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Short communication

Relationships between *Schistosoma malayensis* and other Asian schistosomes deduced from DNA sequences[1]

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At least three *Schistosoma* species can infect humans in South-East Asia. The most widespread of these is *S. japonicum* Katsurada, 1904 which may represent a species complex [1] and occurs in many countries including China, Japan and the Philippines. The second species is *S. mekongi* Voge, Bruckner and Bruce, 1978 which is endemic to a small area near the junction of Laos, Cambodia and Thailand. Most recently described is *S. malayensis* Greer, Ow-Yang and Yong, 1988 from a restricted area of peninsular Malaysia. This is primarily a parasite of rats but has also been found in people [2].

Davis [3], on the basis of snail intermediate host phylogeny and biogeography, proposed that *S. malayensis* and *S. mekongi* are sister taxa relative to *S. japonicum*. The three studies on allozymes among these taxa [4–6] also support this hypothe-

Abbreviations: COI, cytochrome *c* oxidase subunit I; ITS, internal transcribed spacer; ITS1, first internal transcribed spacer; ITS2, second internal transcribed spacer; PCR, polymerase chain reaction.

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[1] *Note*: Nucleotide sequence data reported in this paper is available in the EMBL, GenBank™ and DDJB databases under the accession numbers U82262–U82266, U82282–U82284, U82398.

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Table 1
Sources of material

Species	COI	ITS1	ITS2
S. malayensis[b]	[a]U82262	[a]U82283	[a]U82398
S. mekongi[c]	[a]U82263	[a]U82284	
S. mekongi (Bowles)	U22163 [7,8]	—	U22169 [7]
S. japonicum (Philippines)[d]	—	[a]U82282	—
S. japonicum (Japan)[e]	[a]U82264	—	—
S. japonicum (Bowles, 5 isolates)[f]	U22161 [7,8]		U22167 [7]
S. mansoni[g]	[a]U82265	—	—
S. mansoni (Bowles)	U22162 [7,8]	—	U22168 [7]
S. intercalatum	U22160 [7,8]	Z21717 [10,12]	U22166 [7]
S. haematobium[h]	[a]U82266	Z21716 [10,12]	—
S. haematobium (Bowles)	U22159 [7,8]	—	U22165 [7]
S. mattheei	—	Z21718 [10]	Z21718 [10]
S. hippopotami	—	—	[11]
Fasciola hepatica	M93388 [9]	—	—

Note that corrections have been made to sequences U22167 and U22169 since their publication in [7]
[a] Sequence reported here for the first time.
[b] From mice infected with cercariae from snails (*Robertsiella* sp.) collected at Baling, Malaysia, August 1993.
[c] From Khong Island, Laos (maintained in *Neotricula aperta* for 10 years at Mahidol University, Bangkok, Thailand).
[d] Strain originating at Sorsogon, Philippines (donated by Professor D. McManus, QIMR, Brisbane, Australia).
[e] From Kurume, Japan (strain maintained at Kurume University).
[f] Only a single sequence (from Philippines isolate) was included in analyses.
[g] Puerto Rican strain (maintained at Mahidol University, Bangkok, Thailand).
[h] Kenyan strain (maintained at Fukuoka University, Japan).

sis. Here we set out to test this using DNA sequence data from the nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) and from part of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. Sequences used and origins of material are shown in Table 1 [7–12].

DNA extraction and purification of mtDNA were as described previously [13]. Gene regions were amplified using the polymerase chain reaction (PCR). For the COI region, the primers used were as in [8]. For the ITS2, primers used were BD2 and 3S [7]. An additional primer, A28 (5′GGGATCCTGGTTAGTTTCTTTTCCTCCGC 3′), was sometimes used instead of BD2. For the ITS1 region, primers BD1 (5′GTCGTAACAAGGTTTCCGTA 3′) and 4S (5′TGCGTTCGAA(G/A)TGTCGATG 3′) were used. BD1 primes close to the 3′ end of the small subunit (18S) rRNA gene and 4S within the 5.8S gene.

