

# The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858

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## SUMMARY

Schistosomes are digenean flukes, parasitic of birds, mammals and crocodiles. The family Schistosomatidae contains species of considerable medical and veterinary importance, which cause the disease schistosomiasis. Previous studies, both morphological and molecular, which have provided a good deal of information on the phylogenetics of this group, have been limited in the number of species investigated or the type or extent of molecular data used. This paper presents the most comprehensive phylogeny to date, based on the sequences of 3 genes, complete ribosomal small subunit rRNA and large ribosomal subunit rRNA, and mitochondrial cytochrome oxidase 1, sequenced from 30 taxa including at least 1 representative from 10 of the 13 known genera of the Schistosomatidae and 17 of the 20 recognized *Schistosoma* species. The phylogeny is examined using morphological characters, intermediate and definitive host associations and biogeography. Theories as to the origins and spread of *Schistosoma* are also explored. The principal findings are that *Ornithobilharzia* and *Austrobilharzia* form a sister group to the *Schistosoma*; mammalian schistosomes appear paraphyletic and 2 *Trichobilharzia* species, *T. ocellata* and *T. szidati*, seem to be synonymous. The position of *Orientobilharzia* within the *Schistosoma* is confirmed, as is an Asian origin for the *Schistosoma*, followed by subsequent dispersal through India and Africa.

Key words: interrelationships, character analysis, biogeography, host–parasite associations, Digenea.

## INTRODUCTION

The Schistosomatidae are digenean flukes that parasitize birds, mammals and crocodiles and use gastropod intermediate hosts. Schistosomatids are primarily associated with freshwater habitats and are found in all temperate and tropical regions of the world. There are 14 recognized genera and approximately 100 species of schistosomes (Khalil, 2002) including a number of species of medical and veterinary importance. As the causative agents of schistosomiasis, human schistosomes rank amongst the most important of all metazoan parasites, affecting over 220 million people (WHO, 2001). Other

schistosomatids, such as the avian flukes *Trichobilharzia*, also have implications for human health, as the release of their cercariae can cause severe cercarial dermatitis (e.g. Horak & Kolarova, 2001; Horak, Kolarova & Adema, 2002). A sound framework for the taxonomy of schistosomes may provide a better understanding of the origins, radiation and evolution of schistosomes. The elucidation of the history, present distribution, and the possible future spread of schistosomes, had implications for controlling the diseases they cause. General descriptions of the family and taxonomic histories can be found in Farley (1971) and Gibson, Jones & Bray (2002).

Within the Schistosomatidae, it is the genus *Schistosoma* that contains species that parasitize man. Traditional groupings of *Schistosoma* species, based primarily on egg morphology, intermediate host specificity and biogeography, divided the genus into

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4 groups, represented by the species *S. mansoni*, *S. haematobium*, *S. indicum* and *S. japonicum* (Rollinson & Southgate, 1987). *S. mansoni*, which causes human intestinal schistosomiasis, has lateral spined eggs and uses *Biomphalaria* snails as intermediate hosts. *S. mansoni* is widespread in Africa and is also present in South America and the Caribbean. Other members of this group include *S. rodhaini*, a rodent parasite and also 2 parasites of the hippopotamus, *S. edwardiense* and *S. hippopotami*. *S. haematobium* causes urinary schistosomiasis in man and uses snails of the genus *Bulinus* as its intermediate hosts. This species has terminal spined eggs. Most of the other African species fall into this group, such as *S. intercalatum*, which also infects man and several species that infect mainly cattle and sheep, including *S. bovis*, *S. mattheei* and *S. curassoni*. It has been estimated that at least 165 million cattle worldwide are infected with schistosomiasis (de Bont & Vercruyse, 1997). The third group includes *S. japonicum*, which has a rounded, minutely spined egg. *S. japonicum* is widespread throughout East Asia, although eradicated from Japan by extensive control programmes. Other Asian species in this group are *S. sinensium*, *S. mekongi*, *S. malayensis* and a fourth, recently described, species *S. ovuncatum* (Attwood *et al.* 2002a). Both *S. mekongi* and *S. japonicum* are human pathogens. The *S. indicum* group contains the Indian species *S. incognitum*, *S. spindale* and *S. nasale*, in addition to *S. indicum*. None of these infect man, and they have a variety of egg morphologies. These species have been grouped together for convenience, as much on the basis that they do not fit with the *S. mansoni*, *S. haematobium* and *S. japonicum* groups, as that they are all found in India and parts of S. E. Asia (Rollinson & Southgate, 1987). Indeed, Agatsuma *et al.* (2002) suggested the group may not be monophyletic. Those species which infect man do not fall into a single species group, indicating that they are not closely related and do not share the same morphological features, intermediate host or geographical ranges. Rather, they individually share features with other species that are not infective to man, and this indicates that there have been independent lateral transfers into man from other hosts (Combes, 1990).

#### Taxonomy and systematics

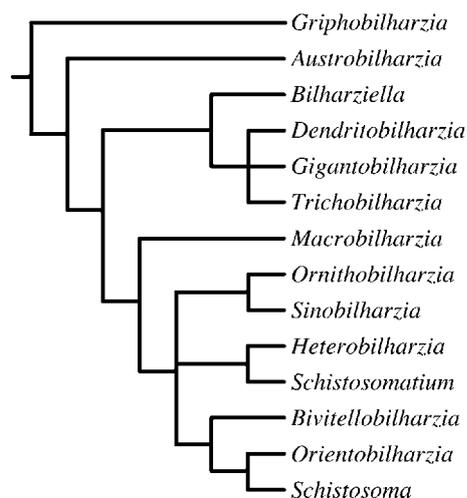
Carmichael (1984) carried out a cladistic analysis of the Schistosomatidae and produced a comprehensive review with a phylogeny based on 24 morphological characters scored for 14 genera. Morand & Müller-Graf (2000) re-analysed these data using modern computational methods, recoded as 37 characters (Carmichael's thesis included a number of multi-state characters). The preferred tree presented by Carmichael was not the most parsimonious as found by a cladistic analysis performed by Morand & Müller-Graf (2000) using the same characters which

provided a more resolved solution (Fig. 1A). There have been a number of molecular attempts to infer schistosomatid phylogenies, with particular emphasis on resolving the interrelationships of species of the medically important genus *Schistosoma*. Rollinson *et al.* (1997) reviewed some of the earliest studies based on mitochondrial and nuclear ribosomal gene sequences (Després *et al.* 1992; Johnston, Kane & Rollinson, 1993; Littlewood & Johnston, 1995), RAPDs (Barral *et al.* 1993; Kaukas *et al.* 1994) and RFLPs of mitochondrial DNA (Després, Imbert-Establet & Monnerot, 1993). The majority of these studies involved only a few exemplar taxa and concentrated on *Schistosoma*. Snyder & Loker (2000) broadened the approach to the Schistosomatidae and used large subunit ribosomal DNA (lsrDNA). Using 12 ingroup taxa (representing 10 genera) and 2 outgroups, and sequencing about a kilobase of lsrDNA encompassing variable domains D1–D2, their solution differed fundamentally from analyses based on morphology by Morand & Müller-Graf (2000) (Fig. 1). With lsrDNA sequence data, *Orientobilharzia* and *Schistosoma* formed a monophyletic (mammalian) clade and the other schistosomatid taxa formed a primarily avian clade, not seen in the morphology tree. The interrelationships of the remaining bird and mammal schistosomes are the same in both analyses, recognizing 3 clades comprising: *Schistosomatium* and *Heterobilharzia*; *Dendrobilharzia*, *Gigantobilharzia*, *Trichobilharzia* and *Bilharziella*; *Ornithobilharzia* and *Austrobilharzia* (called '*Sinobilharzia*' in the tree based on morphology). However, as a result of topological differences, the interpretation of the evolution of the family, including the adoption of intermediate and definitive hosts, also changes. Of particular interest is whether the move from avian to mammalian definitive hosts was a single event. For this to be resolved, the true identity of the sister group to *Schistosoma* must be identified.

While *Schistosoma* has long been a subject of study, a clear phylogeny for the genus has remained elusive. There are discrepancies in our understanding of the radiation of *Schistosoma*, but this stems largely from later efforts building on earlier, relatively poorly sampled attempts, in a fragmented manner. There has rarely been full complementarity between the various studies undertaken, such that some genes are sampled for some taxa but not for all. Johnston *et al.* (1993) and Littlewood & Johnston (1995) used almost complete ssrDNA and partial lsrDNA respectively to estimate the interrelationships of exemplar taxa from the main *Schistosoma* species groups. Barker & Blair (1996) incorporated more species, but used shorter ssrDNA and lsrDNA fragments. Shorter, more variable regions of DNA from nuclear ribosomal internal transcribed spacer region 2 (ITS2) and mitochondrial cytochrome oxidase I (COI), were also used to confirm species groups (Bowles, Blair & McManus, 1995), although again, only a limited number of

## A morphology

Morand &amp; Müller-Graf (2000) after Carmichael (1984)



## B LSU rDNA

Snyder &amp; Loker (2000)

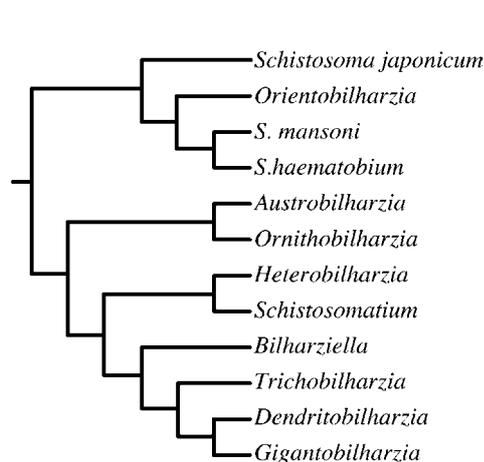


Fig. 1. Previously published phylogenies based on a cladistic analysis of morphology (recoding characters used by Carmichael, 1984; Morand & Müller-Graf, 2000) and a molecular phylogenetic analysis of partial LSU rDNA (Snyder & Loker, 2000).

Note: *Sinobilharzia* refers to *Austrobilharzia odhneri* – One anomaly not explained by Morand & Müller-Graf (2000) concerns this species. In Carmichael's analysis (Carmichael, 1984) *Sinobilharzia* represents a single species that was originally named *Ornithobilharzia odhneri* Faust, 1924, but subsequently reclassified as *Sinobilharzia odhneri* by Dutt & Srivastava (1961). Farley (1971) then placed this species in *Austrobilharzia*, but Carmichael chose to analyse the species separately under the genus *Sinobilharzia*. In their analysis, Morand & Müller-Graf (2000) used Carmichael's morphological matrix to produce a tree, but mapped specific morphological data from another species that they called *Sinobilharzia crecci* – we can find no reference for this species and suggest that they may have mistakenly used *Ĵilinobilharzia crecci* Liu & Bai (1976) in their analysis. Although this has no effect on their analysis of Carmichael's matrix, it should be borne in mind that *Sinobilharzia* in the tree of Morand & Müller-Graf (2000); shown in Fig. 1A refers to *Austrobilharzia odhneri*. Khalil (2002) synonymizes *Sinobilharzia* as *Austrobilharzia*. Indeed, *Sinobilharzia*, which has also been used for *Schistosoma japonicum* by Le Roux (1958), is no longer recognized.

exemplar taxa were included. Most recent studies have added gene fragments or additional taxa to particular clades within *Schistosoma* to test the position of individual taxa (e.g. Agatsuma *et al.* 2001, 2002; Blair *et al.* 1997), or to examine some of the biogeographic hypotheses suggested by Snyder & Loker's (2000) scheme, e.g. Zhang *et al.* (2001) and Attwood *et al.* (2002b). Few molecular studies have focused on the non-*Schistosoma* groups, although molecular methods to differentiate *Trichobilharzia* species are being developed (Dvorak *et al.* 2002).

