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Molecular phylogenetic position of *Schistosoma sinensium* in the genus *Schistosoma*

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Abstract

The status of *Schistosoma sinensium* (samples from Thailand and from Sichuan, China) relative to other species of the genus *Schistosoma* was investigated using DNA sequences from the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene (partial) and the nuclear ribosomal DNA second internal transcribed spacer 2 (ITS2). Trees inferred from these sequences place *S. sinensium* as sister to the *S. japonicum* group and suggest a basal position in the clade utilizing snails of the family Pomatiopsidae. The sequence differences between specimens of *S. sinensium* from China and Thailand are at least as great as between *S. malayensis* and *S. mekongi. Schistosoma sinensium* is probably best regarded as a species complex.

Introduction

Schistosoma species have been arranged in groups based on egg morphology, the genus or family of the intermediate host and geographical origin. The *S. japonicum* group in Asia have eggs with a minute spine and develop in operculate snails of the family Pomatiopsidae. On the other hand, species of the *S. mansoni* and *S. haematobium* groups in Africa (and adjacent regions) and South America (*S. mansoni* only) utilize pulmonate snails. Members of the former group have eggs with a lateral spine, while the *S. haematobium* group has eggs with a terminal spine. In general, therefore, the size and shape of the egg, intermediate host specificity and the geographical origin of isolates are sufficient to identify species infecting man.

Schistosoma sinensium was first isolated from a snail in Szechuan Province, China (Pao, 1959) and a relationship with African species was postulated since the species produces eggs with a lateral spine (Davis, 1980, 1992). Greer *et al.* (1989) redescribed the species based on adults and eggs from north Thailand. They noted that *S. sinensium* resembles *S. mansoni* in having only a few eggs present in the uterus and a posterior caecum in males greater than half of the body length. They also noted that *S. sinensium* resembles *S. japonicum* in several characters, among which are the presence of tegumental tubercles in the male, cercarial flame cell number, and intermediate host species. We have already reported a comparative study on *S. sinensium*, especially on the basis of egg morphology (Kawanaka *et al.*, 1998). However, an intensive phylogenetic analysis has not been carried out. The phylogenetic position of *S. sinensium* in the genus

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Schistosoma is the subject of the present paper. To this end, nucleotide sequences of two DNA regions, mitochondrial CO1 (partial) and nuclear ribosomal ITS2 were determined and analysed.

Material and methods

Collection of S. sinensium

Snails infected with *S. sinensium* were collected from the Fang district in Chengmai Province, Thailand (*Tricula bollingi*, F. Pomatiopsidae), where Baidikul *et al.* (1984) have reported on the parasite, and from Dangling, Chengdu, Sichuan Province, China (*Tricula humida*). Mice were exposed to cercariae and adult worms recovered by perfusion about one month post-infection. Adult worms were maintained at −80°C until used.

DNA extraction and PCR

Genomic DNA was extracted from whole worms. Worms were incubated in extraction buffer (Invitrogen extraction kit) containing proteinase K until the tissues were solubilized. The solubilized samples were twice treated with an equal volume of phenol (approximately pH 8.0), and then treated with an equal volume of chloroform. The extracted DNAs were ethanol-precipitated. ITS2 and CO1 regions were amplified using the polymerase chain reaction (PCR). The PCR conditions were as follows: 94°C for 1 min, 50°C for 2 min, 72°C for 3 min, for 30 cycles. Amplification reactions were performed in a final volume of 50 μl containing primers (3.2 pmol), deoxynucleoside triphosphates (dNTPs, 0.2 mM), and Taq polymerase (1.75 U/reaction). Primers for the ITS2 are 5′-CGG TGG ATC ACT CGG CTC GT-3′ (3S, forward direction) and 5′ -CCT GGT TAG TTT CTT TTC CTC CGC-3′ (A28, reverse direction) (Bowles *et al.*, 1995), and for the CO1 region are 5′-TTT TTT GGG CAT CCT GAG GTT TA-3′ (FH3, forward direction) and 5′-TAA AGA AAG AAC ATA ATG AAA ATA ATC-3′ (FH5, reverse direction) (Bowles *et al.*, 1993). The PCR products were treated with chloroform and purified using Micro-Spin Columns (Pharmacia Biotech). The purified DNA was precipitated with ethanol, resuspended in 20 μl of distilled water and aliquots sequenced using the Bigdye kit (ABI). Polymerase chain reaction primers were used as sequencing primers. The reactions were purified according to the manufacturer's instructions (ABI) and applied to an ABI sequencer (373A).