All COI sequences and all ITS2 sequences except for that from *S. malayensis* were determined directly from the PCR products. Cycle sequencing reactions were run on an ABI 373A automated sequencer. PCR primers were used as sequencing primers. For *S. malayensis*, the entire ITS region including the 5.8S gene was amplified (template DNA pooled from several worms) using primers BD1 and BD2. The PCR product was cloned using a TA cloning kit (Invitrogen, California) and three clones sequenced on an ABI 373A sequencer. Similarly, the ITS1 of *S. mekongi* and of *S. japonicum* were amplified (BD1 and 4S, DNA template from a single worm in each case).

The results are summarised as a series of phylogenetic trees in Fig. 1. Alignments used are available from the corresponding author. A tract near the 5′ end of the ITS1 in all schistosomes was repeated in tandem a number of times which varied among clones sequenced. This region of repeats was therefore omitted from all analyses. Some intra-specific variation was observed among COI sequences (Fig. 1A) and the region of the ITS1 (Fig. 1B) which were analysed.

The trees in Fig. 1 show the 'traditional' arrangement of schistosome taxa [18]. African and Asian species lie within very distinct clades. From the distance tree based on the ITS2 alignment (Fig. 1C), *Schistosoma hippopotami* is the most basal African species as suggested previously [11]. All trees emphatically support the theory that *S. mekongi* and *S. malayensis* are closer to each other than either is to *S. japonicum*.

Intra- as well as inter-specific variation was noted for the COI sequences. The clonal, non-recombinant nature of mitochondrial genomes creates the expectation that their sequences will differ within and among populations [19]. In every case, COI sequences from the same species, but derived independently by Bowles et al. [7,8] and by ourselves, appear as sisters on the tree (Fig. 1A).