Snyder & Loker (2000) found *Orientobilharzia* among the *Schistosoma* lineages, suggesting that *Schistosoma* is paraphyletic. Blair, Davis & Wu (2001) using the same data with the addition of more *Schistosoma* species, showed a single mammalian/avian split but without strong nodal support. A recent summary (Morgan *et al.* 2001) indicated that the *Schistosoma* phylogeny is not resolved leaving many questions unanswered, such as the position of *S. incognitum*. Also, while it now seems likely that the East Asian species are the earliest derived species in the *Schistosoma* clade, the position of *Orientobilharzia*, either within the East Asian clade or basal to the African and Indian species, remains problematic (Attwood *et al.* 2002a; Snyder & Loker, 2000; Zhang *et al.* 2001).

Additional evidence for the phylogeny within *Schistosoma* has been gleaned from investigating complete mitochondrial genomes, and a remarkable split in the genus has been revealed (Le *et al.* 2000). The gene order of *S. mansoni* is quite different to that found in *S. japonicum* and *S. mekongi* and these 2 Asian schistosomes share the same gene order as that found in other trematodes and in cestodes, suggesting that they possess the plesiomorphic pattern. *S. mansoni* not only has a translocation of *atp6* and *nad2* when compared to *S. japonicum*, but also the gene order for *nad3* and *nad1* is reversed, and thus these data confirm the basal status of the East Asian *Schistosoma*. The position of the East Asian species is significant in distinguishing between different theories for the origin and subsequent radiation and dispersal of the schistosomes.

## Biogeography

Two theories of *Schistosoma* origin have been proposed. A Gondwanan-origin (vicariance) hypothesis, based on snail host phylogeny and palaeontology, suggests members of the genus originated in Gondwanaland, with an ancestor rafting on the Indian plate to Asia 70–150 MYA and that *Schistosoma*

transferred to South America 80–120 MYA before continental drift split Gondwanaland (Davis, 1980). The molecular evidence so far refutes this scenario. Firstly, Després *et al.* (1993) using restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA fragments found that the genetic differentiation between African and American populations of *S. mansoni* was no greater than within African populations, suggesting a recent transfer of the parasite to S. America, associated with the slave trade. Secondly, Snyder & Loker (2000) found *S. japonicum* and *Orientobilharzia* at the base of the *Schistosoma* phylogeny and proposed an alternative, Asian, hypothesis. They proposed (and subsequently suggested dates by referring to the historical record of the vertebrate hosts (Morgan *et al.* 2001)), that an ancestral schistosome dispersed to Africa 12–19 MYA via widespread mammal migration from Asia. The *Schistosoma* ancestor remaining in Asia radiated as the *S. japonicum* species group. In Africa the lineage diverged into the *S. mansoni* and *S. haematobium* groups and an *S. indicum* ancestor migrated back to India, possibly with early humans and their animals. *S. mansoni* dispersed to South America about 500 YA via the transport of African slaves (Després *et al.* 1993). Dating such events is highly problematic. Barker & Blair (1996) rejected the use of a molecular clock based on partial lsrDNA, and Snyder & Loker (2000) recognized the whole exercise as highly speculative. Other dates have been proposed for these various splits, but all depend on the acceptance of molecular clocks (Després *et al.* 1992; Morgan *et al.* 2001) that are at best highly erratic and that have been employed without estimating confidence intervals (e.g. Cutler, 2000; Rambaut, 2000). Attwood (2001), Attwood & Johnston (2001) and Attwood *et al.* (2002*b*) also discussed biogeographical predictions for *Schistosoma* which are concordant with an Asian origin, based on intermediate host phylogeography and the late Caenozoic evolution of the main rivers in Asia. Attwood (2002*b*) used partial 18S, 28S and mitochondrial 16S rRNA gene sequences to estimate a phylogeny for the East Asian species which was independent of a molecular clock hypothesis but does rely on, as yet, incomplete palaeogeographical data.

#### *This study*

A robust and comprehensive phylogeny is required to enable us to stabilize the taxonomy, to identify taxonomically useful characters, to investigate the biogeography and the origin of the schistosomes, and to reveal other unique features of this important group, including host-specificity, host-switching, and the evolution of sexual dimorphism. Many of these questions have recently been subjects of investigation and there have been attempts to construct 'super-trees' based on various previous phylogenetic estimates from smaller trees with fewer taxa (Morand

& Müller-Graf, 2000). However, an estimate of the phylogeny based on a fully complementary multi-gene dataset is required. This paper extends on previous studies by using 2 nuclear and 1 mitochondrial gene. Although previous work has resolved some schistosomatid relationships with lsrDNA and ssrDNA sequences treated individually, it is clear that a combination of these data works well among platyhelminth groups in general, and particularly amongst neodermatan flatworms (e.g. Olson *et al.* 2001; Olson & Littlewood, 2002; Olson *et al.* manuscript submitted). However, rather than relying on partial lsrDNA alone, there is growing evidence that combining the complete sequences of both genes adds stability and resolution at a number of levels within and between metazoan taxa (Mallatt & Winchell, 2002; Medina *et al.* 2001), including platyhelminths (Lockyer, Olson & Littlewood, 2003). Additionally, almost complete mitochondrial COI was sequenced in order to provide greater resolution among more closely related taxa. These 3 genes were sequenced from 30 taxa, including at least 1 representative from 10 of the 13 known genera of the Schistosomatidae and 17 of the 20 recognized *Schistosoma* species.

#### MATERIALS AND METHODS

##### *Taxa sampled and choice of outgroup*

Twenty-nine schistosomatid species and one sanguinicolid for outgroup rooting were sampled. Previous morphological and molecular phylogenetic estimates of digenean interrelationships have indicated strongly that the Sanguinicolidae are the sister group to the Schistosomatidae within the superfamily Schistosomatoidea (see Cribb *et al.* 2001). Recent work (D.T.J.L. & P.D.O., unpublished results) has indicated that sanguinicolids are quite divergent from the schistosomatids, exhibiting relatively long branches for both ssrDNA and lsrDNA. Nevertheless, each selected gene partition was sequenced from the basal sanguinicolid *Chimaerohemecus trondheimensis*, its position based on analyses of digenean interrelationships using full ssrDNA and partial lsrDNA (D. T. J. L., P. D. O., unpublished results). An, as yet undescribed, sanguinicolid, used elsewhere for ssrDNA and lsrDNA analyses of platyhelminth relationships (Lockyer, Olson & Littlewood, 2003), added additional information for outgroup rooting. Although the COI fragment could not be amplified from this second outgroup taxon, all ingroup topologies of ssrDNA and lsrDNA trees were identical with one or two outgroups, so the analyses were restricted to rooting against *C. trondheimensis* alone. If suggestions that the Spirorchidae are in fact the sister group to the Schistosomatidae (Platt & Brooks, 1997) can be confirmed, additional molecular sampling from this family may be worthwhile. No spirorchids were available for the present analysis.

All major schistosomatid genera were sampled except *Macrobilharzia*, *Bivitellobilharzia*, *Jilinobilharzia* and *Griphobilharzia*. These taxa are parasites of protected or rare vertebrate hosts and one, *Griphobilharzia*, has remained elusive since its original description from the freshwater crocodile (Platt *et al.* 1991). Among the genus *Schistosoma*, every species was sampled except *S. hippopotami* and *S. edwardsiense*, both parasites of *Hippopotamus amphibius* L., another protected species. Unfortunately, there were insufficient female (most readily identifiable) specimens of *S. ovuncatum* (Attwood *et al.* 2002a) available for the present study. The full classification of the Schistosomatidae according to the latest keys (Khalil, 2002) is replicated in Table 1. The same table gives full details of the taxa sampled here, including authorities and sources.

#### DNA extraction and gene sequencing

Total genomic DNA was extracted from liquid nitrogen-frozen or ethanol-preserved specimens using standard proteinase K, phenol-chloroform extraction techniques (Sambrook, Fritsch & Maniatis, 1989) or DNeasy™ Tissue kit (Qiagen) according to the manufacturer's protocol. The 25 µl amplifications were performed with 3–5 µl of genomic extract (~10 ng) using Ready-To-Go PCR beads (Amersham Pharmacia Biotech) each containing 1.5 U *Taq* Polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM each dNTP and stabilisers including BSA; and 0.4 µM of each PCR primer. The complete lsrDNA was amplified in 3 overlapping sections using the primer combinations U178 + L1642, U1148 + L2450 and U1846 + L3449 (see Table 2). PCR conditions used were: 2 min denaturation at 94 °C; 40 cycles of 30 sec at 94 °C, 30 sec at 52 °C and 2 min at 72 °C; followed by a final 7 min extension at 72 °C. Where necessary to obtain a product, the stringency was reduced by adding MgCl<sub>2</sub> to a final concentration of 2.5 mM or by reducing the annealing temperature to 50 °C. Amplification of mitochondrial cytochrome oxidase subunit 1 (CO1) was performed using the primers Cox1\_Schist\_5' and Cox1\_Schist\_3' (see Table 2) with the same PCR conditions as above. Complete sequencing of ssrDNA was performed as described previously (Littlewood *et al.* 1999).

PCR products were purified with Qiagen Qiaquick columns, cycle-sequenced directly using ABI Big-Dye chemistry, ethanol-precipitated and run on an ABI prism 377 automated sequencer. A variety of internal primers were used to obtain the full sequence of each fragment from both strands (see Table 2). Sequences were assembled and edited using Sequencher ver 3.1.1 (GeneCodes Corp.) and submitted to EMBL/GenBank (see Table 1 for accession numbers). In all cases complete lsrDNA, ssrDNA and CO1 were sequenced, except for conserved

regions at both 5' and 3' ends that were targeted for primer design.

#### Sequence alignment and phylogenetic analyses

ssrDNA and lsrDNA sequences were each aligned initially with the aid of ClustalX using default parameters (Jeanmougin *et al.* 1998), and alignments then refined by eye with MacClade ver. 4.03 (Maddison & Maddison, 2000). CO1 sequences were aligned with reference to the open-reading frame and the inferred amino acid sequences. Individual gene alignments were concatenated in MacClade, ambiguously aligned positions excluded and data partitions defined.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP\* ver. 4.0b10 (Swofford, 2002) and the resulting networks rooted with the outgroup taxon. Each gene was analysed both independently and combined using MP, ML and also Bayesian inference (BI) using the program MrBayes (Huelsenbeck, 2000). Mitochondrial COI sequences were analysed only as nucleotides but were investigated to see whether trees differed in topology when using only first and second codon positions or all 3 positions, in order to best reflect the signal at nonsynonymous sites. Analyses by MP were performed using a heuristic search strategy (1000 search replicates), random-addition of sequences and tree-bisection-reconnection (TBR) branch-swapping options. All characters were run unordered and equally weighted. Gaps were treated as missing data. Nodal support was assessed by bootstrap resampling in MP (1000 replicates) and ML (100 replicates). Nodal support from majority-rule consensus trees found with BI were also utilized. In order to test whether there was significant conflict between the data partitions prior to combining them the criteria of conditional combination of independent data sets (Huelsenbeck, Bull & Cunningham, 1996; Cunningham, 1997) were examined using the incongruence length-difference (Farris *et al.* 1995) test as implemented in PAUP\*. The test was performed with maximum parsimony, 10 heuristic searches (random sequence addition, TBR branch-swapping) each for 100 homogeneity-replicates on informative sites only (Lee, 2001).