Alignment and tree analysis

Analyses for multiple sequence alignments were done using the programs CLUSTAL V (Higgins *et al.*, 1992) and GENETYXMAC ver. 6.0 (Software Development Co., Tokyo, Japan). Codon usage was derived from a report of Blair *et al.* (1999a) in DNASIS ver. 3.2 (Hitachi Software Engineering Co., Japan 1994). Phylogenetic analysis was performed using distance and parsimony methods in MEGA (ver. 1.01) and PAUP (ver. 3.1.1), respectively. Many sequences used in the present study have been reported elsewhere (Blair *et al.*, 1997) and are given in table 1.

Table 1. Source of materials of species of the genus, *Schistosoma* and *Clonorchis*.

Species	CO1	ITS2
S. japonicum	U82264	U22167
S. malayensis	U82262	U82398
S. sinensium China	present study	present study
S. sinensium Thailand	present study	present study
S. mekongi	U82263	U22169
S. mansoni	U82265	U22168
S. haematobium	U82266	U22165
S. hippopotami	—	Després *et al.* (1995)
C. sinensis	Agatsuma (1999)	Agatsuma (1999)

Results and Discussion

The CO1 alignment was 372 bp long. The Thai and Chinese isolates of *S. sinensium* differed from each other at 40 nucleotide sites (fig. 1) and at three amino acids sites (fig. 2). This is comparable with the number of differences between *S. malayensis* and *S. mekongi* (see discussion below). Table 2 shows pairwise differences among CO1 nucleotide sequences. Values above the diagonal are transitions/transversions. Those below are amino acid differences. The value of the ratios in this table indicates saturation at the species level, showing approximately 0.673 on average. However, the ratios for *S. malayensis*/*S. mekongi* and for the two isolates (China/Thailand) of *S. sinensium* are quite large (3.143 and 1.667, respectively), indicating that the isolates do not reach saturation. The average ratios of the *Schistosoma* species presented here are different from those between species of the genus *Paragonimus* which are all much higher (Blair *et al.*, 1999b). The NJ method (Saitou & Nei, 1987) was used for constructing a phylogenetic tree which shows that the two isolates of *S. sinensium* form a clade to the exclusion of all other species (fig. 3). The two African species form a clade sister to all Asian species. Basically the same topology was obtained by using the parsimony method. The single most-parsimonious tree constructed using all sites in the alignment had a consistency index of 0.805 (0.693 when uninformative characters were excluded). The same topology was found by bootstrapping with high bootstrap values (data not shown).

A length of 294 bases at the ITS2 region in *S. sinensium* was aligned with those of the other species (fig. 4). Table 3 provides pairwise differences among ITS2 nucleotide sequences (excluding gaps). The Thai and Chinese isolates of *S. sinensium* differed at two sites from each other. The two isolates of this species share several gaps with the three African species, and share one gap at a different site with the *S. japonicum* group. The NJ method (Saitou & Nei, 1987) was also used for constructing a phylogenetic tree (fig. 5) which showed that the two isolates of *S. sinensium* are sisters as shown in the CO1 tree. *Schistosoma hippopotami* was placed as a sister to the remaining African species in this analysis. The parsimony method gave a similar topology, yielding two most-parsimonious trees with a consistency index of 0.857 (0.756). The two trees differed from each other in that *S. hippopotami* was placed as a sister to the African species or as a sister of *S. sinensium*.

