

A NEW SPECIES OF BLOOD FLUKE (DIGENEA: SPIRORCHIIDAE) FROM THE MALAYAN BOX TURTLE, *CUORA AMBOINENSIS* (CRYPTODIRA: GEOMYDIDAE) IN THAILAND

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ABSTRACT: *Spirhapalum siamensis* n. sp. obtained from the heart of the Malayan box turtle, *Cuora amboinensis*, from western Thailand is described. This blood fluke differs from the 2 previously known species of *Spirhapalum* in its larger overall body size, constricted mid-body, greater number of pre-ovarian testes, ceca that loop anteriorly from the cecal bifurcation before turning to the posterior, and the relatively smaller size of its ventral sucker. A phylogenetic analysis using molecular characters places the new species in the basal clade of tetrapod blood flukes, more derived than *Spirhapalum polesianum*, but less derived than *Spirorchis* spp.

Estimates of the percentage of turtle species that have been examined for spirorchiid blood flukes range between 15% (Smith, 1997) and 25% (Platt, 1992). It is clear, however, that even turtle species that have been commonly reported as spirorchiid hosts can harbor novel species of blood flukes (e.g., Platt, 1988, 1990). The Malayan box turtle, *C. amboinensis* (Daudin, 1802), is the type host of *Spirhapalum elongatum* Rohde, Lee, and Lim 1968, the second recognized species of this genus. Herein, we describe a third species of *Spirhapalum* from *C. amboinensis* in Thailand.

MATERIALS AND METHODS

In April 2004, the internal organs of *C. amboinensis* were recovered from a turtle butchered for food in Mae Sot, Thailand. Specimens of a new digenean species belonging to *Spirhapalum* were recovered from the heart of this turtle. Live worms were rinsed in saline, killed with hot water, and fixed in 70% ethanol. Specimens were stained with aqueous alum carmine or Mayer's hematoxylin, dehydrated in a graded ethanol series, cleared in methyl salicylate or clove oil, and mounted permanently in Damar balsam.

Measurements were taken using a compound microscope using digital imaging and Rincon measurement software (v. 7.1.2, Imaging Planet, Goleta, California). Mean, standard deviation, and coefficient of variation (CV) were calculated according to Steel and Torrie (1980). The CV is a percentage value of the ratio of the standard deviation to the mean of a particular metric character. Characters with lower CV have values that are more stable around the mean than are those with higher CV.

Sequences of DNA of spirorchiid species were taken from GenBank for use in a phylogenetic analysis to include the new species: *Spirorchis artericola* (AY604712, AY604704), *Spirhapalum polesianum* (AY604713, AY604705), *Unicaecum* sp. (AY604719, AY604711), and *Vasotrema robustum* (AY604714, AY604706). Outgroup sequences were taken from GenBank and included a clinostomid, *Clinostomum* sp. (AY222176, AY222095), a diplostomid, *Alaria alata* (AF184263, AY222091), and a leucochloridiid, *Leucochloridium perturbatum* (AY222169, AY222087).

Genomic DNA for molecular analysis was isolated from 1 specimen of the new species and from 1 *Spirorchis haematobius* (Stunkard, 1922) (ex *Chelydra serpentina*, Fremont County, Iowa) according to Tkach and Pawlowski (1999). A fragment of a single worm was used for each DNA extraction upon preliminary morphological identification. DNA fragments of approximately 3,300 base pairs and spanning the entire 18S nuclear rDNA and 5' end of the 28S gene (including variable domains D1–D3) were amplified and sequenced according to Snyder

(2004). Contiguous sequences were assembled and edited using Sequencher (GeneCodes Corp., ver. 4.1.4) and submitted to GenBank: *S. haematobius* (FJ481163, FJ481165), *Spirhapalum siamensis* n. sp. (FJ481165, FJ481166). A voucher specimen of *S. haematobius* was deposited in the Harold W. Manter Laboratory (HWML 49030).