A suitable nucleotide substitution model was estimated using Modeltest (Posada & Crandall, 1998), which showed a general time reversible (GTR) model including estimates of invariant sites (*I*) and among-site rate heterogeneity (*G*) for each individual and combined data set. In calculating maximum likelihood trees, values of *I* and *G* were set to those estimated by Modeltest but substitution rate parameters were free to vary and nucleotide frequencies used were empirical.

Bayesian inference (BI) of phylogeny was estimated using the following nucleotide substitution

Table 1. List of taxa and sequences used in this study and their geographical origin

(Avian (A) or mammalian (M) vertebrate host indicated. See Fig. 5 for list of mollusc hosts. Previously unreported sequences are marked §.)

Classification	Source	Vertebrate host		GenBANK Accession No.		
		A	M	COI	ssrDNA	lsrDNA
Schistosomatidae Stiles & Hassall, 1898						
Schistosomatinae Stiles & Hassall, 1898						
<i>Austroilharzia</i> Johnston, 1917						
<i>Austroilharzia terrigalensis</i> Johnson, 1916	ex <i>Batillaria australis</i> ; Rodd Point, Iron Cove, Sydney Harbour, NSW, Australia.	✓		AY157195§	AY157223§	AY157249§
<i>Austroilharzia variglandis</i> (Miller & Northup, 1926)	ex <i>Larus delawarensis</i> ; Delaware, USA.	✓		AY157196§	AY157224§	AY157250§
<i>Bivitellobilharzia</i> Vogel & Minning, 1940	No species sampled		✓			
<i>Heterobilharzia</i> Price, 1929						
<i>Heterobilharzia americana</i> Price, 1929	ex <i>Mesocricetus auratus</i> ; (experimental infection) NHM-409 original isolate from Louisiana, USA.		✓	AY157192§	AY157220§	AY157246§
<i>Macrobilharzia</i> Travassos, 1922	No species sampled.	✓				
<i>Orientobilharzia</i> Dutt & Srivastava, 1955						
<i>Orientobilharzia turkestanicum</i> (Skrjabin, 1913)	ex <i>Ovis aries</i> ; Iran.		✓	AY157200§	AF442499	AY157254§
<i>Ornithobilharzia</i> Odhner, 1912						
<i>Ornithobilharzia canaliculata</i> (Rudolphi, 1819)	ex <i>Larus delawarensis</i> ; Donley County, Texas, USA.	✓		AY157194§	AY157222§	AY157248§
<i>Schistosomatium</i> Tanabe, 1923						
<i>Schistosomatium douthitti</i> (Cort, 1915)	ex <i>Mesocricetus auratus</i> ; (experimental infection) Indiana, USA.		✓	AY157193§	AY157221§	AY157247§
<i>Schistosoma</i> Weinland, 1858						
<i>Schistosoma bovis</i> (Sonsoni, 1876)	ex <i>Mus musculus</i> ; (experimental infection) original isolate from Iranga, Tanzania.		✓	AY157212§	AY157238§	AY157266§
<i>Schistosoma curassoni</i> Brumpt, 1931	ex <i>Mesocricetus auratus</i> ; (experimental infection) original isolate from Dakar, Senegal.		✓	AY157210§	AY157236§	AY157264§
<i>Schistosoma haematobium</i> (Bilharz, 1852)	ex <i>Mesocricetus auratus</i> ; (laboratory infection) NHM-3390, Village 10, Nigél delta, Mali.		✓	AY157209§	Z11976	AY157263§
<i>Schistosoma incognitum</i> Chandler, 1926	ex <i>Bandicota indica</i> ; Phitsanulok, Thailand.		✓	AY157201§	AY157229§	AY157255§
<i>Schistosoma indicum</i> Montgomery, 1906	ex <i>Bos taurus</i> ; Mymensingh, Bangladesh.		✓	AY157204§	AY157231§	AY157258§
<i>Schistosoma intercalatum</i> Fisher, 1934	ex <i>Mus musculus</i> ; (laboratory infection) NHM-3188, San Antonio, São Tomé.		✓	AY157208§	AY157235§	AY157262§
<i>Schistosoma japonicum</i> Katsurada, 1904	ex <i>Mus musculus</i> ; (experimental infection) isolate S15/90–19. Original isolate from the Philippines.		✓	AF215860	AY157226§	AY157607§
<i>Schistosoma leiperi</i> Le Roux, 1955	ex <i>Mesocricetus auratus</i> ; (experimental infection) original isolate from South Africa.		✓	AY157207§	AY157234§	AY157261§
<i>Schistosoma malayensis</i> Greer <i>et al.</i> 1988	ex <i>Mus musculus</i> ; (experimental infection) original isolate from Baling, Kedah, Malaysia		✓	AY157198§	AY157227§	AY157252§
<i>Schistosoma mansoni</i> Sambon, 1907	ex <i>Mus musculus</i> ; (experimental infection) isolate NHM-3454/5/6.		✓	AF216698	M62652	AY157173§
<i>Schistosoma margrebowiei</i> Le Roux, 1933	ex <i>Mus musculus</i> ; (experimental infection) lab strain isolate NHM-3295. Original isolate from Lochinvar, Zambia.		✓	AY157206§	AY157233§	AY157260§

<i>Schistosoma mattheei</i> Veglia & Le Roux, 1929	ex <i>Mus musculus</i> ; (experimental infection) original isolate from Denwood Farm, Nr Lusaka, Zambia.	✓	AY157211§	AY157237§	AY157265§
<i>Schistosoma mekongi</i> Voge, Buckner & Bruce, 1978	ex <i>Mus musculus</i> ; (experimental infection) originally isolated from <i>Neotricula aperta</i> , Khong Island, Laos.	✓	AY157199§	AY157228§	AY157253§
<i>Schistosoma nasale</i> Rao, 1933	ex <i>Capra hircus</i> ; Sri Lanka.	✓	AY157205§	AY157232§	AY157259§
<i>Schistosoma rodhaini</i> Brumpt, 1931	ex <i>Mus musculus</i> ; (experimental infection) lab strain (NHM).	✓	AY157202§	AY157230§	AY157256§
<i>Schistosoma sinensium</i> Bao, 1958	ex <i>Mus musculus</i> ; (experimental infection) originally isolated from <i>Tricula</i> sp., Mianzhu, Sichuan, China.	✓	AY157197§	AY157225§	AY157251§
<i>Schistosoma spindale</i> Montgomery, 1906	ex <i>Mus musculus</i> ; (experimental infection) NMH 1630 original isolate from <i>Indoplanorbis exustus</i> from Sri Lanka.	✓	AY157203§	Z11979	AY157257§
Griphobilharziinae Platt, Blair, Purdie & Melville, 1991					
<i>Griphobilharzia</i> Platt, Blair, Purdie & Melville, 1991	No species sampled.				
Bilharziellinae Price, 1929					
<i>Bilharziella</i> , Looss, 1899					
<i>Bilharziella polonica</i> (Kowalewski, 1895)	ex <i>Anas platyrhynchos</i> ; Kherson Oblast, Ukraine.	✓	AY157186§	AY157214§	AY157240§
<i>Jilinobilharzia</i> Liu & Bai, 1976	No species sampled.	✓			
<i>Trichobilharzia</i> , Skrjabin & Zakharow, 1920					
<i>Trichobilharzia ocellata</i> (La Valette, 1855)	ex <i>Lymnaea stagnalis</i> ; Germany.	✓	AY157189§	AY157217§	AY157243§
<i>Trichobilharzia regenti</i> Horak, Kolarova & Dvorak, 1998	ex <i>Radix peregra</i> ; (experimental infection); Horak Lab., Prague, Czech Rep.	✓	AY157190§	AY157218§	AY157244§
<i>Trichobilharzia szidati</i> Neuhaus, 1952	ex <i>Lymnaea stagnalis</i> ; (experimental infection); Horak Lab., Prague, Czech Rep.	✓	AY157191§	AY157219§	AY157245§
Gigantobilharziinae Mehra, 1940					
<i>Dendritobilharzia</i> Skrjabin & Zakharow, 1920					
<i>Dendritobilharzia pulverulenta</i> (Braun, 1901)	ex <i>Gallus gallus</i> ; (experimental infection), Bernalillo County, New Mexico, USA.	✓	AY157187§	AY157215§	AY157241§
<i>Gigantobilharzia</i> Odhner, 1910					
<i>Gigantobilharzia huronensis</i> Najim, 1950	ex <i>Agelaius phoeniceus</i> ; Winnebago County, Wisconsin, USA.	✓	AY157188§	AY157216§	AY157242§
Sanguinicolidae					
<i>Chimaerohemecus trondheimensis</i> van der Land, 1967	ex <i>Chimaera monstrosa</i> ; Korsfiorden, near Bergen, Norway.		AY157185§	AY157213§	AY157239§

Table 2. Primers used for PCR amplification and sequencing of complete *lsrDNA* and COI(See Littlewood *et al.* (1999) for *ssrDNA* amplification and sequencing primers.)

Amplification and sequencing	Primer sequence (5'–3')
<i>lsrDNA</i>	
U178	GCACCCGCTGAAYTTAAG
L1642	CCAGCGCCATCCATTTTCA
U1148	GACCCGAAAGATGGTGAA
L2450	GCTTTGTTTTAATTAGACAGTCGGA
U1846	AGGCCGAAGTGGAGAAGG
L3449	ATTCTGACTTAGAGGGCGTTCA
COI	
Cox1_schist_5'	TCTTTTRGATCATAAGCG
Cox1_schist_3'	TAATGCATMGGAAAAACA
<i>Additional sequencing primers</i>	
<i>lsrDNA</i>	
300F	CAAGTACCGTGAGGGAAAGTTG
300R	CAACTTTCCCTCACGGTACTTG
EDC2	CCTTGGTCCGTGTTTCAAGACGGG
900F	CCGTCTTGAAACACGGACCAAG
1200F	CCCGAAAGATGGTGAACATATGC
1200R	GCATAGTTCACCATCTTTTCGG
1600F	AGCAGGACGGTGGCCATGGAAG
U2229	TACCCATATCCGCAGCAGGTCT
L2230	AGACCTGCTGCGGATATGGGT
U2562	AAACGGCGGGAGTAACATATGA
L2630	GGGAATCTCGTTAATCCATTCA
U2771	AGAGGTGTAGGATARGTGGGA
L2984	CTGAGCTCGCCTTAGGACACCT
U3119	TTAAGCAAGAGGTGTCAGAAAAGT
U3139	AAGTTACCACAGGGATAACTGGCT
LSU3_4160	GGTCTAAACCCAGCTCACGTTCCC
L3358	AACCTGCGGTTCCCTCTCGTACT
COI	
CO1560Fa	TTTGATCGTAAATTTGGTAC
CO1560Fb	TTTGATCGGAATTTTGGTAC
CO1560R	GCAGTACCAAATTTACGATC
CO1800F	CATCATATGTTTATGGTTGG
CO1800Ra	CCAACCATAAACATATGATG
CO1800Rb	CCAACCATAAACATGTGATG

parameters: lset nst=6, rates=invgamma, ncat=4, shape=estimate, inferrates=yes and basefreq=empirical, that approximates to a *GTR+I+G* model as above. Posterior probabilities were approximated over 200 000 generations, log-likelihood scores plotted and only the final 85% of trees where the log-likelihood had reached a plateau were used to produce the consensus tree.

