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Research Brief

Orientobilharzia turkestanicum is a member of *Schistosoma* genus based on phylogenetic analysis using ribosomal DNA sequences

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rDNA, ribosomal DNA
SDS, sodium dodecyl sulfate
PCR, polymerase chain reaction
NJ, neighbor-joining
MP, maximum parsimony
Cattle
Sheep
Cashmere goat
Goat
Phylogenetic analysis

ABSTRACT

In the present study, samples representing *Orientobilharzia turkestanicum* from cattle, sheep, cashmere goat and goat in Heilongjiang Province, China, were characterized and grouped genetically by sequences of internal transcribed spacer (ITS, including ITS-1 and ITS2) and 28S ribosomal DNA (28S rDNA). The ITS and 28S rDNA were amplified by polymerase chain reaction (PCR) and then sequenced and compared with that of other members of the Schistosomatidae available in GenBank™, and phylogenetic relationships between them were re-constructed using the neighbor-joining and maximum parsimony methods. The lengths of ITS-1, ITS-2 and 28S rDNA sequences for all *O. turkestanicum* samples from different hosts were 384 bp, 331 bp and 1304 bp, respectively. While the ITS-1 sequences of *O. turkestanicum* from each of the four different hosts, and ITS-2 of *O. turkestanicum* from cattle, sheep and cashmere goat were identical, respectively, the ITS-2 of *O. turkestanicum* from goat differed from that of *O. turkestanicum* from cattle, sheep and cashmere goat by one nucleotide. The 28S rDNA sequences of *O. turkestanicum* from sheep and cashmere goat were identical, but differed from that of *O. turkestanicum* from cattle and goat by two nucleotides, with the latter two also having identical 28S rDNA sequence. Phylogenetic analyses based on the combined sequences of the ITS-1 and ITS-2, or the 28S rDNA sequences placed *O. turkestanicum* within the genus *Schistosoma*, and it was phylogenetically closer to the African schistosome group than to the Asian schistosome group. These results should have implications for studying the origin and evolution of *O. turkestanicum* and other members of the Schistosomatidae.

Adult worms of *Orientobilharzia turkestanicum* (Trematoda: Schistosomatidae) live in the portal veins or intestinal veins of cattle, sheep, goat and other mammals, causing orientobilharziasis. Cattle and sheep that are infected with *O. turkestanicum* often display chronic disease, presenting the syndrome of emaciation, anemia and diarrhea, and may have deciduous mucosae and blood in the faeces. Other symptoms include pale mucous membranes, edema in jaw and abdomen. The female animals may suffer from acyesis or abortion, and the young animals grow slowly.

Prevalence of orientobilharziasis has been reported in a number of countries, including China, India, Mongolia, Pakistan, Iraq, and Iran in Asia and Russia and Turkey in Europe (Kumar and de Burbure, 1986). In China, *O. turkestanicum* has been reported in 20 provinces, with severe infections in numerous ruminants, such as cattle and sheep, causing significant economic losses to the livestock industry (Wang et al., 2002). More importantly, the cercariae of *O. turkestanicum* can also infect humans and often cause cercarial dermatitis, and are considered the major pathogen of cercarial dermatitis in the Caspian Sea area of Iran (Sahba and Malek, 1979) and several provinces of the People's Republic of China (Bai et al., 1963; Li and Li, 1980; Tian and Li, 1980), posing significant public health problem in a number of countries. However, limited studies have been done in the classification and phylogenetic studies of *O. turkestanicum*, and there is controversy as to the phylogenetic position of *O. turkestanicum* (Snyder and Loker, 2000; Attwood et al., 2002; Brant and Loker, 2005).

It has becoming a common practice to use sequences of different DNA regions for inferring evolutionary relationships among parasites because different DNA regions evolve at different rates (McDonnell et al., 2000; Barta, 2001). Our recent study using sequences of mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 gene (*nad*1) proposed that *O. turkestanicum* be considered a member of the genus *Schistosoma* (Li et al., 2008). Extending that previous investigation, the objectives of the present study were to sequence the internal transcribed spacer (ITS, including ITS-1 and ITS-2) and 28S ribosomal DNA (rDNA) of *O. turkestanicum* from

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cattle, sheep, cashmere goat and goat in China, compare them with that of other members of the Schistosomatidae, and examine the phylogenetic relationships of *O. turkestanicum* with other members of the Schistosomatidae using the combined sequence of the ITS-1 and ITS-2, and the 28S rDNA sequences.

Five adult trematodes were collected, respectively, from the portal veins or intestinal veins of three individual animals (cattle, sheep, cashmere goats and goats) which were naturally infected and slaughtered in our laboratory in Daqing, Heilongjiang Province, China in 2006. They were washed extensively in physiological solution, identified morphologically as *O. turkestanicum* according to existing keys and descriptions (Dutt and Srivastava, 1961), and fixed in 70% ethanol before DNA extraction. Then, the genomic DNA was prepared using the method reported previously (Li et al., 2008) and the DNA samples were stored at −20 °C until further use.

The ITS was amplified by polymerase chain reaction (PCR) from each trematode DNA sample using primers p1 (forward; 5′-GTCGT AACAAGGTTTCCGTAGGTG-3′) and p2 (reverse; 5′-TATGCTTAA ATTCAGCGGGTAATC-3′) designed based on the 18S and 28S rDNA sequences of blood flukes available in GenBank™ (Accession Nos. AY197343 and AY197344), and the 28S rDNA amplified using primers p3 (forward; 5′-TATCACTAAGCGGAGGAAAAGA-3′) and p4 (reverse; 5′-AGGGAACCAGCTACTAGATGG-3′) designed based on the 28S rDNA sequence of *O. turkestanicum* available in GenBank™ (Accession No. AJ313461), respectively. For the amplification each rDNA region, PCR reactions were performed using the protocols reported previously (Li et al., 2008). Two microliters (10–20 ng) of genomic DNA was added to each PCR reaction. One fifth of each reaction product was analyzed by electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide and photographed using a gel documentation system.