Sequences were initially aligned using Clustal W as implemented in the BioEdit program, version 7.0.1 (Hall, 1999) and manually refined using BioEdit. Ambiguously aligned bases were excluded from the analysis. Maximum parsimony analysis of these data was performed using the exhaustive search, random sequence addition, and TBR branch-swapping options of PAUP* (v. 4.0b10) (Swofford, D.L., 2001. PAUP*: Phylogenetic Analysis Using Parsimony [and other methods]), Version 4.0b10. Sinauer, Sunderland, Massachusetts). Gaps were treated as missing data and characters were unordered with equal weight. Nodal support was assessed using bootstrap resampling (Felsenstein, 1985) (1,000 bootstrap replicates, 100 heuristic searches per replicate).

Measurements of the holotype are given in the text; measurements of the entire type series are given in Table I. Measurements are given in μm .

DESCRIPTION

Spirhapalum siamensis n. sp.

(Fig. 1)

Diagnosis: Based on 4 complete and 3 partial adult specimens. Body elongate, 4,410; divided into distinct forebody and hind body by long constriction at level of ventral sucker. Forebody 571 wide, 352 at level of ventral sucker. Tegument rugose, with numerous papillae; papillae longest and most dense in middle part of body. Oral sucker, 124×96 . Esophagus surrounded by glandular tissue, sinuous, 470 long. Plicate organ surrounding posterior region of esophagus 87×150 . Cecal bifurcation 565 from anterior end of body. Cecal loop anteriorly from cecal bifurcation before turning posteriorly. Cecal straight to slightly sinuous, extending to near posterior body end. Ventral sucker round, smaller than oral sucker, 50×56 , 1,580 from anterior body end, 2,560 to posterior body end.

Testes 13, intercecal, 12 pre-ovarian, 1 post-ovarian, irregular in shape, sometimes strongly lobed. Pre-ovarian testes usually with overlapping posterior and anterior margins. Anterior-most testis 519 posterior of ventral sucker. Posterior-most pre-ovarian testis 765 from posterior end. Post-ovarian testis posterior to seminal receptacle, near posterior body end. Short seminal ducts from each anterior testis join common seminal duct running ventrally from anterior-most testis to cirrus sac. Seminal duct from posterior testis joins external seminal vesicle at short distance from proximal end of cirrus sac. External seminal vesicle elongate, situated between posterior-most pre-ovarian testis and cirrus sac, ventral to ovary, often overlapping sinistral margin of ovary and posterior-most pre-ovarian testis. Cirrus sac thick-walled, length 164, width at base 58, sinistral to ovary, containing small internal seminal vesicle, pars prostatica and ejaculatory duct. Proximal margin of cirrus sac originating posterior to seminal vesicle at approximate midpoint of ovary. Cirrus sac originates dorsally and runs ventro-posteriorly toward sinistral margin, displacing left cecum. Cirrus sac terminates between ceca in ventral, sinistral common genital pore.

Ovary immediately posterior to testicular column, deeply lobed, 302×194 , intercecal, displacing ceca. Short oviduct originates from posterior lobe of ovary and forms oviductal seminal receptacle. Seminal

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TABLE I. Metric data for *Spirhpalum siamensis* n. sp.