In order to include more sites and test further the interrelationships of the *Schistosoma* species, a subset of the entire dataset comprising only the *Schistosoma* (but including *Orientobilharzia*) was analysed rooting against the most basal, East Asian clade, as determined in the full analyses.

Final tree topologies were tested against previous hypotheses of interrelationships by using ML alone on the combined data set to find the best constrained trees, and then applying the Shimodaira-Hasegawa test (Shimodaira & Hasegawa, 1999) as implemented

in PAUP\* with full optimization and 1000 bootstrap replicates, testing within and between the constrained and unconstrained topologies.

#### Character mapping and interpretation

The morphological character matrix of Carmichael (1984) (based on personal observations of many schistosomatid species, as well as on an extensive review of literature including Farley (1971)) was adapted, in order to interpret our molecular estimate of phylogeny in the context of morphology. Carmichael's matrix of 24 characters was taken in its entirety, but taxa not sampled in this study, namely Old and New World *Macrobilharzia* and *Bivitellobilharzia* and '*Sinobilharzia*', were omitted (see Fig. 1 legend). Characters that changed unambiguously were mapped on our phylogeny using MacClade

Table 3. Maximum likelihood parameter estimates

(All estimates are based on a general time reversible model of nucleotide substitution incorporating estimates of among-site rate variation (ASRV), estimated proportion of invariant sites (Inv-E), transition rates (Ts), transversion rates (Tv) and alpha shape parameter estimate of the gamma distribution ( $\alpha$ ). COI<sub>12</sub> and COI<sub>123</sub> indicate analyses using only the first two codon positions for cytochrome oxidase 1, and that using all 3 positions, respectively.)

Data partition	ASRV		Ts		Tv			
	$\alpha$	Inv-E	AG	CT	AC	AT	GC	GT
All taxa								
ssrDNA	0.652	0.539	5.021	7.039	0.901	1.842	0.660	1.000
lsrDNA	0.639	0.494	4.260	5.755	0.508	1.955	0.342	1.000
COI <sub>12</sub>	0.421	0.453	8.798	8.779	0.639	1.980	2.441	1.000
COI <sub>123</sub>	0.449	0.370	18.815	25.656	1.723	2.421	8.636	1.000
ssrDNA + lsrDNA + COI <sub>123</sub>	0.351	0.589	5.929	2.879	0.276	2.401	0.339	1.000
<i>Schistosoma</i> only								
ssrDNA + lsrDNA + COI <sub>123</sub>	0.438	0.743	7.101	2.648	0.133	2.762	0.263	1.000

(Maddison & Maddison, 2000) and treated as unweighted and unordered but were not recoded.

To further interpret the phylogeny, the Host-Parasite Database (H-PD) of The Natural History Museum (Gibson & Bray, 1994), see [www.nhm.ac.uk/zoology/hp-dat.htm](http://www.nhm.ac.uk/zoology/hp-dat.htm), was used to code the snail genera used as intermediate hosts by the taxa studied here as well as those snail genera used by the other schistosomatid species not available in our molecular study. Using a variety of literature, including Carmichael (1984), Farley (1971) and the H-PD, the biogeographical distribution of species and genera included in our phylogenetic estimates was also examined. It should be noted that relying on the literature may incorporate certain errors, particularly where authors have misidentified parasite or host taxa. The best test for host associations is full, experimentally determined, life-cycle information but this is unavailable for most taxa. Finally, mitochondrial gene arrangements, based on published and unpublished observations of taxa used in this study were coded or inferred according to phylogenetic position and mapped on the phylogeny.

## RESULTS

Accession numbers for each gene sequenced are shown in Table 1. Only ML solutions are presented for each gene, as BI methods yielded identical tree topologies throughout and MP produced only minor deviations in some cases. The full *GTR+I+G* model parameters for each data partition are shown in Table 3. Major associations for each individual gene are presented below, but the full detail of species interrelationships is restricted to the combined evidence solution (Fig. 5 below).

### Cytochrome oxidase I

A total of 1139 sites were available for alignment, of which 1122 were unambiguously aligned. Of the

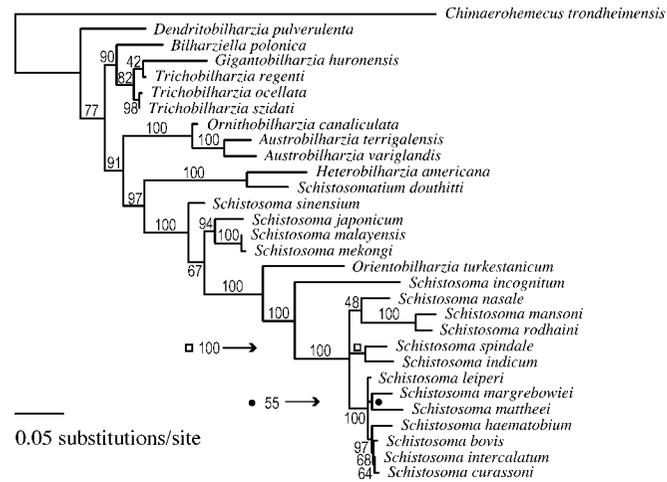
aligned positions 524 were constant and 498 parsimony informative. Removing third codon positions resulted in 748 included positions, of which 496 were constant and 180 parsimony informative. Phylogenetic estimates using the first 2 and all 3 codon positions are shown in Fig. 2A and B, respectively. The trees are almost identical in topology, suggesting none or insignificant levels of bases saturation, but with longer branch lengths for all taxa and greater resolution among the more derived *Schistosoma* taxa when all 3 positions were included (Fig. 2B). *Dendrobilharzia* falls as the most basal taxon with other bird schistosomes radiating first with a (*Bilharziella* + *Gigantobilharzia* + *Trichobilharzia*) clade and then the (*Ornithobilharzia* + *Austrobilharzia*) clade. The mammalian schistosomes are split into 2 major clades, namely (*Heterobilharzia* + *Schistosomatium*) and (*Schistosoma* + *Orientobilharzia*).

Where multiple exemplars of genera were sampled, only *Schistosoma* appears non-monophyletic, due to the placement of *Orientobilharzia*. All schistosome species appear to be well differentiated from one another, in terms of molecular distance, except *Trichobilharzia szidati* and *T. ocellata*, which are almost identical. For COI these taxa differ in 9 bases out of 1125 bp (0.008) and all differences are at synonymous sites. Poorly resolved nodes, as judged by relatively low Bayesian support, include the relative placement of the bird schistosome genera, the (*Ornithobilharzia* + *Austrobilharzia*) clade, and the most derived members of the African *Schistosoma*. Otherwise the gene provides a high proportion of informative positions, at least as judged by parsimony, for its relatively short length.

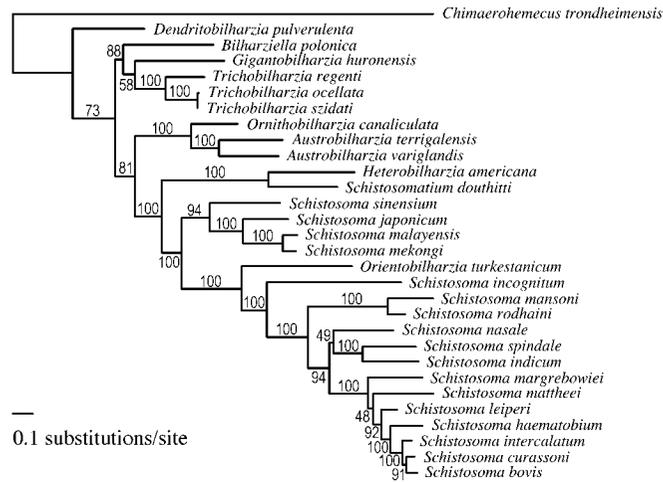
### ssrDNA

A total of 1937 sites were available for alignment, of which 1831 were unambiguously aligned. Of the aligned positions 1526 were constant and 145 parsimony informative. The phylogenetic estimate

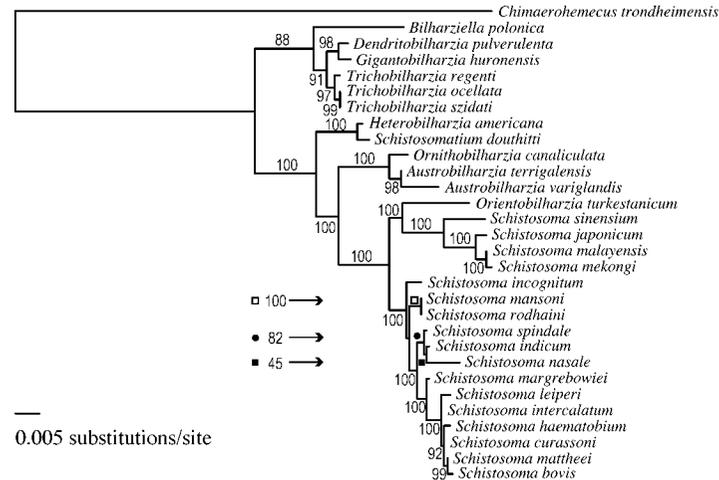
A CO1 (12)



B CO1 (123)



C SSU rDNA



D LSU rDNA

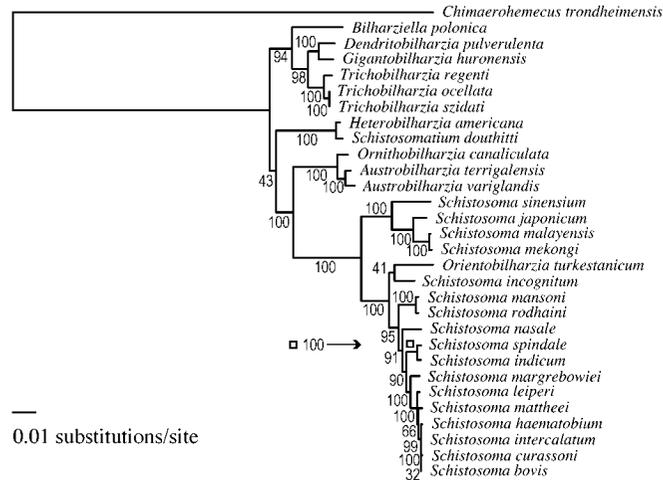


Fig. 2. Maximum likelihood estimates of the interrelationships of the Schistosomatidae from individual gene fragments. (A) Mitochondrial CO1 using only the first 2 codon positions. (B) Mitochondrial CO1 using all 3 codon positions. (C) Nuclear small subunit rDNA. (D) Nuclear large subunit rDNA. Nodal support values are posterior probabilities (expressed as percentages) from Bayesian analyses for each of the same data partitions.

afforded by this gene is shown in Fig. 2C. In contrast to COI, *Dendritobilharzia* did not appear as the most basal taxon. Instead, a larger clade including (*Dendritobilharzia* + *Bilharziella* + *Gigantobilharzia* + *Trichobilharzia*) occupies this position. Also in contrast to COI, the next clade is (*Heterobilharzia* + *Schistosomatium*) making the mammal schistosomes paraphyletic and giving (*Ornithobilharzia* + *Austrotilharzia*) as the sister group to (*Schistosoma* + *Orientobilharzia*). It is noteworthy that the interrelationships within *Schistosoma* are almost identical to COI although *Orientobilharzia* falls in the same clade as the East Asian *Schistosoma* (*S. sinensium*, *S. japonicum*, *S. malayensis* and *S. mekongi*), rather than basal to the African and Indian species. In the case of *Trichobilharzia*, *T. szidati* and *T. ocellata* differ by just 1 base change in 1868 bp (0.0005).