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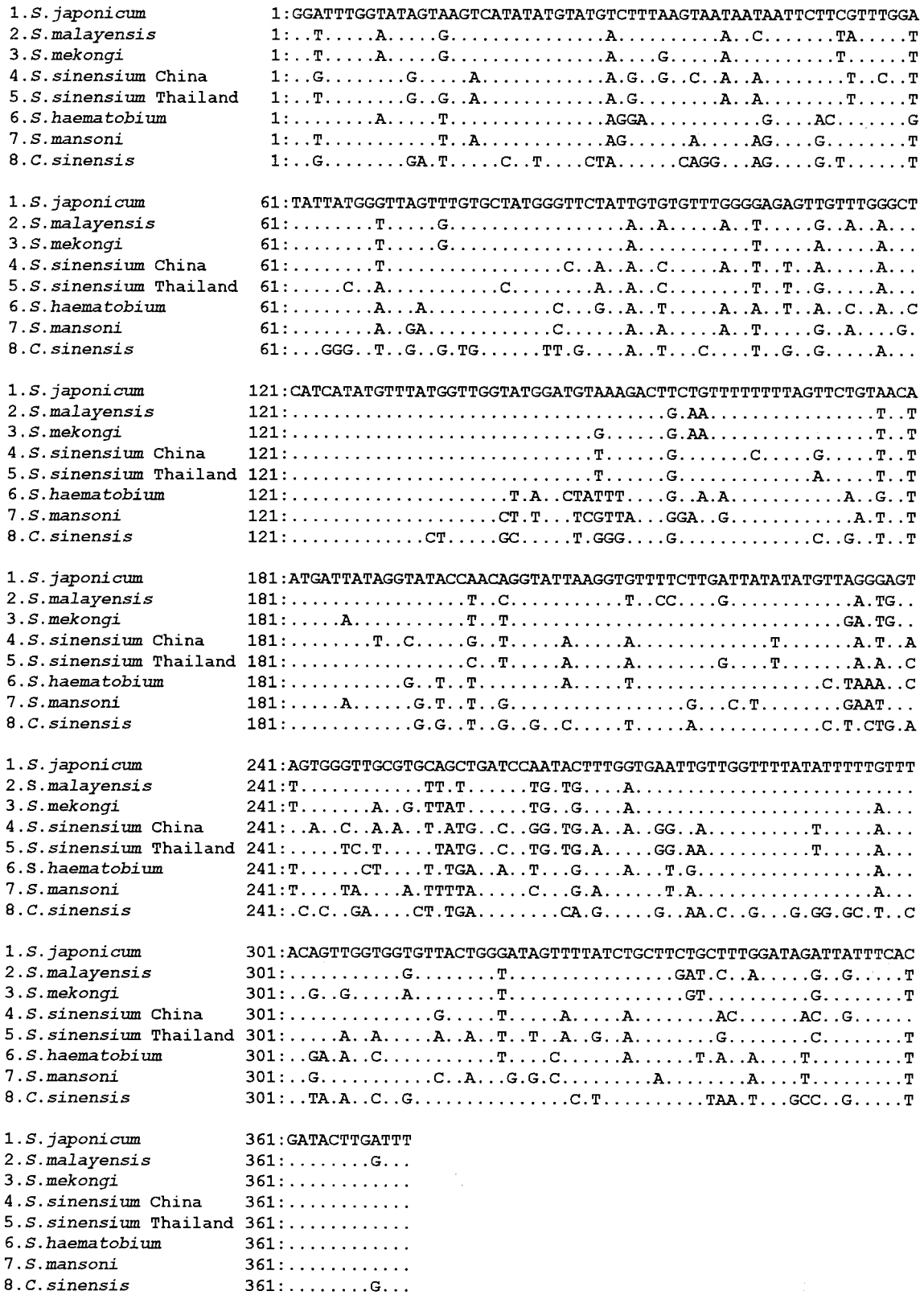

Fig. 1. Nucleotide sequence alignment of mitochondrial cytochrome c oxidase subunit 1 (CO1) region among *Schistosoma* species. A dot indicates identity with base on top line.

