogenetic analyses were performed using the maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980). and the evolutionary history was inferred using the maximum parsimony method in MEGA version 7.0.14 (Kumar et al., 2016).

2.5. Parasite infection experiment

Individual snails were exposed to 5–10 miracidia hatched from eggs of infected tree shrew. Five 5–10-month-old schistosome-free female tree shrews were each infected with approximately 150 cercariae shed by laboratory-reared snails following the protocol described by Greer et al. (1989).

3. Results

3.1. Morphology of schistosomes

Fig. 1 shows the gross appearance of the egg, miracidium, cercaria, and adult.

3.1.1. Male (N = 4) (Fig. 2A)

In this study, the average length of the male worm after 10 weeks was 3113 μm, ranging from 2590–3680 μm, the maximum body width of 218 μm, this is similar the 3200–3650 μm reported by Bao (Pao, 1959). Males appeared to have a smooth tegument, lacked tubercles or spines. The diameters of the oral and ventral suckers were 73–90 μm, and 147–248 μm respectively. The testes were 8 in number, and it were often found to overlap to some degree. The intestinal bifurcation in our isolate occurred just anterior to the ventral sucker, then reunite near the end of the worm. The constriction just posterior to the ventral sucker was most pronounced and similar to Attwood et al. (2002b).

3.1.2. Female (N = 3) (Fig. 2B)

The average length of the female worm after 10 weeks was 3787 μm, ranging from 3320–4170 μm, the mean maximum body width of 112 μm, similar to that recorded by Attwood et al. (2002b). The diameters of the oral and ventral suckers were 21–23 μm, and 43–46 μm respectively.