Characters	n	Min-Max	Mean	SD	CV
Body length	4	3,336–4,489	4,082.0	517.8	12.7
Anterior end to ventral sucker	5	1,420–1,811	1,560.2	159.5	10.2
Ventral sucker to posterior end	4	1,913–2,922	2,486.8	419.6	16.9
Forebody width	4	470–850	617.2	146.1	23.7
Body width at ventral sucker	4	270–381	318.3	57.0	17.9
Oral sucker length	6	106–131	122.0	8.5	6.9
Oral sucker width	6	80–103	92.5	8.2	8.9
Plicate organ length	6	65–90	83.5	9.4	11.3
Plicate organ width	6	110–154	129.2	19.0	14.7
Esophagus	6	385–494	457.8	38.0	8.3
Cecal bifurcation to anterior end	5	510–707	576.4	75.8	13.2
Anterior testis to ventral sucker	5	328–580	449.6	110.1	24.5
Ventral sucker length	6	50–65	57.3	5.7	9.9
Ventral sucker width	6	42–70	54.8	9.4	17.2
Cirrus sac length	6	113–211	176.2	34.6	19.6
Cirrus sac width at base	7	52–64	58.7	4.8	8.2
Vitelline field-anterior end	5	407–494	445.2	33.2	7.5
Ovary length	7	196–354	292.9	58.3	19.9
Ovary width	7	171–245	208.4	27.4	13.2
Seminal receptacle length	5	43–106	76.4	30.0	39.3
Seminal receptacle width	5	39–88	60.8	23.0	37.9
Posterior pre-ovarian testis to posterior end	6	495–966	760.3	163.0	21.4

CV, coefficient of variation; Min-Max, minimum-maximum; SD, standard deviation.

receptacle 45 × 83, tubular, elongate, dextral, between ovary and post-ovarian testis. Ootype at level or slightly anterior and sinistral to vitelline reservoir. Very short uterus opens into common genital pore. Laurer's canal opens dorsal to posterior testis. Vitellarium consisting of numerous, irregularly shaped, densely packed follicles forming 2 lateral fields extending from approximate level of cecal bifurcation to posterior end. Follicular density greatest in forebody and hindbody, only scattered follicles surround ceca in mid-body constriction. Follicles in forebody and hindbody extend from lateral body margin to just medial of ceca, barely overlapping reproductive organs in hindbody. Vitelline fields merge posterior to post-ovarian testis. Sinistral and dextral common vitelline ducts merge to form transverse median vitelline reservoir situated between ovary and post-ovarian testis. Egg observed in 1 paratype, 84 long.

Excretory bladder V-shaped, with short arms reaching only posterior ends of ceca; excretory ducts originate at anterior margins of bladder arms. Excretory pore terminal.

Taxonomic summary

Type host: *Cuora amboinensis* (Cryptodira: Geomydidae) (Malayan box turtle).

Type locality: Mae Sot, Thailand; 16°42'N, 98°34'E.

Site of infection: Heart, between atria and ventricle.

Prevalence and intensity of infection: One turtle was infected with 8 specimens.

Specimens deposited: Holotype: Harold W. Manter Laboratory no. HWML49027.

Paratypes: Harold W. Manter Laboratory nos. HWML49028–49029. All labeled identically: ex *Cuora amboinensis*, Mae Sot, Thailand, April 2004. Coll. V. Tkach.

Etymology: The species refers to the former name of Thailand, Siam.

Remarks

Spirhpalum siamensis n. sp. differs from the 2 previously described species of *Spirhpalum* Ejsmont, 1927 in having a larger number of pre-ovarian testes, with 12 in the new species versus 4–6 (Platt, 2002) in the type species, *S. polesianum*, and 6 (Rohde et al., 1968) in *S. elongatum*. The new species also possesses ceca that loop anteriorly from the cecal bifurcation before turning posteriorly. The other members of the genus are characterized by a T-shaped cecal bifurcation

(Platt, 2002). The new species is considerably longer (mean length = 4,082 µm) than *S. elongatum* (mean length = 1,530 µm) (Rohde et al., 1968) and *S. polesianum*, the longest of which is 2,000 µm (Ejsmont, 1927). Neither of the 2 previously named species has the lateral constriction characteristic of *S. siamensis* n. sp. In addition, the ventral sucker in *S. elongatum* and *S. polesianum* is equal to, or slightly larger than, the oral sucker (Ejsmont, 1927; Rohde et al., 1968), while the ventral sucker is distinctly smaller than the oral sucker in the new species (Table I).