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1.S.japonicum            1:GFGIVSHICMSLSNNNSSFGYYGLVCAMGSIVCLGSVVWAHHMFMVGMDVKTSVFFSSVT
2.S.malayensis           1:.........T......L.................................AI......
3.S.mekongi              1:.........T......L.................................AI......
4.S.sinensium China      1:...M......T.....................................A.......
5.S.sinensium Thailand   1:...M......T.....................................A.......
6.S.haematobium          1:...........I...D.P......I...A.................L.YL.AI......
7.S.mansoni              1:..............D........I...A.........G.......F.SL.G.....I.
8.C.sinensis             1:...MI....TT.TG.D.L...G...L..FA.............T..L.LG.A.......

1.S.japonicum           61:MIIGIPTGIKVFSWLYMLGSSGLRAADPILWWIVGFIFLFTVGGVTGIVLSASALDSLFH
2.S.malayensis          61:............P.....SGC...VV..VV.....................WS......
3.S.mekongi             61:..................SGC...VI..VV.....................S.......
4.S.sinensium China     61:..............F..S....HVM..VV..VI.....................N...
5.S.sinensium Thailand  61:..............F..S......M..VV..VI.....................
6.S.haematobium         61:..................N.C.S.VWE..V..L........I......A....S..I...
7.S.mansoni             61:....V............N.C.M.VL...V..L.............VA.......I...
8.C.sinensis            61:....V............AGTRE.LW...M...I..VV...I...........VI.A...

1.S.japonicum           121:DTWF
2.S.malayensis          121:....
3.S.mekongi             121:....
4.S.sinensium China     121:....
5.S.sinensium Thailand  121:....
6.S.haematobium         121:....
7.S.mansoni             121:....
8.C.sinensis            121:....
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Fig. 2. Amino acid sequence alignment of mitochondrial cytochrome c oxidase subunit 1 (CO1) region among *Schistosoma* species. A dot indicates identity with base on top line.

Table 2. Pairwise differences among CO1 sequences in species of the genus *Schistosoma* and *Clonorchis*.

Species	1	2	3	4	5	6	7	8
S. japonicum	*	23/29	19/28	29/38	22/43	29/45	27/47	41/63
S. malayensis	14	*	22/7	29/37	25/38	27/48	33/42	43/60
S. mekongi	12	3	*	22/38	23/37	22/47	29/38	43/60
S. sinensium China	13	14	12	*	25/15	33/51	37/55	48/63
S. sinensium Thailand	10	13	11	3	*	34/46	32/50	41/68
S. haematobium	22	21	19	25	25	*	26/44	47/62
S. mansoni	20	25	23	24	24	15	*	48/60
C. sinensis	33	33	31	33	32	34	37	*

Values above the diagonal are transitions/transversions. Those below are amino acid differences.

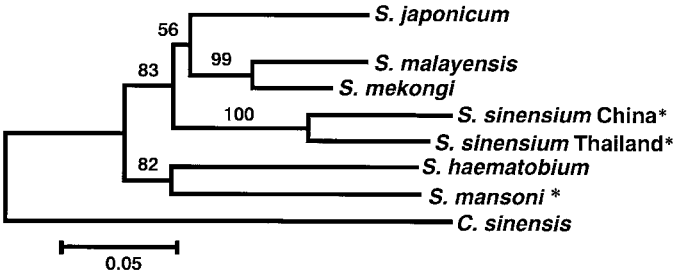

Fig. 3. Phylogenetic tree depicting relationships among *Schistosoma* species inferred from CO1 nucleotide data. A distance matrix was calculated using the Kimura 2-parameter model and the tree constructed using neighbour-joining approach in MEGA. The data set was bootstrapped 1000 times and the appropriate bootstrap values placed on each branch. Branch lengths shown are those assigned by MEGA. *Clonorchis sinensis* was used as an outgroup taxon. An asterisk indicates species which produces lateral spined eggs.