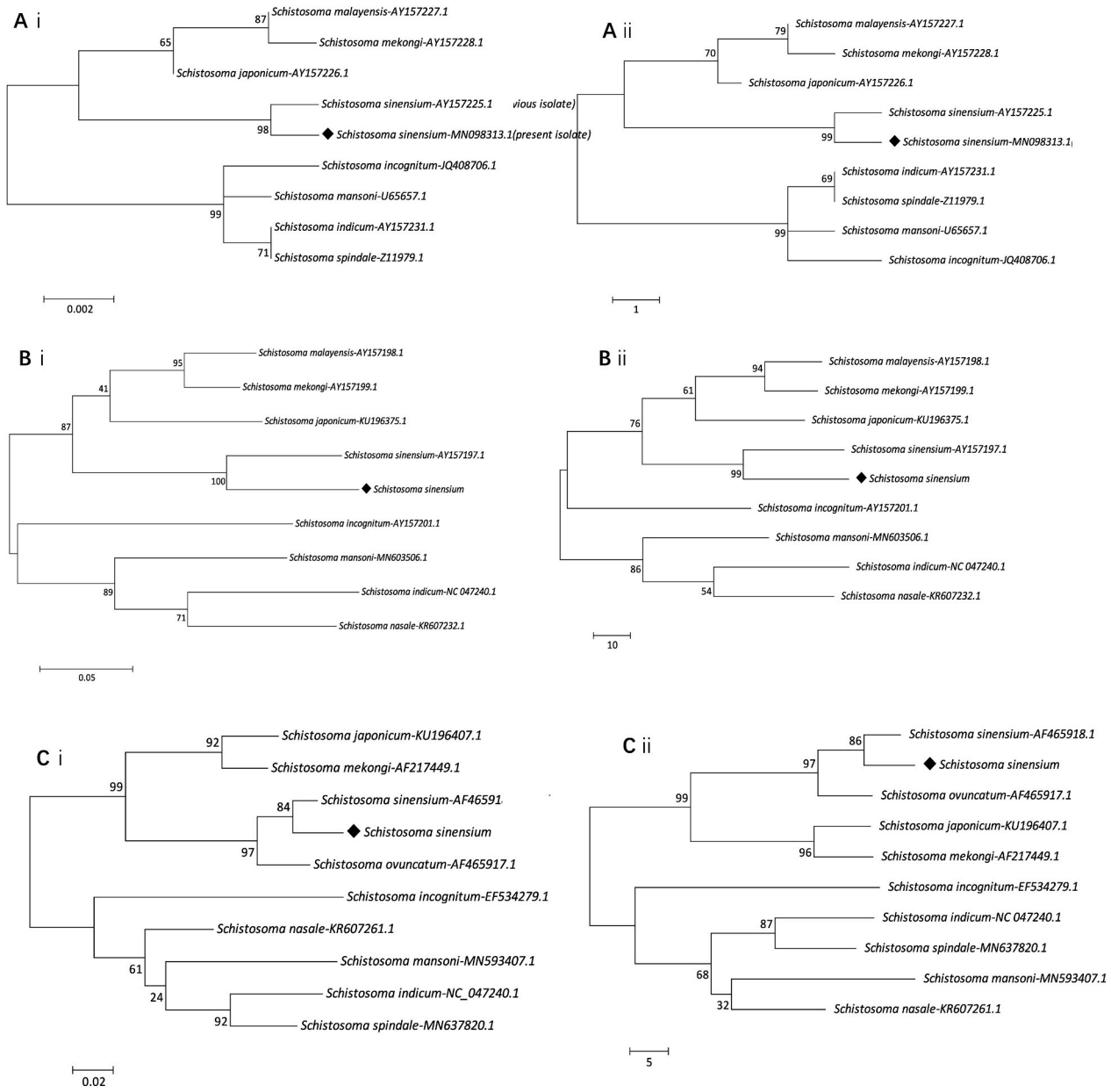

Fig. 3. Molecular phylogenetic analyses of the present *Schistosoma sinensium* (◆) by the maximum likelihood method (ML) and maximum parsimony method (MP). Phylogenetic tree depicting relationships among *Schistosoma* species inferred from:
A. 18S nucleotide data analyses using ML (Ai) and MP (Aii).
[*Schistosoma malayensis AY157227.1, Schistosoma mekongi AY157228.1, Schistosoma sinensium AY157225.1, Schistosoma indicum AY157231.1* and *Schistosoma japonicum AY157226.1* were cited from Lockyer et al. (2003); *Schistosoma incognitum JQ408706.1* was cited from Webster and Littlewood (2012); *Schistosoma.spindale Z11979.1* was cited from Johnston et al. (1993)].
B. CO1 nucleotide data analyses using ML (Bi) and MP (Bii).
[*Schistosoma malayensis AY157198.1, Schistosoma mekongi AY157199.1, Schistosoma sinensium AY157197.1* and *Schistosoma incognitum AY157201.1* were cited from Lockyer et al. (2003); *Schistosoma indicum NC_047240.1* was cited from Jones et al. (2020); *Schistosoma nasale KR607232.1* was cited from Devkota et al. (2015)].
C.12S nucleotide data analyses using ML (Ci) and MP (Cii).
[*Schistosoma mekongi AF217449.1* was cited from Le et al. (2000); *Schistosoma sinensium AF465918.1* and *Schistosoma ovuncatum AF465917.1* were cited from Attwood et al. (2002a); *Schistosoma incognitum EF534279.1* was cited from Attwood et al. (2007); *Schistosoma nasale KR607261.1* was cited from Devkota et al. (2015); *Schistosoma mansoni MN593407.1* was cited from Catalano et al. (2020); *Schistosoma indicum NC_047240.1* and *Schistosoma spindale MN637820.1* were cited from Jones et al. (2020)].
Other sequences were from GenBank.

Tegument smooth, lacking tubercles or spines. In worm, the posterior extremity was broader. The ovary was located in the front 1/3 of the worm. The vitellarium accounts for a large proportion, extending from behind the ovary to the posterior end of the worm. The intestines with obvious black substance bifurcated in the ventral sucker, converged behind the ovary.

3.1.3. Egg (N = 100) (Fig. 1A)

Eggs were elongated-oval and presented a lateral spine, with a length of 85.12–117.61 μm, width of 34.21–53.22 μm, and lateral spine length of 15.08–26.02 μm, which agree with previous analyses (Greer et al., 1989; Pao, 1959).

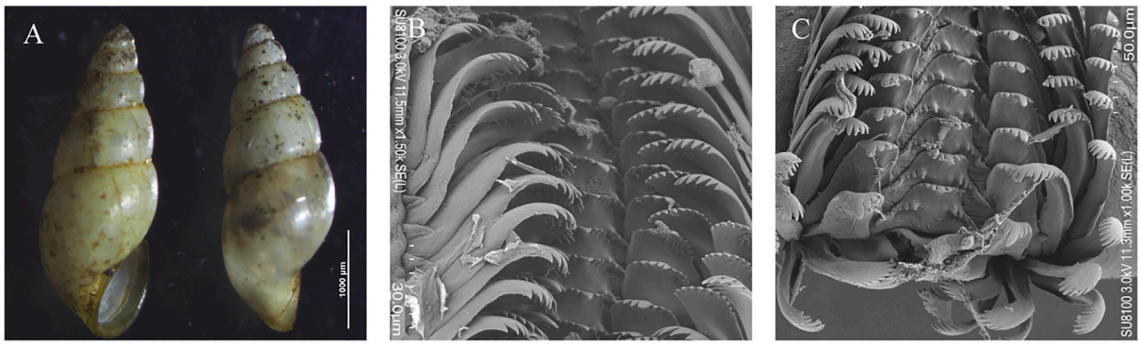

Fig. 4. Shell morphology of *Tricula* sp. LF. **A.** Shape. **B–C.** Scanning electron micrograph of the radula.