DISCUSSION

A single most parsimonious tree was produced by analysis of the combined SSU and LSU DNA data (Fig. 2). The clade of *Vasotrema robustum* (Stunkard, 1928), *S. polesianum*, *S. siamensis*, and 2 species of *Spirorchis* MacCallum, 1919 is strongly supported by bootstrap analysis. An independent phylogenetic analysis not reported here supported the overall topology of Snyder (2004) and the position of the *Vasotrema* Stunkard 1926, *Spirhpalum*, *Spirorchis* clade as basal among tetrapod blood flukes. In a morphology-based phylogeny, Platt (1992) found *Spirhpalum* to be basal to both the Indian *Plasmiorchis* (Mehra, 1934) and the North American *Spirorchis*. *Plasmiorchis* was not available for the current study, but both *Spirhpalum* and *Plasmiorchis* possess a ventral sucker, as does the basal *Vasotrema* (Fig. 2) of the current study. Both studies suggest that the loss of a ventral sucker is a derived trait of *Spirorchis*.

Although the 2 species of *Spirorchis* used in the current examination are sister to one another (Fig. 2), the 2 species of *Spirhpalum* are not. *Spirhpalum polesianum* is basal to *S. siamensis*, which is, in turn, basal to the *Spirorchis* clade. The phylogenetic position of *S. siamensis* first suggested to us that this species represents a novel genus. However, morphological examination of these worms provided no novel generic-level