#### lsrDNA

A total of 3950 sites were available for alignment, of which 3765 were unambiguously aligned. Of the aligned positions 2900 were constant and 470 parsimony informative. The tree is shown in Fig. 2D and is almost identical in topology to that of ssrDNA except in one important aspect. As with COI, *Orientobilharzia* groups with *S. incognitum*, although with poor nodal support. Poor nodal support also characterizes the relative position of the (*Heterobilharzia* + *Schistosomatium*) clade and the interrelationships of the most derived African *Schistosoma*. In the case of *Trichobilharzia*, *T. szidati* and *T. ocellata* differ by 7 base changes in 3856 bp (0.0018).

#### Combined COI, ssrDNA and lsrDNA

The partition homogeneity test (ILD; incongruence length difference test), using 100 test replicates including parsimony informative sites only, indicated no significant difference in signal between the 3 genes for the ingroup ( $P=0.09$ ), and therefore passed a test for conditional combination of independent datasets. Considering that this test has been demonstrated to fail in detecting congruence when dealing with heterogeneous datasets, such as mitochondrial and nuclear gene sequences, the fact that no significant difference was found, adds greater confidence in combining our data (Dowton & Austin, 2002). The combined data were analysed in full, and also for the (*Schistosoma* + *Orientobilharzia*) clade alone (ILD;  $P=0.65$ ). Results of the full analysis are shown in Fig. 3. The avian clade is the same as with ssrDNA and lsrDNA alone, and the interrelationships of these schistosomes remains (*Bilharziella* (*Trichobilharzia*, (*Dendritobilharzia*, *Gigantobilharzia*))). The next 2 major clades appear as with ssrDNA and lsrDNA individually, but very poor nodal support means that the true position of (*Heterobilharzia* + *Schistosomatium*) may not be fully resolvable with these data

alone. However, even with this node unresolved, it appears that mammalian schistosomes are paraphyletic and, as with the individual rRNA genes, the full analysis including COI resolves the bird schistosome clade (*Ornithobilharzia* + *Austrotilharzia*) as the sister group to the (*Schistosoma* + *Orientobilharzia*) clade. It is clear that *Orientobilharzia turkestanicum* is a member of the *Schistosoma* clade. The *Schistosoma* split into 2 lineages, the East Asian species and the rest. Within the East Asian clade, *S. sinensium* was the first to diverge, followed by *S. japonicum*. *Orientobilharzia* and *S. incognitum* separate the East Asian *Schistosoma* from the remaining schistosomes, but the relatively poor nodal support for *S. incognitum* suggests it may occupy a clade with *Orientobilharzia* (as suggested, also weakly, by the lsrDNA analysis). Of the remaining taxa, *S. mansoni* and *S. rodhaini* form a well-supported clade as do *S. spindale* and *S. indicum*. *S. nasale* is very weakly supported (by bootstrap analysis) as the sister group to *S. spindale* and *S. indicum* in the full analysis and its position remains unresolved with these and the clade of more derived taxa in the analysis of *Schistosoma* taxa alone. Indeed, little resolution was gained in analysing *Schistosoma* alone (results not shown). Only an additional 127 sites were re-included in the alignment (3 for COI; 46 for ssrDNA; 78 for lsrDNA) and the topology within the (*Schistosoma* + *Orientobilharzia*) clade remained essentially the same as with the full analysis except that the relationships between the 3 most derived taxa were marginally better supported as (*S. intercalatum* (*S. curassoni*, *S. bovis*)) by both bootstrap analysis using maximum likelihood and the proportion of best Bayesian trees supporting the nodes.

#### Constraint analyses

Constraint analyses were performed in order to test whether the combined data set argued significantly against specific topologies that were different from the fully resolved, unconstrained solution provided by ML, MP and BI (shown in Fig. 3). In particular to test: (a) the avian schistosomes as a monophyletic clade; (b) the mammalian schistosomes as monophyletic; (c) the major avian clade including *Bilharziella* as the sister group to *Schistosoma*; (d) the (*Heterobilharzia* + *Schistosomatium*) clade as the sister group to *Schistosoma* (a slight variation on simply holding mammalian schistosomes as monophyletic); (e) *Orientobilharzia* and *S. incognitum* as a monophyletic group, as suggested by lsrDNA alone (Fig. 2D); (f) *Orientobilharzia* belonging in a clade with the East Asian *Schistosoma* as suggested by ssrDNA alone (Fig. 2C); (g) the 'indicum' species group as monophyletic. Results are shown in Table 4. Of all of these permutations 2 hypotheses are clearly rejected by the full implementation of the Shimodaira-Hasegawa test. These were that *Orientobilharzia* falls in a clade

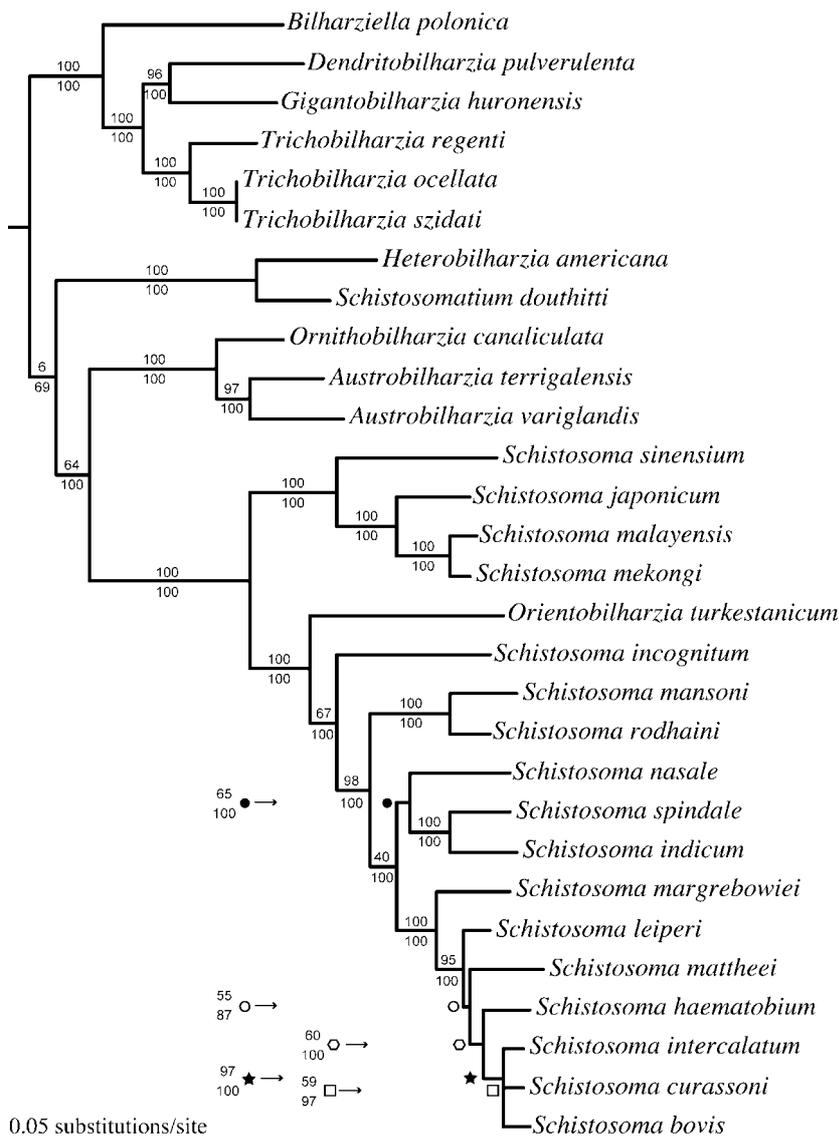


Fig. 3. Maximum likelihood estimate of the interrelationships of the Schistosomatidae from the combined analysis of data partitions (ssrDNA, lsrDNA and CO1 employing all 3 codon positions). Nodal support values are bootstrap values shown above ( $n=100$ ), and Bayesian (posterior) probabilities expressed as percentages, shown below branches.

with the East Asian *Schistosoma* and that the 'indicum' species group is monophyletic; *S. incognitum* does not cluster with the other 'indicum' group species, but lies basal to the schistosomes of Africa and the Indian subcontinent.

#### Mapping morphological characters

Six morphological characters from Khalil (2002) were taken as synapomorphies for the Schistosomatidae and a seventh character inferred from our tree, namely the presence of a gynaecophoric canal; Morand & Müller-Graf (2000) also recognized this character as plesiomorphic for the Schistosomatidae. All other characters were taken directly from Carmichael (1984) and mapped onto the combined evidence topology shown in Fig. 3 (see Fig. 4; character

numbers corresponding to the codes used by Morand & Müller-Graf (2000) are also indicated). Carmichael's thesis deals only at the genus level and so the combined evidence tree is reduced to reflect this. At this level, all clades are supported by at least two synapomorphies except the union of (*Ornithobilharzia* + *Austrobilharzia*), which is strongly supported by all molecular data but no obvious morphological traits. Support for the sister group status between (*Ornithobilharzia* + *Austrobilharzia*) and (*Schistosoma* + *Orientobilharzia*) emerges from 2 synapomorphies; vitelline follicles paired along a common caecum and the female common caecum being long and straight. Four synapomorphies unite *Schistosoma* and *Orientobilharzia*; the absence of Laurer's Canal and a cirrus; weak coiling of the ovary and possession of a small, globular seminal vesicle.

Table 4. Results of constraint analyses on the combined data set testing for the likelihood of accepting alternative tree topologies

(See text for further details. Log likelihood values, their differences with respect to the unconstrained solution and the significance of the constraints tested by the Shimodaira-Hasegawa test as implemented in PAUP\* on ML trees are indicated;  $P < 0.05$  indicates a significantly different topology (†).)

Constraint*	−lnL	diff. −lnL	P
Unconstrained	34170.47		
Avian schistosomes monophyletic	34180.50	10.03	0.522
Mammalian schistosomes monophyletic	34179.01	8.54	0.564
<i>Bilharziella</i> clade <sup>1</sup> as sister to <i>Schistosoma</i>	34180.50	10.03	0.522
<i>Heterobilharzia</i> clade <sup>2</sup> as sister to <i>Schistosoma</i>	34179.01	8.54	0.564
<i>Orientobilharzia</i> + <i>S. incognitum</i> monophyletic	34186.67	16.20	0.356
<i>Orientobilharzia</i> + E. Asian <i>Schistosoma</i> monophyletic <sup>3</sup>	34212.00	41.53	0.035†
' <i>indicum</i> ' species group monophyletic <sup>4</sup>	34228.01	57.54	0.006†

\* <sup>1</sup>*Bilharziella*, *Dendritobilharzia*, *Gigantobilharzia* and *Trichobilharzia*; <sup>2</sup>*Heterobilharzia* and *Schistosomatium*; <sup>3</sup>*S. sinensium*, *S. japonicum*, *S. malayensis*, *S. mekongi*; <sup>4</sup>*S. incognitum*, *S. spindale*, *S. nasale* and *S. indicum*.