Table 2
Geographical location and morphological analyses of *Tricula* sp. LF, *T. bambooensis*, and *T. ludongbini*.

	Tricula sp. LF	*Tricula bambooensis* (Davis et al., 1986)	*Tricula ludongbini* (Davis et al., 1986)
Geographical position	25°04′50″ N, 102°19′30″ E	25°06′N, 99°45′ E	25°06′N, 99°45′ E
Whorls	6	6	5.5–6
Shell length (mm)	2.99-4.27	3.48-4.00	3.08-3.68
Shell width (mm)	1.18-1.66	1.64-1.80	1.48-1.72
Radular teeth formulae	$\frac{3-1-3}{3-3}$; 4 – 1 – 3; 10 – 11; 9 – 11	$\frac{3(2)-1-(2)3}{2(3)-(3)2}$; 4(5) – 1 – 3; 11 – 13; 10 – 12	$\frac{3-1-3}{3-3}$; 3 – 1 – 3; 10 – 12; 10 – 12
Shell shape and appearance	Ovate-turreted; Glassy	Ovate-conical or ovate-turreted; Glassy	Ovate-conical; Chalky

3.1.4. Miracidia (N = 5) (Fig. 1B)

The size of living miracidia varies greatly. The front has a conical protuberance. The body surface was covered with abundant cilia. There are two glandular cells in front of miracidia.

3.1.5. Cercaria (N = 5) (Fig. 1C)

The average body length is 160 μm, width is 66 μm, tail length is 140 μm. Cercariae have eight flame cells and rest on the water surface, these morphology and features are similar to those recorded by Bao (1959) and Attwood et al. (2002b).

3.2. Schistosome phylogeny

18S (Fig. 3A), CO1 (Fig. 3B) and 12S (Fig. 3C) gene sequencing results revealed a high degree of similarity (>99%) with the target sequence. Phylogenetic analysis showed that our sample was similar to another Chinese strain of *S. sinensium*. Two specimens of *S. sinensium* form a clade to the exclusion of all other species. Furthermore, it is a sister group to another Asian schistosome species, and is related to *Schistosoma mansoni*, *S. nasale, S. incognitum, S. spindale,* and *S. indicum*. Better concordance was observed between the phylogenies estimated by the two methods for the 18S, CO1 and 12S loci (Fig. 3).

3.3. Snail morphology

The snails were 2.99–4.27 mm in height and 1.18–1.66 mm in width, with six slightly convex whorls, and ovate-turreted, glassy shells (when clean) with a smooth surface. The sutures were deep. The peristome was complete and had an oval aperture (Fig. 4A). The radular formula was: $\frac{3-1-3}{3-3}$; 4 – 1 – 3; 10 – 11; 9 – 11 (Fig. 4B and C). Shell size and shape, and radular formula were slightly different from those of *T. bambooensis* and *T. ludongbini* (Table 2).

3.4. Snail phylogeny

The analysis of 16S (516bp) and 28S (764bp) gene sequences revealed a high degree of similarity (>90%) with other triculine snails. The obtained sequences were deposited in GenBank under accession numbers MT573225 and MT573250. A phylogenetic tree was constructed and showed that *Tricula* sp. LF formed a clade with *T. bambooensis* and *T. ludongbini* (Fig. 5). Both results of 28S (Figs. 5A) and 16S (Fig. 5B) data were consistent by using the maximum likelihood and maximum parsimony methods. The same basic topology were obtained by using two methods.

3.5. Schistosome infection results

Cercariae were released from infected *Tricula* sp. LF 50 days post-infection. Schistosome eggs were discovered in the feces of all five experimentally infected female tree shrews at 25–45 days post-infection.

4. Discussion

Of 290 wild-caught tree shrews, three (1.03%) were infected with *S. sinensium*, and the morphology and 18S, 12S and CO1 gene sequences of these specimens were analyzed.