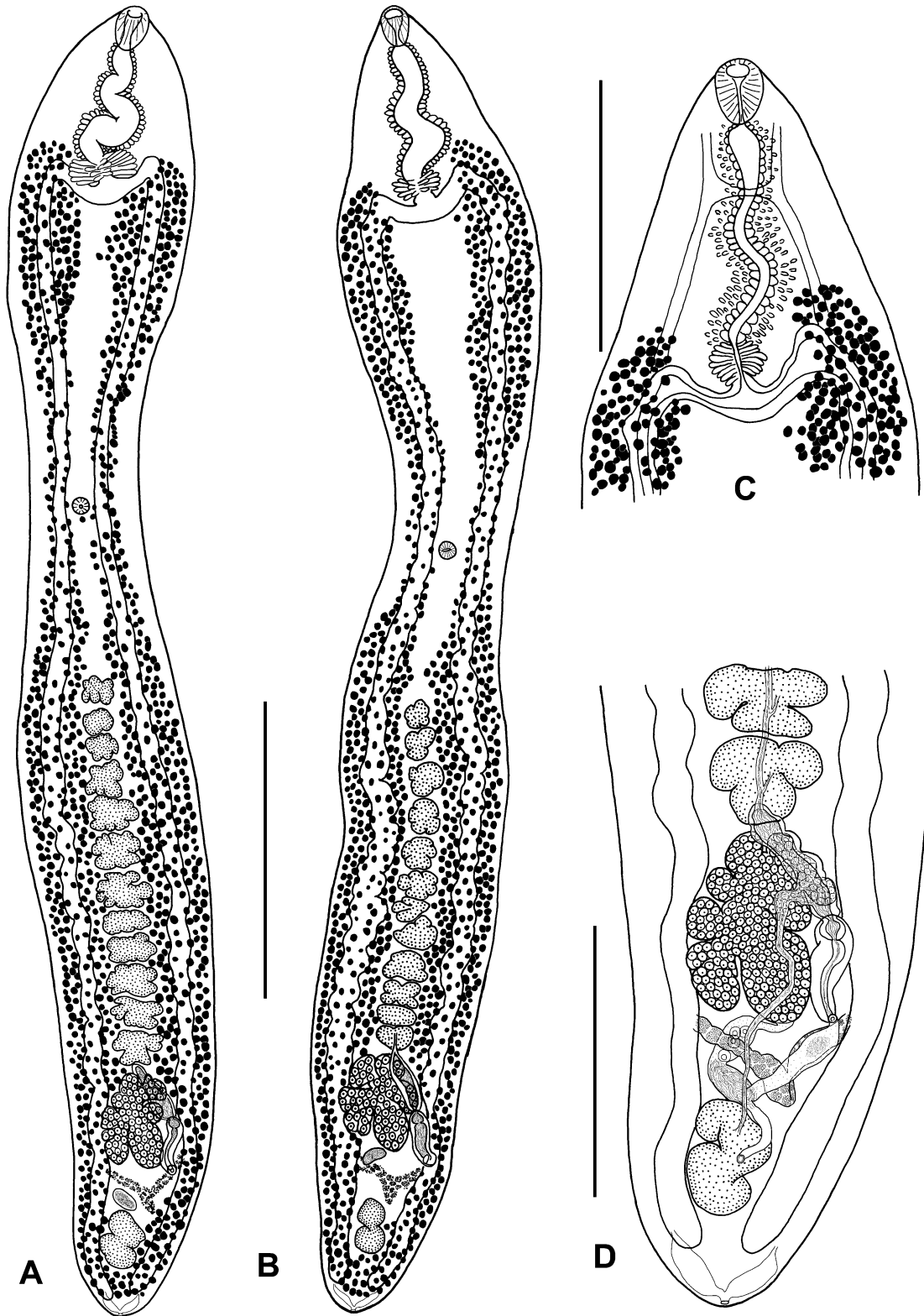


FIGURE 1. *Spirhpalum siamensis* n. sp. (A) Ventral view of holotype. (B) Ventral view of a paratype. (C) Anterior end of a paratype. (D). Posterior end of holotype. Scale bars: A, B = 1,000 μ m, C, D = 500 μ m.

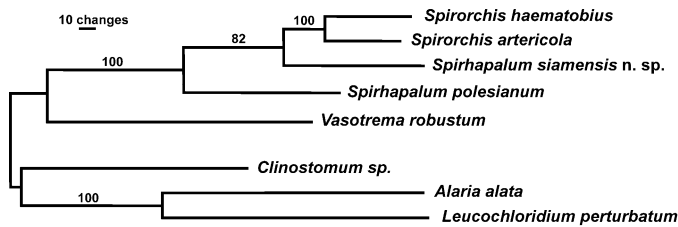


FIGURE 2. Phylogenetic tree showing relationships among *S. siamensis* n. sp. and other taxa of blood flukes. Numbers above nodes show bootstrap support from maximum parsimony analysis in PAUP (1,000 bootstrap replicates, 100 heuristic searches per replicate).

characters. The ceca loop anteriorly in the new species is a characteristic of the closely related *Plasmiorchis* (see Platt, 1992), but members of *Plasmiorchis* do not possess the post-ovarian testis characteristic of *Spirhpalum*. The new species is much larger than its congeners, but body size is variable within most digenean genera and the greater number of testes in *S. siamensis* n. sp. compared with other members of the genus does not, of itself, suggest elevation to generic status. In fact, testis number in *Spirorchis* ranges from 5 to 10, and both morphological (Platt, 1993) and molecular (S. Snyder, unpubl. obs.) data support the monophyly of this genus. The paraphyletic nature of *Spirhpalum* in the current molecular phylogeny is not supported by any substantial differences in morphological characters and may reflect insufficient taxon sampling within the genus.

Spirhpalum siamensis is the second species of the genus reported from the geomydid (=batagurid) turtle, *C. amboinensis*. *Spirhpalum elongatum* was reported from these turtles in central-western Malaysia (Rohde et al., 1968), approximately 1,500 km from the type locality of the new species. *Spirhpalum elongatum* inhabits the mesenteric arteries of the host (Rohde et al., 1968) and *S. polesianum* was recovered from the heart (Ejmsmont, 1927). The new species was also found in the heart, extending between the atria and ventricle. Curiously, the mid-body constriction of *S. siamensis*, not found in other members of the genus, is the region of the body that threads through the

atrioventricular valve. Further studies may illustrate whether the positioning of these worms in the heart is consistent across hosts.

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