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1.S.japonicum          1:GGGTCGGCTTTTCATCTATCACGACGCACATTAAGTCGTGGATTGGCCGAGTGCCTGCCGGCATGT--TTGATGCGCTCGTATATATGAATGCGGGTTGCTGCTGGTCAA
2.S.malayensis         1:.....................................................A.AT.............G...C--..C...................
3.S.mekongi            1:.....................................................A.AT.............G...C--..C...................
4.S.sinensium China    1:.....................................A.................AA.---------..C....C--..C...T...............
5.S.sinesnium Thai     1:.....................................A.................AA.---------..C....C--..C...T...............
6.S.hippopotami        1:.....................................G.......-.........A.AT---.CA.A.C.......C--..C..........---.....
7.S.haematobium        1:.....................G.......G.............G.......G.......G...AT-------A.C..C....C--..C..........---...G.
8.S.mansoni            1:.....................................G..............G.T.AA-------A.C..C....C--..C..........---....G

1.S.japonicum        111:AGGCTCCGTCCGAATAATCCGACCACAGCCTAGTCCGGTCTAGATAGCCAGATTAAGATGCTGCAGTGGGTTGTGCTCGAGTCATGGCTTAATGATAATTTCCTTTATAC
2.S.malayensis       111:.............A.......T.....................A.......G.....................G..............A-.T.......
3.S.mekongi          111:.............A.......T.....................A.......G.....................G..............A-.T.......
4.S.sinensium China  111:..........T.........G.............A.........A.......G.........G..A.............G..........G.--------.....
5.S.sinensium Thai   111:.........T.T.........G.............A.........A.......G.........G..A.............G..........G.--------.....
6.S.hippopotami      111:...T.......T.......--G.................TC.T..GA.......G.........G.................G............-------.....
7.S.haematobium      111:..........T....G....G.....................GA.TT...CG.........G.................G...........C.-------.....
8.S.mansoni          111:..........T.........G.............T.........GA.TT....G.........G.................G...........C.-------.....

1.S.japonicum        221:ATGCTCGAGAAGAA-CACACCTACCGTAAGCTAAGTTAATCACTTAATCATGGCTCTATGATTTGGTCTATGGTTTGTATCGAGGGTGTGTGC
2.S.malayensis       221:..............-.G...T...T...C......C.....................C.....................A.........
3.S.mekongi          221:..............-.G.......T...C......C.....................C.....................A.........
4.S.sinensium China  221:..........GA..-.......G.T...T....T.G.GGAT............T.....................C....T.........
5.S.sinensium Thai   221:..........GA..-.......G.T...T....T.G..GAT............T.....................C....T.........
6.S.hippopotami      221:.......G..GT..A...G....T....T....T...........G...T..........G.................C...A........T
7.S.haematobium      221:GC.....G......T.G......T....C....C...GG......G...T..T.......G..C......C........C...T........T
8.S.mansoni          221:.C.....G..TA.TT.G......T....T....C...........G...T..T...--..G.................C...TT.......T
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Fig. 4. Nucleotide sequence alignment of internal-transcribed spacer region 2 (ITS2) among *Schistosoma* species. A dot indicates identity with base on top line, and a hyphen, gaps.