Schistosoma classification is based on egg morphology and intermediate host specificity. Egg morphology is an important conserved characteristics of schistosomes. In the present study, egg size and shape, and the presence of lateral spines agreed with a previous study. Moreover, adults lack tegumental tubercles, and cercariae have eight flame cells and rest on the water surface. Display of worms are similar to *S. sinensium* previous recorded (Pao, 1959; Attwood et al., 2002b). Further research shows that the cercariae shed from laboratory-reared snails can reinfect captive-bred tree shrews in the laboratory. In addition, considering rodents act as definitive host of *S. sinensium* based on the initial report, we tried to infect two ICR mice in the laboratory. *Schistosoma* worms were recovered from the exposed mice. This result is expected as the ancestral host of the *S. sinensium* group is thought to be a rodent. The worm size obtained from the mice at the same time post-exposure was slightly smaller than that obtained in the tree shrews; these data are not to be reported elsewhere.

The 18S rDNA,12S rDNA and cytochrome oxidase subunit 1 (CO1) genes are commonly used for schistosome identification. In the present study, the three loci sequencing results revealed a high degree of similarity with the related genes of *S. sinensium*. Phylogenetic analysis by using the maximum likelihood method and maximum parsimony method, that showed that our sample was highly similar to another Chinese strain of *S. sinensium* and was a sister group to other Asian schistosome species, such as *S. ovuncatum*, *S. mekongi, S. malayensis* and