## DISCUSSION

The combination of 3 genes has provided a phylogeny of the Schistosomatidae that is reasonably well resolved at all levels within the tree. The full data set estimated a tree that is unique when compared to the estimates from the individual nuclear ribosomal DNA and mitochondrial cytochrome oxidase I gene trees, but the same broad patterns emerge from each. The main avian clade includes *Bilharziella*, *Trichobilharzia*, *Dendritobilharzia*, and *Gigantobilharzia*; hereafter referred to as the *BTDG* clade. Its sister clade includes the mammalian taxa, but also some avian schistosomes. Within this clade, the next group to diverge is less certain, based on nodal support alone. The nuclear ribosomal genes both suggest the mammalian clade (*Heterobilharzia* + *Schistosomatium*) (the *HS* clade) but COI supports the remaining avian genera (*Ornithobilharzia* + *Austrobilharzia*). The full analysis reflects the individual ribosomal solutions. Intuitively it might be expected that avian clades gave rise to a monophyletic mammalian clade, with the adoption of a mammalian vertebrate host as a single evolutionary event. Constraint analyses failed to reject either of these two clades as the true sister group to *Schistosoma* and so phylogenetic analyses were conducted without the use of an outgroup (results not shown) since the poor support for these internal deep nodes may be related to the long-branching outgroup spuriously polarizing these basal taxa (Felsenstein, 1978). However, the unrooted phylograms from all analyses reflected the same patterns in each rooted analysis and the final, full data set indicated a very short branch length between the *BTDG* and *HS* clades that, in turn, resulted in relatively low bootstrap support at this critical node. Although support for the relative positions of the *BTDG* and *HS* clades remains problematic when assessed in isolation, Bayesian inference strongly recognized *Ornithobilharzia* + *Austrobilharzia* as the sister group to the *Schistosoma* clade, and we consider

the full analysis to be the best available estimate. Indeed, the clade is also well supported morphologically (see below). The topology from our combined evidence solution provides the foundation for the following discussion. Specific implications suggested by this new topology are discussed below.

### Systematics and taxonomy

The overall topology of the schistosomatid genera is identical to that presented by Snyder & Loker (2000), based on partial *lsrDNA*, except for the position of the root. Snyder & Loker (2000) resolved *Schistosoma* + *Orientobilharzia* as a sister clade to the other schistosomes, but all other relationships are the same. Thus, this study confirms that at least one higher taxonomic group is now clearly challenged. The Schistosomatidae has been subdivided into 4 subfamilies. Leaving aside *Griphobilharziinae*, for which no samples were obtained, of the others *Gigantobilharziinae* (Mehra, 1940) as amended by Farley (1971) comprises the genera *Dendritobilharzia* and *Gigantobilharzia* and remains a valid taxon in our scheme. Likewise, the Schistosomatinae Stiles & Hassall, 1926, which includes all remaining taxa other than *Bilharziella* and *Trichobilharzia*, also remains monophyletic in this study. However, *Bilharziella* and *Trichobilharzia* do not form a monophyletic clade and therefore the subfamily *Bilharziellinae* Price, 1929 can be rejected. Although Carmichael (1984) listed 2 characters that appeared synapomorphic for the *Bilharziellinae*, namely the presence of a gynaecophoric canal only surrounding the genital pore (Carmichael's character 4.2), and the male genital pore well behind the acetabulum and caecal reunion (Carmichael's character 20.2), neither Carmichael (1984) nor Morand & Müller-Graf (2000) favoured the relationship in their final trees. A further consequence of rejecting the *Bilharziellinae* is that it is not possible to speculate on the position of *Jilinobilharzia*, within which there are only 2 species,

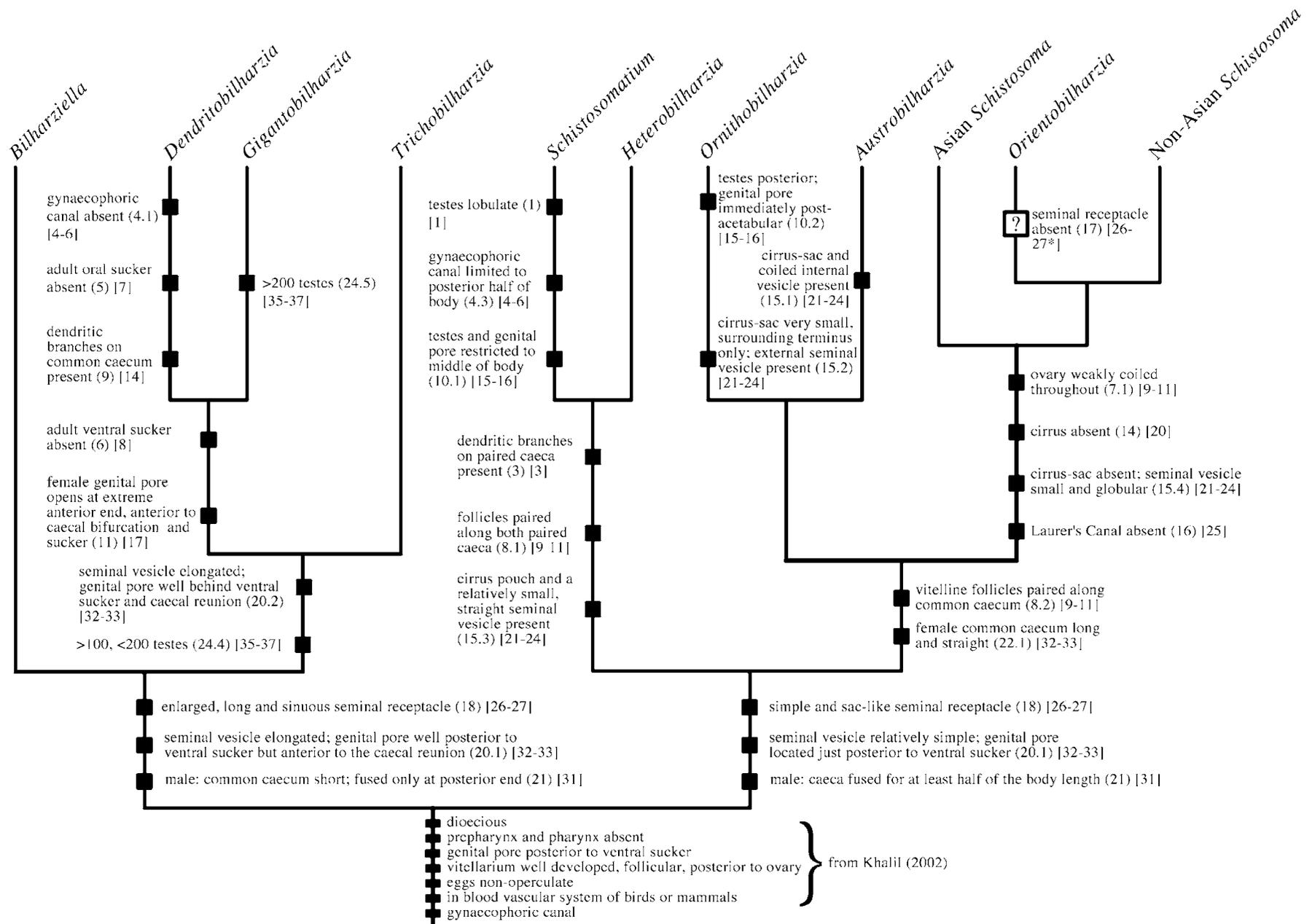


Fig. 4. For legend see facing page.

beyond suggesting that it will likely fall within the (BTDG) clade.

In order to evaluate our combined evidence tree in the light of morphology, Carmichael's characters (Carmichael, 1984) were mapped on to the tree; the codes used by Morand & Müller-Graf (2000) are also shown. Highlighting only those characters changing unambiguously (i.e. with a consistency index of 1) the character changes are indicated in Fig. 4. In spite of short branch lengths and relatively poor nodal support at the deeper nodes within our tree, a satisfying number of morphological synapomorphies exist at critical nodes throughout the tree. The Schistosomatinae is supported by 3 characters (Carmichael's characters 18, 20·1, 21), and the sister group status of (*Ornithobilharzia* + *Austrotilharzia*) to *Schistosoma* is supported by 2 characters concerning the arrangement of the vitelline follicles (8·2) and the length and shape of the female common caecum (22·1). Four characters (7·1, 14, 15·4, and 16) support the re-inclusion of *Orientobilharzia* within *Schistosoma*, a relationship originally proposed until revised by Dutt & Srivastava (1961). More recently this relationship was resurrected by Snyder & Loker (2000) using partial lsrDNA; supported by ITS2 (Zhang *et al.* 2001) and again partial lsrDNA (Attwood *et al.* 2002b); and is confirmed by the present study. *Orientobilharzia* is traditionally distinguished from *Schistosoma* by the large number of testes (usually >50) present in *Orientobilharzia* (Farley, 1971; Khalil, 2002). Morand & Müller-Graf (2000) demonstrated that testes number varied widely and not systematically throughout the family, suggesting this is not a good character for phylogenetic purposes. The only autapomorphy for the genus *Orientobilharzia* (the absence of a seminal receptacle, character 17) may be erroneous, as Carmichael (1984), based on preliminary observations, reported that further study may well reveal the presence of the structure in the 'genus'. It is clear, however, that the genus *Orientobilharzia* is synonymous with *Schistosoma*. A revision of the genus *Orientobilharzia*, ideally supported by molecular evidence, is required.

Within *Schistosoma* the traditional species groups, based partly on egg morphology, are only marginally compromised by our scheme. The 'japonicum', 'mansoni' and 'haematobium' species groups remain intact but the position of *S. incognitum* renders the 'indicum' group either redundant, or restricted to *S. nasale*, *S. spindale* and *S. indicum*. Egg morphology within the group, with or without *S. incognitum*, is still highly divergent. Of those *Schistosoma* species

not available to us, the position of *S. hippopotami* and *S. edwardiense* remains problematic. Although nominally included in the 'mansoni' species group on the basis of lateral-spined eggs, in the single molecular study that used ITS2 from *S. hippopotami* (Després *et al.* 1995), it failed to cluster with the *S. mansoni* group. *S. hippopotami* does show morphological similarities to *S. incognitum* at both egg and adult stages (Thurston, 1963) although the intermediate snail host is not known. Since our results remove *S. incognitum* from the 'indicum' group, it seems likely that these two species could show a close affiliation. No sequence data have ever been obtained from *S. edwardiense* and its position is undetermined, although this species is believed to use *Biomphalaria* as a snail host (as do *S. mansoni* and *S. rodhaini*) (Pitchford & Visser, 1981).