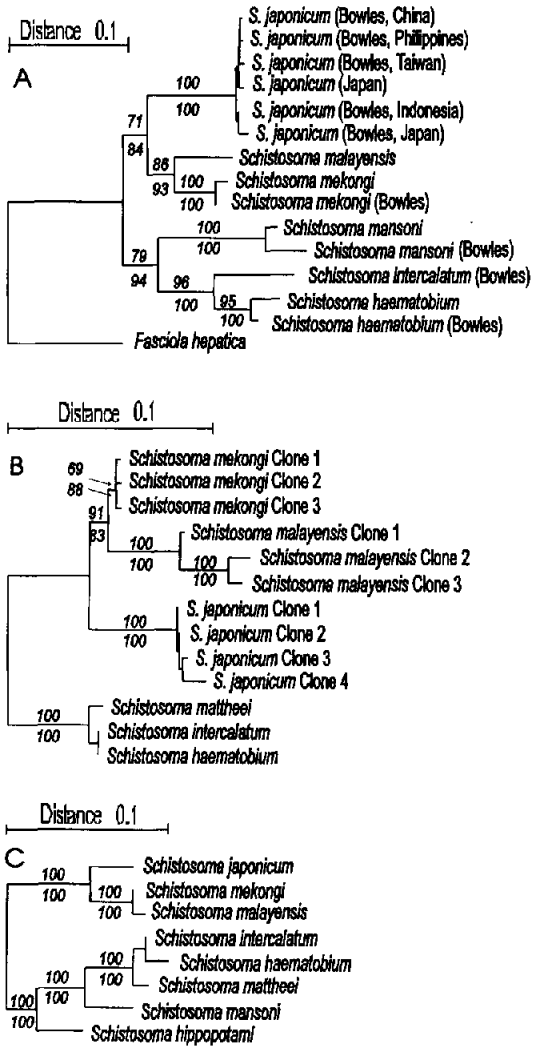

Fig. 1. Phylogenetic trees summarising results. Sequences were aligned using the sequence editor ESEE [14]. Parsimony and distance analyses were done using PAUP [15] and TREECON [16] respectively. In TREECON, pairwise distances between sequences were calculated using the Kimura 2-parameter method, gaps were not taken into account in construction of the matrices and trees were drawn using the neighbor-joining method. In PAUP, gaps were treated as missing data. Whenever bootstrapping was done in either program, 1000 cycles were performed. Bootstrap values below each internode are those found using TREECON. Those above are from PAUP. (A) Distance tree based on COI DNA alignment. The alignment used was 375 bases in length and included 160 variable sites and 110 sites informative for parsimony analyses (ambiguities being ignored). Regions undetermined for the sequences reported in [8] were omitted from analyses. Three equally-parsimonious trees were found in PAUP (length 301, consistency index 0.781, consistency index excluding uninformative sites 0.732). Apart from some rearrangements among the *S. japonicum* sequences, these were identical to the distance tree. The translated amino acid sequence of 125 residues (not shown) included 50 variable sites and 31 informative sites. Amino acid residues for the COI region were inferred using codon tables in [9], except that the codon ATA was translated to isoleucine rather than methionine [17]. Analyses of amino acid sequences were done using PAUP only and found 14 equally most-parsimonious trees (length 82, consistency index 0.854, consistency index excluding uninformative sites 0.812). These differed from each other (and from Fig. 1a) in the relationships among the *S. malayensis* and *S. mekongi* sequences and in the relationships among *S intercalatum* and *S. haematobium* sequences. (B) Distance tree based on ITS1 alignment. The alignment consisted of 375 sites (of which 5 were omitted from analyses) starting just 3′, of the repeat region. Of the remaining sites, 110 were variable and 90 informative for parsimony methods (ignoring ambiguities). Fourteen equally most-parsimonious trees found by PAUP from the ITS1 alignment have a length of 91, consistency index of 0.945, consistency index excluding uninformative sites of 0.938. The trees vary only in the arrangement among *S. japonicum* clones and are otherwise identical to the distance tree. (C) Distance tree based on ITS2 alignment. The ITS2 alignment had 358 sites of which 27 were omitted from analyses. Of the remaining sites, 88 were variable and 53 informative (ignoring ambiguities). The single most-parsimonious tree found by PAUP was identical to the distance tree, had a length of 88, consistency index of 0.943 and consistency index excluding uninformative sites of 0.919.

In contrast to mitochondrial genes, ribosomal gene clusters are expected to experience homogenising processes [20] which will cause even non-coding regions to be relatively conserved. Despite this expectation, intra-specific variation has been noted, primarily in the ITS 1, in some flatworms. For example, in the cestode genus *Echinococcus*, some ITS1 variants from a single isolate most closely resembled variants from other isolates or species [21]. The ITS1 sequences from schistosomes exhibit much more modest variation [12], and all variants from a single Asian species are sisters to one another on the tree (Fig. 1B). This is consistent with levels of ITS1 variation among African schistosomes [12].

In contrast with ITS1, two previous studies [8,22] have noted little or no intra-specific variation in schistosome ITS2 sequences. Such as has been noted previously might be due to gel reading errors [7]. One of our three clones of *S. malayensis* differed from the other two at a single site which was otherwise invariant among all taxa examined. This case might represent PCR misincorporation.

The close relationship between *S. mekongi* and *S. malayensis* inferred from DNA sequences is entirely consistent with previous studies. Intermediate hosts of both are very closely related and cross infections are possible experimentally [3]. The intermediate host of *S. japonicum* is only distantly related to these and experimental cross-infections have not proved possible [3]. Allozyme studies [6] have suggested that *S. mekongi* and *S. malayensis* are no more distantly related than are some geographical strains within *S. japonicum*. However, our molecular studies [8] detected little geographic structure within *S. japonicum* even though they found considerable differences among the three Asian species.

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