PCR products were purified using the BioSpin gel extraction kit (Bioer Technology) according to the manufacturer's recommendations. The purified products were ligated with pMD 18-T vector (TaKaRa) and transformed into the JM109 competent cells. The recombinant bacterium was screened, identified by PCR amplification and enzymatic digestion, and then sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. At least two clones for each DNA region per trematode sample were sequenced.

Sequence variation in the ITS-1 and ITS-2, and the 28S rDNA of *O. turkestanicum* from different hosts obtained in the present study was examined, and then the sequences were compared with the corresponding sequences of *Orientobilharzia* spp., and that of other schistosomes available in GenBank™. Phylogenetic relationships of *O. turkestanicum* with other members of the Schistosomatidae were re-constructed using the combined ITS-1 and ITS-2 sequences, as well as the 28S rDNA sequences, using *Fasciola hepatica* as outgroup. The 5.8S rDNA sequences and the regions of repeat sequence in the ITS-1 of *Schistosoma* spp. were not included for phylogenetic analyses. The DNA sequences were aligned using Clustal X under default setting (the sequence alignments are available from the authors upon requests). Neighbor-joining (NJ) method and maximum parsimony (MP) method were performed using the MEGA3 (Kumar et al., 2004). Branch supports were estimated by bootstrap analysis of 3000 replicates for NJ analysis.

There was no difference in length and nucleotides between the three sequences of the ITS-1, ITS-2 and 28S rDNA representing *O. turkestanicum* from the same definitive host. Also, there was no difference in length of the ITS-1, ITS-2 and 28S rDNA sequences of *O. turkestanicum* from different definitive hosts, respectively. Eventually four ITS-1 sequences of 384 bp, four ITS-2 sequences of 331 bp and four 28S rDNA sequences of 1304 bp were obtained for *O. turkestanicum* from cattle, sheep, cashmere goat and goat in Daqing District, Heilongjiang Province, China, respectively. These sequences have been deposited in GenBank™ under Accession Nos. EU436659–EU436666.

Comparison of these sequences revealed that there was only subtle variation (0–0.3% for ITS-1 and ITS-2, and 0–0.2% for 28S rDNA) among the sequences from different hosts. While the ITS-1 sequences of *O. turkestanicum* from all four different hosts, and ITS-2 sequences of *O. turkestanicum* from cattle, sheep and

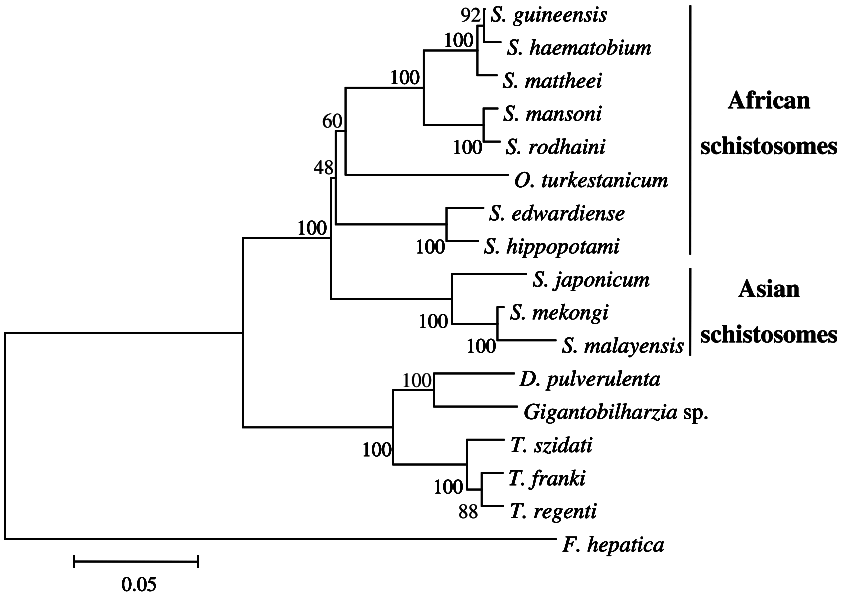

Fig. 1. Phylogenetic relationships of *O. turkestanicum* with other members of the Schistosomatidae re-constructed by neighbor-joining method based on the combined sequences of the ITS-1 and ITS-2, using *Fasciola hepatica* (GenBank™ Accession No. AM709498) as the outgroup. The following members of the Schistosomatidae are used: *Schistosoma guineensis* (Z21717), *Schistosoma haematobium* (Z21716), *Schistosoma mattheei* (Z21718), *Schistosoma mansoni* (AY446079), *Schistosoma rodhaini* (AY446078), *Orientobilharzia turkestanicum* from cashmere goat (EU436665), *Schistosoma edwardiense* (AY197344), *Schistosoma hippopotami* (AY197343), *Schistosoma japonicum* (U97533 and U22167), *Schistosoma mekongi* (U89871 and U22169), *Schistosoma malayensis* (U82283 and U82398), *Dendritobilharzia pulverulenta* (AY713962), *Gigantobilharzia* sp. (AY713963), *Trichobilharzia szidati* (AY713972), *Trichobilharzia franki* (AY713973) and *Trichobilharzia regenti* (AF263829). Number at the branch nodes indicates percentage bootstrap support for 3000 replicates.