Attwood *et al.* (2002a) described *S. ovuncatum* of northern Thailand as a new species, distinct from *S. sinensium* of Sichuan, China, on the basis of clear morphological difference; the eggs of *S. ovuncatum* bear a small hook-like subterminal spine. Attwood *et al.* (2002b) provided partial lsrDNA and mitochondrial 12S rDNA sequences for specimens from the type population of *S. ovuncatum* and these indicated that this taxon is the sister species of *S. sinensium* from China (0·8% of sites were polymorphic at the 28S locus and 5·3% at the 12S). Denser sampling within *Schistosoma* species may indicate high genetic divergence, as found within *S. sinensium* sampled between Thailand and China (Agatsuma *et al.* 2001) and for *S. intercalatum* using RAPD data (Kaukas *et al.* 1994). Divergence levels within *S. sinensium* exceed the divergence between *S. malayensis* and *S. mekongi* and it seems likely that further species will be described. In contrast, sampling of large fragments of mitochondrial DNA within *S. malayensis* and *S. mekongi* populations has shown limited variation (Le, Blair & McManus, 2002b).

Our tree suggests one other taxonomically important taxonomic feature. The very close relationship, and almost identical nucleotide sequences between *Trichobilharzia ocellata* and *T. szidati*, suggests they may be synonymous. A wider study including denser molecular sampling of populations and a re-evaluation of purported morphological differences will help to confirm this.

#### Host identity and host associations

Schistosomatids use snails from across a wide phylogenetic range within the Gastropoda and appear to

Fig. 4. Interpretation of the evolutionary radiation of the Schistosomatidae with the acquisition and loss of key features. Numbers in rounded brackets are the characters from Carmichael (1984), while those in square brackets the numbers assigned for the same characters by Morand & Müller-Graf (2000). Character 17 of Carmichael (1984), is marked as '?'; although absence of a seminal receptacle is a characteristic of *Orientobilharzia*, Carmichael (1984), suggested that such a structure may have been visible in one specimen he observed (USNHC #45820). \* – Morand & Müller-Graf (2000) coded the seminal receptacle using two characters (26 and 27); for *Orientobilharzia* it was coded as 'simple and sac-like' rather than absent.

have switched intermediate host across considerable phylogenetic distances (Blair *et al.* 2001). Our own review of the literature, to assess mollusc families indicated as intermediate hosts to schistosomatids in the wild, is recorded in Fig. 5. Data for individual species used in this study and the genera as a whole are indicated. As a literature review, this figure reflects the completeness and accuracy of the published data. This is not without its difficulties due to the problems in identifying mollusc species and emerging cercariae and it is recognized that the list may include anomalous associations that require at least resampling and at best experimental verification. Our review of this literature suggested that the schistosomatids are generalists in their use of intermediate snail hosts. The same data are presented in Fig. 6 with phylogenies of the molluscs and parasites interlinked by their recorded associations. The basic mollusc phylogeny is adapted from the tree presented by Blair *et al.* (2001). The interrelationships of the cerithioidean gastropods are adapted from the molecular systematic analysis of Lydeard *et al.* (2002). Snail host and parasite phylogenies and associations were drawn with the aid of TREEMAP (Page, 1995) in an attempt to identify any patterns of cospeciation. A heuristic search, superimposing the parasite tree on the host tree resolved only 1 cospeciation event and 101 sorting events (see Page, 1994). Intermediate host associations provide little evidence as to the interrelationships of the parasites at the generic level, and it seems likely that there have been several host-switching events into related taxa. However, the 'tanglegram' indicates the huge variety of snails used by schistosomatids and also indicates how clades within *Schistosoma* have radiated predominantly within 3 families of snails. The East Asian clade is restricted to the Pomatiopsidae (Gastropoda: Caenogastropoda), while all other species are restricted to the Planorbidae with the exception of *S. incognitum* and *Orientobilharzia*. These species both use snails of the Lymnaeidae. Only the other mammalian clade (*Heterobilharzia* + *Schistosomatium*) shows a restricted use of snail hosts (Lymnaeidae) whereas it is the avian schistosomes that appear to be transmitted by the greatest diversity of snails. Caenogastropod hosts are only used by the East Asian *Schistosoma* clade, the (*Ornithobilharzia* + *Austrotilharzia*) clade, *Gigantobilharzia* and 1 species of *Trichobilharzia* (*T. corvi*), and again, it is the *Schistosoma* that are restricted to a single snail family (Pomatiopsidae). Blair (2001) considered pulmonates to be the ancestral hosts for the schistosomatids, with individual host switching events accounting for those using caenogastropod hosts, but the basal position of *Austrotilharzia* and *Ornithobilharzia* in their tree meant that they could not be certain. Our tree, with *Ornithobilharzia* + *Austrotilharzia* as sister group to the *Schistosoma*, adds weight to his argument that association with pulmonate host is the plesiomorphic condition.

Finally, Fig. 5 highlights situations in which schistosomes can become established in new regions. A case in point is the occurrence of an *S. haematobium*-like species infecting humans in India, which may be transmitted by *Ferrissia tenuis*, an ancyloid snail (Southgate & Agrawal, 1990). Although this parasite has never been formally identified and other, perhaps more suitable, hosts are present in the reported focus, this unusual occurrence readily demonstrates the ease with which schistosomes can establish themselves when suitable hosts are present.

#### *Geographical distribution and historical biogeography*

As with intermediate host identity, the geographical distribution of taxa studied here, and other members of the genera, is indicated in Fig. 5, again produced from reviewing published work. Not surprisingly, avian schistosomes have a very broad distribution. The most basal avian clade has achieved an almost global distribution with species found in all regions except South America. The wide dispersal of bird parasites is easy to envisage, but the avian schistosomatids' success must also be due to their ability to utilize a variety of molluscan hosts.

With respect to the possible origin and spread of the mammalian genera, *Schistosoma* and *Orientobilharzia* (assuming that current distribution is indicative of past events) the Asian origin has been confirmed, with the 'japonicum' group clearly basal to the African and Indian schistosomes. Fig. 7A is a synthesis of Snyder and Loker's hypothesis as reviewed by Morgan *et al.* (2001). Most interestingly *S. incognitum*, found in India as well as Thailand and Indonesia, diverges early in the phylogeny suggesting that ancestors of the *Schistosoma* may have entered the Indian subcontinent before Africa; see Fig. 7B for a diagram of our interpretation; Attwood *et al.* (2002b) provide finer-scale hypotheses on the movement of Asian *Schistosoma* into and from the Indian subcontinent. Our results suggest that there has been movement east across Asia giving rise to *Orientobilharzia* and *S. incognitum* followed by colonization of Africa from India. There followed a reinvasion of the Indian subcontinent by the ancestor to the 'indicum' species group, which radiated to form *S. nasale*, *S. spindale* and *S. indicum*, whilst members of the 'haematobium' group continued to radiate within Africa. Such a scenario is dependent upon the position of *S. nasale* within a monophyletic 'indicum' group suggested by the combined evidence. The most recent and most easily dated dispersal (~500 YA) was *S. mansoni* to S. America via the African slave trade (Després *et al.* 1993), where its subsequent establishment was due to the presence of suitable snail hosts (Campbell *et al.* 2000; DeJong *et al.* 2001). The occurrence of 2 lineages within Africa has been recognized for a long time and each lineage has independently given rise to schistosomes that infect

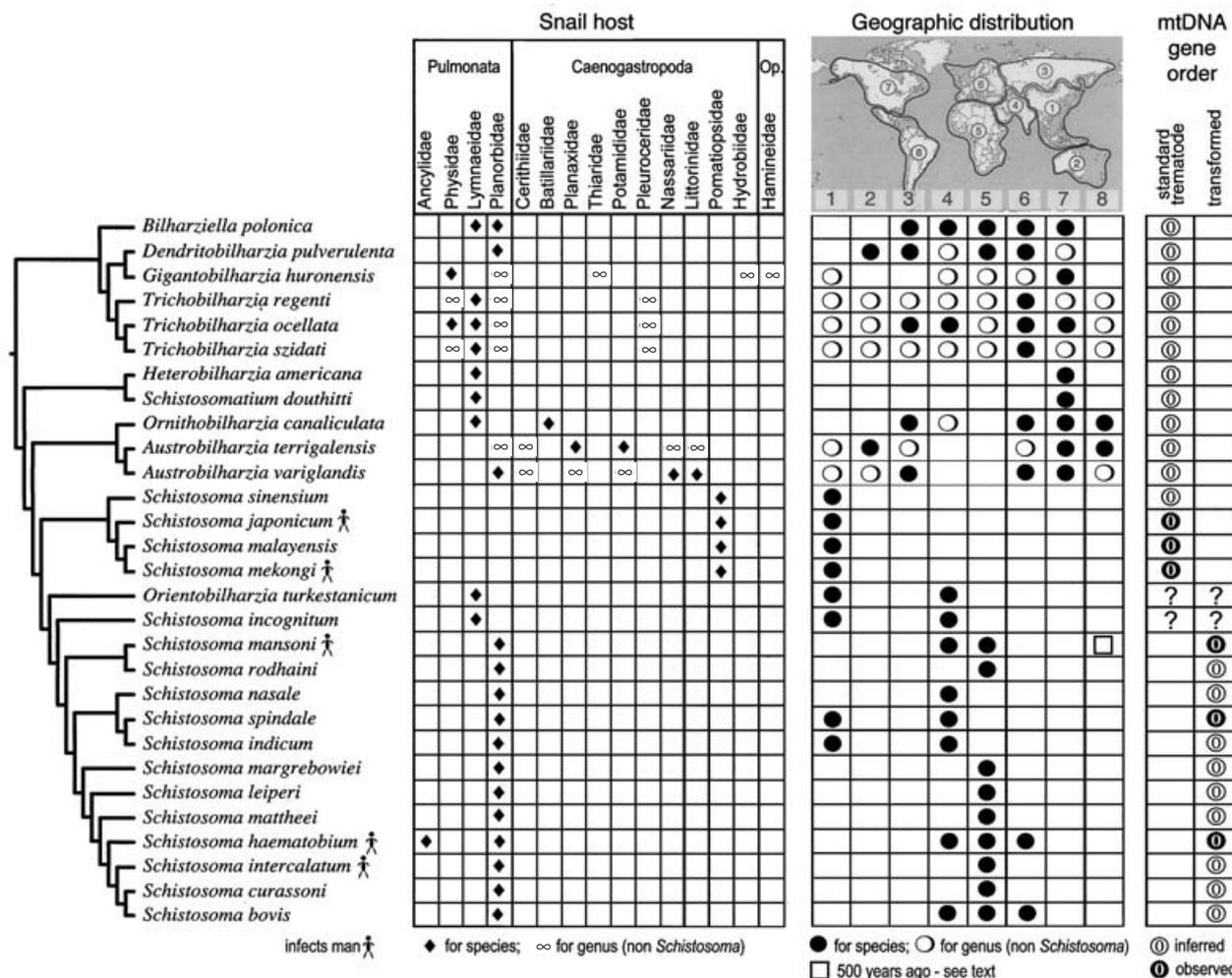


Fig. 5. Intermediate host associations, geographical range and mitochondrial gene order for species studied here, and for all members of each genera (not *Schistosoma*) as determined from the literature (predominantly the NHM's Host-Parasite Database) or, in the case of mtDNA, inferred from experimental data. Parasites that infect humans are indicated. Op, Opisthobranchia. Snail host associations: (◆) indicates that the named species uses that snail family as host; (∞) indicates that other species within the genus associate with the host family. Geographical distribution: (●) indicates that the named species occupies the specific geographic range indicated; (○) indicates that other species within the genus occupy the range indicated.

man, suggesting multiple lateral transfers between hosts, in the case of *S. mansoni* from rodents and for *S. haematobium* and *S. intercalatum* from ungulates (Combes, 1990). A minimum of 3 independent origins of schistosomes in humans can be scored from our phylogeny. To answer the question posed in the title of Zhang *et al.*'s study (2001), there were at least 1 Asian and 2 African evolutionary origins for human schistosomes. Once associated with humans, the dispersal of schistosomes may have occurred via early humans and their domesticated animals. The fact that schistosomes infecting man seem to be the result of a number of lateral transfers in different lineages indicates that schistosomes were already widespread before the evolution and spread of *Homo*. This suggests that non-human mammal migration is most likely to be responsible for the continental dispersal of *Schistosoma* species. Attwood *et al.* (2002a, b)

regarded climate driven dispersal of rodents and other small mammals, rather than human involvement, as a key factor in the early radiation of *Schistosoma*.