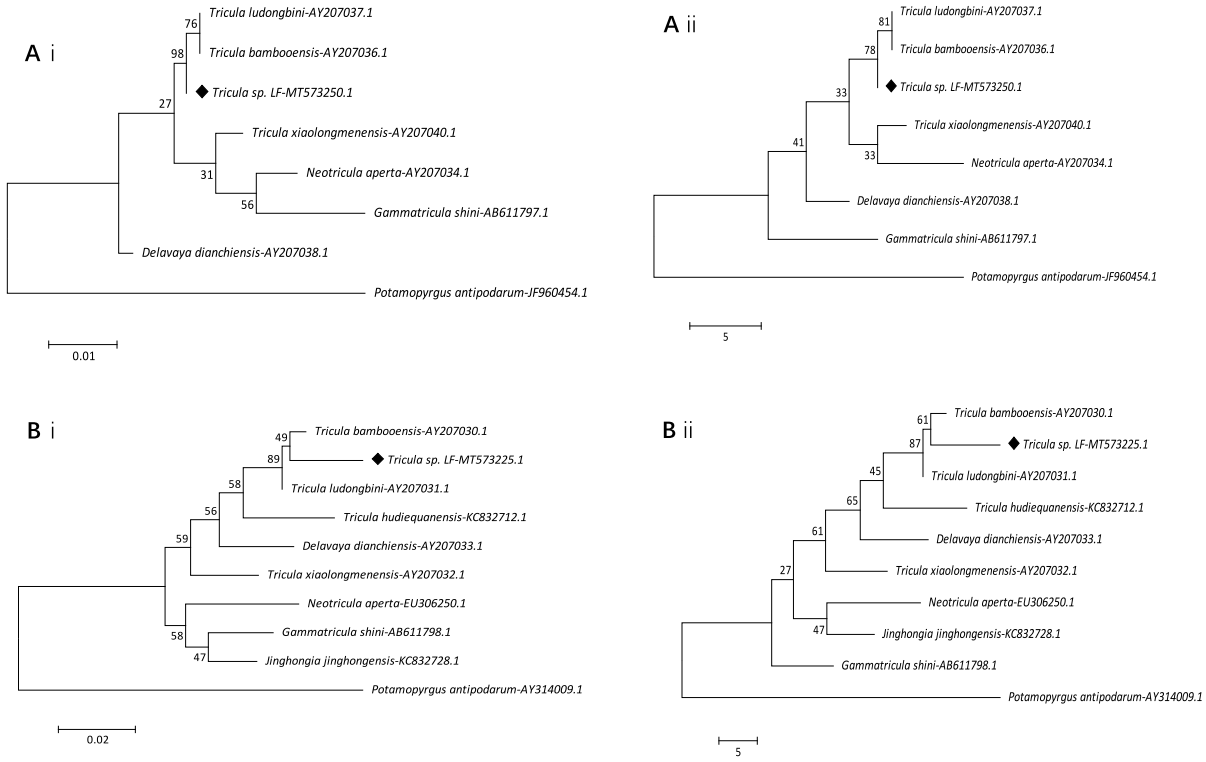

Fig. 5. Molecular phylogenetic analyses of the *Tricula* sp. LF (◆) by the maximum likelihood method (ML) and maximum parsimony method (MP). Phylogenetic tree depicting relationships among *Tricula* species inferred from:
A. 28S nucleotide data analyses using ML (Ai) and MP (Aii).
[*Tricula ludongbini AY207037.1, Tricula bambooensis AY207036.1, Tricula xiaolongmenensis AY207040.1, Neotricula aperta AY207034.1* and *Delavaya dianchiensis AY207038.1* were cited from Attwood et al. (2004); *Gammatricula shini AB611797.1* was cited from Kameda and Kato (2011)].
Outgroup taxon *Potamopyrgus antipodarum-JF960454.1*.
B. 16S nucleotide data analyses using ML (Bi) and MP (Bii).
[*Tricula ludongbini AY207031.1, Tricula bambooensis AY207030.1, Delavaya dianchiensis AY207033.1* and *Tricula xiaolongmenensis AY207032.1* were cited from Attwood et al. (2004); *Tricula hudiequanensis KC832712.1* and *Jinghongia jinghongensis KC832728.1* were cited from Liu et al. (2014); *Neotricula aperta EU306250.1* was cited from Attwood et al. (2008); *Gammatricula shini AB611798.1* was cited from Kameda and Kato (2011)].
Outgroup taxon *Potamopyrgus antipodarum-AY314009.1*.
Other sequences were from GenBank.

S. japonicum. Furthermore, our sample was related to *S. mansoni, S. nasale, S. incognitum, S. spindale,* and *S. indicum*. The results are similar to those previous studies (Attwood et al., 2002a). *S. ovuncatum* was considered to be the closest known relative of *S. sinensium,* which was first collected by Baidikul et al. (1984) in northwest Thailand and described by Attwood et al. (2002b). However, the eggs of *S. ovuncatum* are significantly smaller than those of *S. sinensium*, with a length of 70 (65–80) μm, width of 45 (40–45) μm, and spine length of 5 μm. Only the *S. ovuncatum* 12S sequence was analyzed in phylogene due to the limited data from GenBank. Sequence alignment between the 18S sequence of *S. ovuncatum* (GenBank accession no. AF465929.1) and our sample was performed using DNAMEN version 6, and the results showed very low overlop.

The geographical distribution of schistosomes depends on the distribution of snail intermediate hosts (Colley et al., 2014). *Schistosoma sinensium* is transmitted by freshwater *Tricula* snails (Pomatiopsidae: Triculinae). A previous study indicated that triculine snails originated in the highlands of Tibet and Yunnan (Liu et al., 2014). Yunnan Province is located in southwest China and has different biomes, including tropical and subtropical forests. *Tricula* is extensively distributed and abundant in this area (Attwood et al., 2004; Davis et al., 1986). Davis predicted that new species of snails would be discovered in Asia, and these snails would be species of *Tricula* or close relatives (Davis, 1980). In this report, the location where we found the *Tricula* snail is close to previous reported distribution area (Attwood et al., 2004).

The morphology of the shell and radula serves as the basis for classifying mollusks, this is evidenced by DNA-sequence-based phylogenies (partial nuclear and mitochondrial genes). Comparative studies were performed to improve identification. The results showed that there were slight differences in shell and radula. *Tricula* sp. LF shows ovate-turreted on shell shape. The shell is longer than *T. bambooensis* and *T. ludongbini* and narrower than both, the radular formula is difference that represent fewer number of inner and outer margin teeth. Morphology is closer *T. bambooensis* than *T. ludongbini* (Table 2). Moreover, 16S and 28S genes sequencing data suggest that *Tricula* sp. LF collected in our study site (25°04′ N, 102°19′ E) is a sister species of *T. bambooensis* and *T. ludongbini* collected from another site (25°06′ N, 99°45′ E) (Davis et al., 1986). This result confirms that the newly described pomatiopsid is a member of the genus *Tricula* (Triculinae). In addition, this study is the first to perform a phylogenetic analysis of *Tricula* sp. LF using 16S and 28S data.

The factors driving host diversity and co-evolution with *Schistosoma* are not fully understood (Liu et al., 2014). The reasons why the host range of *S. sinensium* has expanded, and whether this parasite has co-evolved with snails over a long period, more work is required in the future. These discoveries may help elucidate the evolution of Asian *Schistosoma* and the phylogeography of triculine snails.

5. Conclusions

We report for the first time that *Tupaia belangeri* (Tupaiidae) is a natural definitive host for *Schistosoma sinensium*, and this relationship was first identified in Lufeng, Yunnan Province, China (latitude, 25°04′50″ N; longitude, 102°19′30″ E; altitude, 1820 m). The snail intermediate host, *Tricula.* sp. LF, was also found in this area. The parasites and snail intermediate host were described, partial DNA sequences were used to estimate phylogenies. These findings may improve our understanding of the distribution and historical biogeography of *S. sinensium* and its co-evolution with snails.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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