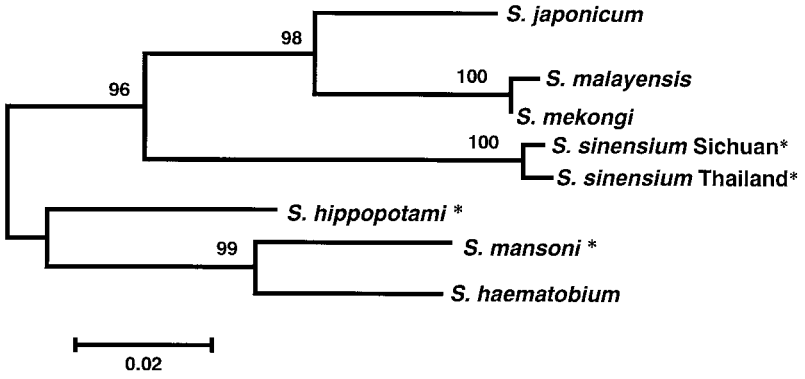

Fig. 5. Phylogenetic tree depicting relationships among *Schistosoma* species inferred from ITS2 nucleotide data. A distance matrix was calculated using the Kimura 2-parameter model and the tree constructed using neighbour-joining approach in MEGA, and midpoint rooted because of the lack of an appropriate outgroup. The data set was bootstrapped 1000 times and the appropriate bootstrap values placed on each branch. Branch lengths shown are those assigned by MEGA. An asterisk indicates species which produces lateral spined eggs.

Table 3. Pairwise differences among ITS2 sequences in species of the genus *Schistosoma*.

Species	1	2	3	4	5	6	7	8
S. japonicum	*	16	15	28	28	28	39	35
S. malayensis		*	1	29	29	31	38	36
S. mekongi			*	28	28	30	37	35
S. sinensium China				*	2	30	39	34
S. sinensium Thailand					*	31	42	35
S. hippopotami						*	29	22
S. haematobium							*	18
S. mansoni								*

Based on total number of 286 bp nucleotides, ignoring seven gaps of each 1–12 bases long.

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Davis (1980, 1992) suggested that *S. sinensium* constitutes a species complex, because of geographical differences in snail host specificity. Our previous morphological study showed a large difference in egg sizes between the two isolates from China and Thailand (Kawanaka *et al.*, 1998). In the present study, a large number of base substitutions was observed in the CO1 sequence between the isolates from the two countries. The extent of the differences corresponds to that between *S. malayensis* and *S. mekongi*. Therefore the present study supports the hypothesis that a species complex exists.

The phylogenetic distribution of species with lateral-spined eggs suggests that this condition is ancestral within the genus. On the basis of coevolution of snail hosts and *Schistosoma*, David (1980, 1992) stated that the genus *Schistosoma* is of African origin, and an ancestor of *S. japonicum* might have drifted from Africa to Asia during the Tertiary on the Indian plate. *Schistosoma sinensium* may have retained the ancestral egg form, and from its lineage the *S. japonicum* group might have arisen long ago. Our phylogenetic trees do not exclude this scenario. On the other hand, our recent chromosomal studies, based on C-banding patterns of chromosome 2, have indicated that *S. japonicum* could represent the plesiomorphic type from which the remaining species have derived. The chromosomes of *S. sinensium* have features of both the Asian and African types, suggesting an origin from the proto-African lineage after this had started to diverge from the ancestral form (Hirai *et al.*, 2000). Additional molecular data are available to support this view. The distribution of gaps in the present ITS2 data provides one line of evidence. Another is that the mitochondrial gene order in *S. japonicum* is almost identical with that of a number of other trematodes and cestodes, while in *S. mansoni* and other African species the gene order is highly derived (Le *et al.*, 2000). Further, recent ribosomal RNA 28S gene data suggested that *Schistosoma* appears to have originated in Asia (Snyder & Loker, 2000). It should also be noted that several studies have already observed that some Asian cattle species of *Schistosoma* have affinity with the African species in nucleotide sequences of CO1 and ITS2 genes (Agatsuma, 1999) and of the ribosomal RNA gene (Barker & Blair, 1996). Apparently, more molecular data of the Asian cattle species and some African species such as *S. hippopotami* would provide an important information to answer the above question.

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