*Mitochondrial genomes*

Le *et al.* (2000) reported a remarkable difference in mitochondrial gene order for *S. mansoni* when compared to other parasitic platyhelminths, and indeed other *Schistosoma* species. Comparing sequenced and characterized mitochondrial genomes for 4 species of *Schistosoma*, 2 other digeneans and 4 cestodes, Le *et al.* (2001; Le, Blair & McManus, 2002a) revealed that *S. mansoni* exhibited a major translocation involving the genes *atp6*, *nad2* and *trnaA* and a rearrangement of *nad3* and *nad1*. All the East Asian schistosomes (*S. japonicum*, *S. mekongi* and *S. malayensis*) essentially exhibit the same gene

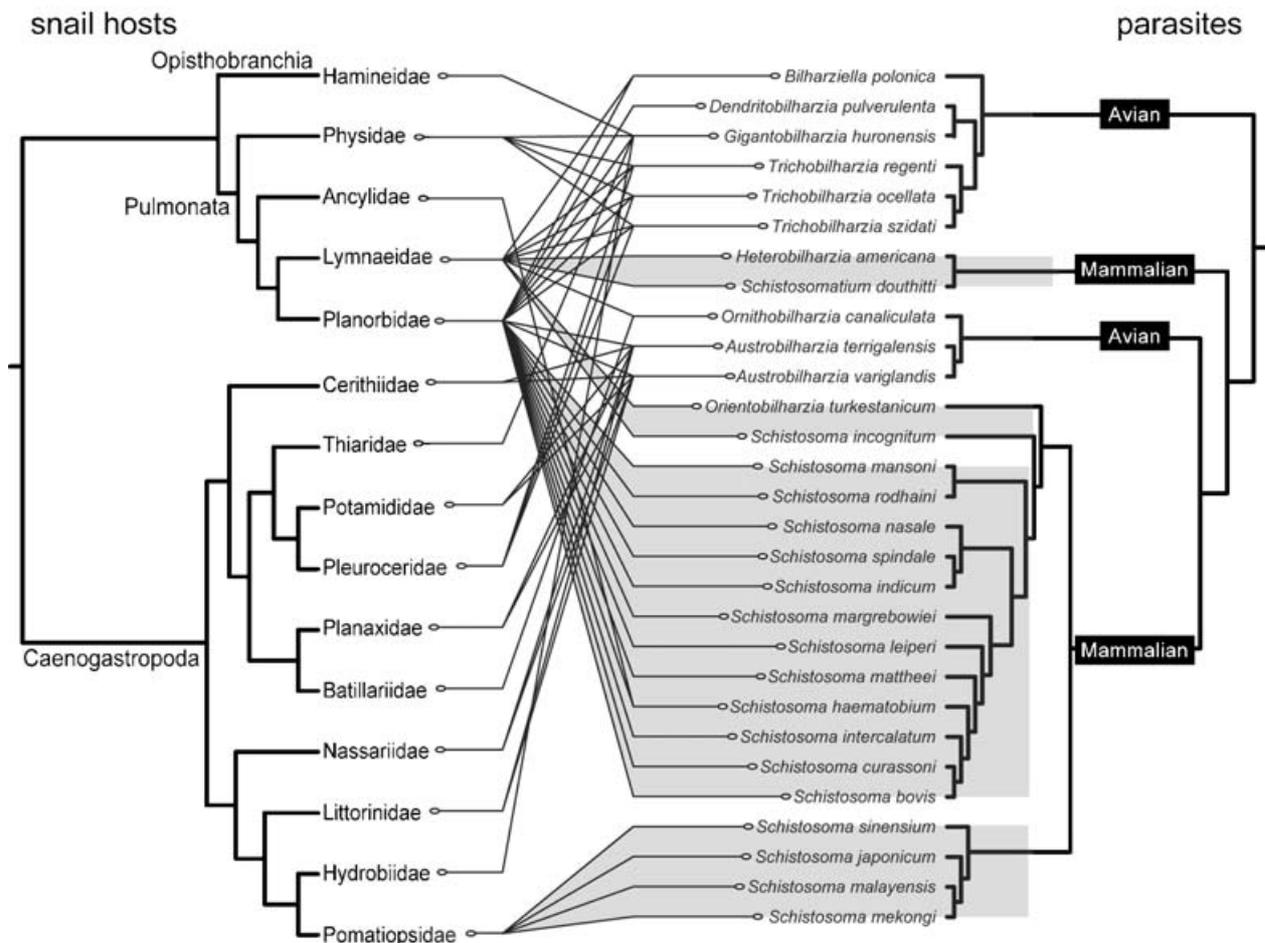


Fig. 6. TREE-MAP (Page, 1995) plot of snail host and schistosome parasite associations in relation to their respective phylogenies. Avian and mammalian schistosome lineages are indicated. Shaded areas represent snail family associations with the mammalian schistosome clades.

order as other digeneans and cestodes examined to date, suggesting that they have the plesiomorphic condition for the Digenea. It can be inferred therefore, that taxa basal to the East Asian schistosomes also have the plesiomorphic condition and it has recently been confirmed that *S. spindale* and *S. haematobium* have the same gene order as *S. mansoni* (unpublished results). This same gene order is also suggested for 6 other African schistosome species (*S. bovis*, *S. curassoni*, *S. intercalatum*, *S. margrebowiei*, *S. mattheei* and *S. rodhaini*), based on length and preliminary sequencing of a 2.6 kb fragment of mitochondrial DNA amplified from all 6 species using universal primers (unpublished results). It is therefore inferred that all taxa on our tree from *S. mansoni* to the most derived African taxa have this same gene order. However, the gene order of *Orientobilharzia* and *S. incognitum* is unknown and since they are likely to hold the key as to when and, based on biogeography, perhaps where the major rearrangement and translocation events occurred, we are currently characterizing these genomes.

The phylogeny presented here, based on 1 mitochondrial protein-coding and 2 nuclear ribosomal

RNA genes, provides a robust assessment of inter-relationships within the Schistosomatidae and, in particular, within 18 species of a revised genus *Schistosoma* that includes *Orientobilharzia*. Four major clades radiated within the family, reflecting 2 radiations within birds and 2 within mammals. Mammalian schistosomes are relatively restricted in their biogeographical distribution and their use of snail intermediate hosts. Avian schistosomes are more widespread and use a far greater diversity of snail families. There is little evidence for cospeciation between snail families and parasites, although finer scale phylogenies within snail families hosting mammalian schistosomes may subsequently reveal such patterns. We resolved an avian clade, including *Austrobilharzia* and *Ornithobilharzia* as sister group to *Schistosoma*, showing that colonization of mammalian host was not a single event. Within *Schistosoma*, the East Asian taxa are most basal, confirming an Asian origin for the genus. Subsequent movement of *Schistosoma* eastwards across Asia resulted in an invasion of Africa, suggested by the position of *S. mansoni* and *S. rodhaini*, a re-invasion of central Asia and the Indian subcontinent, suggested by the positions of

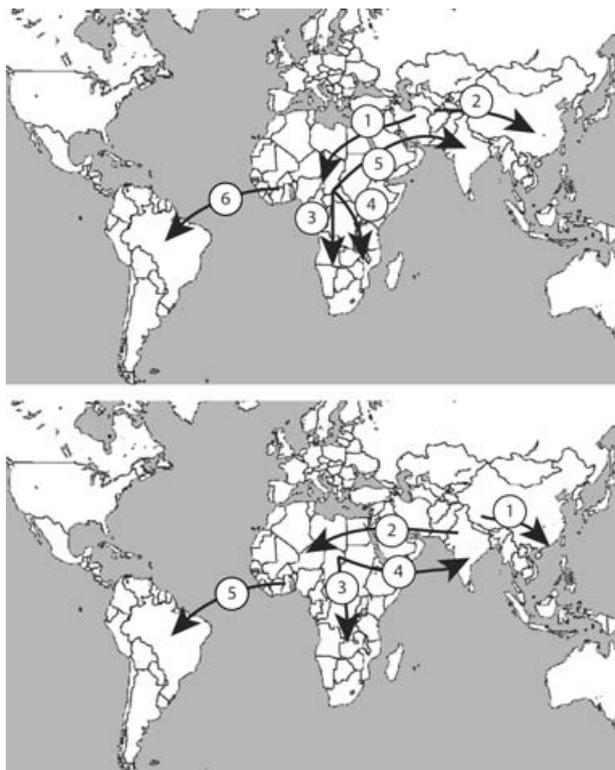


Fig. 7. Hypotheses on the Asian origin and dispersal of *Schistosoma*. (A) Asian origin (after Morgan *et al.* 2001; Snyder & Loker, 2000). (1) The ancestral Asian *Schistosoma* moves to Africa via widespread mammal migration. (2) The *Schistosoma* ancestor remaining in Asia becomes the *S. japonicum* species group. (3 and 4) The African *Schistosoma* ancestor diverges to form the *S. mansoni* and *S. haematobium* species groups. (5) An *S. indicum* ancestor also diverges from the African ancestor and migrates back to India, probably with early humans and their animals. (6) *Schistosoma mansoni* disperses to South America via the transport of African slaves. (B) Revised Asian hypothesis (this study). (1) The ancestral Asian *Schistosoma* radiates into the *S. japonicum* group. (2) Meanwhile ancestral schistosomes disperse to Africa via India and form the *S. mansoni* group. (3) A subsequent split forms the *S. haematobium* group which radiates through Africa, and (4) the *S. indicum* group which has reinvaded the Indian subcontinent. (5) *Schistosoma mansoni* disperses to South America via the transport of African slaves.

*S. nasale*, *S. spindale* and *S. indicum*, and an additional radiation of species within Africa among the 'haematobium' species group. Non-human mammal migration is most likely to be responsible for the earlier continental dispersal of *Schistosoma* species. A suite of morphological characters supports the molecular tree and a number of morphological synapomorphies are recognized for all but a few clades. We reject the subfamily Bilharziellinae, suggest that *Trichobilharzia ocellata* and *T. szidati* may be the same species, and advise a revision and renaming of *Orientobilharzia* to reflect its unquestioned position within the genus *Schistosoma*. Our tree supports a split in the pattern of mitochondrial genome

organization between the East Asian *Schistosoma* and the more derived taxa, but it remains to be seen whether *S. incognitum* and/or *Orientobilharzia* have the plesiomorphic or derived unique patterns of mitochondrial gene arrangement exhibited by *S. mansoni*.

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